mRNA diversity represents a major theme of neuronal nitric-oxide synthase (nNOS) gene expression in somatic cells/tissues. Given that gonads often express unique and biologically informative variants of complex genes, we determined whether unique variants of nNOS are expressed in the testis. Analysis of cDNA clones isolated from human testis identified a novel, testis-specific nNOS (TnNOS) mRNA transcript. A predicted 3294-base pair open reading frame encodes an NH2-terminal truncated protein of 1098 amino acids. Measurement of calcium-activated \textsuperscript{14}C-citrulline formation and nitric oxide release in CHO-K1 cells stably transfected with the TnNOS cDNA indicates that this protein is a calcium-dependent nitric-oxide synthase with catalytic activity comparable to that of full-length nNOS. TnNOS transcripts exhibit novel 5' mRNA sequences encoded by two unique exons spliced to exon 4 of the full-length nNOS. Characterization of the genomic structure indicates that exonic regions used by the novel TnNOS are expressed from intron 3 of the NOSI gene. Although lacking canonical TATA and CAAT boxes, the 5'-flanking region of the TnNOS exon 1 contains multiple putative cis-regulatory elements including those implicated in testis-specific gene expression. The downstream promoter of the human nNOS gene, which directs testis-specific expression of a novel NH2-terminal truncated nitric-oxide synthase, represents the first reported example in the NOS gene family of transcriptional diversity producing a variant NOS protein.

The nitric-oxide synthases (NOS)\textsuperscript{*} constitute a family with at least three distinct human isoforms: neuronal (nNOS), inducible, and endothelial constitutive. These apoenzymes are cytochrome P450-like hemeproteins that require tetrahydrobiopterin, calmodulin, FMN, and FAD as cofactors. NOS enzymes function as homodimers and catalyze the NADPH-dependent five-electron oxidation of L-arginine to form L-citrulline and NO (1–4). The three isoforms are structurally similar, sharing in their primary amino acid sequences binding sites for heme, calmodulin, FMN, FAD, and NADPH (1, 5, 6). However, nNOS differs from the other two isoforms in that it exhibits a unique NH2-terminal extension containing a PDZ/GLGF motif of \textasciitilde100 amino acids. PDZ/GLGF motifs participate in protein-protein interactions in a variety of proteins (7, 8). In fast twitch skeletal muscle fibers, for example, nNOS is engaged to the sarcolemma dystrophin complex via this PDZ/GLGF motif (9). In a variety of neurons, subcellular localization of nNOS in synapses involves PDZ/GLGF anchoring to postsynaptic density 95 (PSD-95) and the related protein PSD-93 (10). Furthermore, recent work has demonstrated that nNOS also contains within the NH2-terminal region a unique binding domain for PIN (protein inhibitor of nNOS) (11). Binding of PIN to this domain results in significant inhibition of nNOS enzymatic activity.

The biological roles for nNOS continue to expand. The enzyme is implicated in the regulation of neuronal cell biology and neurotransmission (1, 12, 13), as a major nonadrenergic-noncholinergic transmitter in enteric nerves (14, 15), in the neuroendocrine biology of the hypothalamus and pituitary (16–18), in modifying skeletal muscle contractile force and development (19, 20), in the control of total body sodium content and body fluid homeostasis via its expression in the macula densa and distal nephron of the kidney (21–23), and in male sexual function (24), among other roles. Targeted disruption of the nNOS gene in mice by homologous recombination was recently reported (25). Studies using this genetic model have indicated involvement of the enzyme in neurotoxicity secondary to ischemia reperfusion injury in the central nervous system (26), in behavior (27), and in the development and normal function of the gastric pyloric sphincter (28). Significantly, a recent study in 27 families with inherited infantile pyloric stenosis has identified nNOS as a susceptibility gene in this human disorder (28).

The structural organization of the human nNOS gene has been described (29). NOS1 is a complex locus consisting of 29 exons and 28 introns, localized to 12q24.2, spanning a region greater than 160 kb as a single copy in the haploid human genome. The full-length open reading frame of 4302 bp encodes a protein of 1434 amino acids. Translation initiation and termination sites are located in exons 2 and 29, respectively. Structural and allelic mRNA diversity represents a major theme in the regulated expression of this gene in somatic...
tissues and cells, including cDNA clones from human cDNA libraries (29), multiple examples of exon 1 (30), and alternate polyadenylation signal usage (29). The functional relevance of the diversity of mRNA transcripts remains to be elucidated. Similar mRNA diversity has been observed in other species. An alternative splicing event between exons 1 and 3 was detected in wild type mouse and in mice in which exon 2 was disrupted by homologous recombination, resulting in an NLS-terminal truncated protein, nNOSy (10). Furthermore, a cassette exon insertion between exons 16 and 17 was shown to exist in the skeletal muscle of rat (31).

Cardiovascular and neuroendocrine genes commonly exhibit expression patterns in cells of germ-lineage. In many cases, unique mRNA transcripts are expressed. These variants are often informative with respect to enzyme structure and function. Examples in which a novel protein is expressed that differs from its somatic counterpart include angiotensin-converting enzyme (32, 33), Ca2+/calmodulin-dependent protein kinase (34), A-type lamin (35), glutamic acid decarboxylase (36), propionelarnocortin (37), and type β platelet-derived growth factor receptor (38), among others. Alternate promoter usage commonly underlies this mRNA and protein diversity. In each of these cases, characterization of the protein expressed in germ cells has led to a better understanding of the biology and biochemistry of the protein involved and provided important new insight into the regulation of the gene. Furthermore, where 5′ mRNA diversity exists in the 5′-UTR of mRNA sequences of germ-cell transcripts, profound effects on translational efficiency have been described, as in proenkephalin (39), copper-zinc superoxide dismutase (40), GATA-1 (41), cytochrome c (42), γ-glutamyl transpeptidase (43), and e-mos (44).

In this study, we report the cloning from human testis of a novel nNOS cDNA (TnNOS) resulting from transcriptional activation of a downstream promoter. This novel transcript encodes an NH2-terminal truncated protein that is an analogue of the mouse nNOS. The human nNOSy possesses NOS enzymatic activity comparable to that of the full-length nNOS.

**MATERIALS AND METHODS**

cDNA Cloning and Sequencing

**Library Screening—**An oligo(dT) + random primed agt10 human testis 5′ stretch cDNA library and an oligo(dT) primed agt11 human testis cDNA library (CLONTECH Laboratories, Palo Alto, CA) were screened with restriction enzyme fragments derived from a full-length human nNOS cDNA previously described (45): 1344-bp BamHI fragment, exons 2–6; 1476-bp BamHI fragment, exons 6–17; 867-bp BamHI/Xhol fragment, exons 17–23; 2153-bp XhoI/BamHI fragment, exons 23–29. Bacteriophage were plated and transferred to nitrocellulose filters. Probes were labeled to a specific activity of 5 × 10⁶ cpm/μg with [γ-32P]ATP (DuPont NEN; specific activity, 3000 Ci/mmol) using the random primer method. Hybridization and post-hybridization washes were carried out at 65 °C. Cross-hybridizing bacteriophage were isolated by plaque purification prior to DNA sequence analysis as described (45).

**Rapid Amplification of 5′ cDNA Ends (5′-RACE Protocol)—**Modifications of the RACE protocol were utilized to isolate the 5′-termini of mRNA transcripts for human nNOS in human testis total cellular RNA (45). Briefly, total cellular RNA derived from normal human testis (100 μg) (CLONTECH) was reverse transcribed with a gene-specific antisense primer (P1, exon 12, 5′-GCA CGA TCC ACA CCC AGT-3′; antisense primer, 5′-TGG TTA TGT GAC CCT CGT TG-3′) using SuperScript II reverse transcriptase (Life Technologies, Inc.). First round PCR was performed for 35 cycles with primer annealing at 57 °C (Perkin Elmer 480 thermocycler) (generic sense primers 5′-GAC TCG AGT CGA AGA ATT CAA TGT, 2.5 pmol; 5′-GAC TCG AGT CGA AGA ATT CAA-3′, 25 pmol; generic sense antisense primer P2, exon 8, 5′-CGT CCT CGT CTG TCC T-3′, 25 pmol). Second round of amplification was performed for 35 cycles with primer annealing at 57 °C (generic sense primer 5′-GAC TCG AGT CGA AGA ATT CAA-3′, 26 pmol; nested gene-specific antisense primer P3, exon 7, 5′-CTT TGG TGG CAT ACT TGA-3′, 25 pmol). PCR products were subcloned into pBluescript II SK(−) and subjected to DNA sequence analysis.

A second 5′-RACE analysis was performed to independently assess putative start sites of transcription using an additional set of gene-specific reverse primers and total cellular RNA derived from multiple independent sources of normal human testis and brain (CLONTECH). Reverse transcription was carried out with a gene-specific antisense primer (P4, exon 5, 5′-CTT TGG CGA GAG GAG AGA G-3′). First round PCR amplification was performed with primer annealing at 51 °C (gene-specific antisense primer P5a, exon 4, 5′-CAA GTG CCT TCT GTG ACA-3′ or P5b, exon 4, 5′-5-GAC ACT TGG CAT AC-3′). Second round amplification was performed with primer annealing at 49 °C (nested gene-specific antisense primer P6, testis exon 2, 5′-TGG TTA TGT GAC CCT GTG TG-3′). PCR products were subcloned into the pCRII vector (TA cloning kit, Invitrogen, San Diego CA) and subjected to DNA sequence analysis.

**Characterization of Genomic Organization**

A 15-kb EcoRI fragment was subcloned from a genomic cosmid sublibrary of the human nNOS previously reported (29). Plasmid subclones were analyzed with restriction enzyme mapping and Southern blotting with oligonucleotides end-labeled with [γ-32P]ATP (DuPont NEN; specific activity, 3000 Ci/mmol). All exonic sequences and exon-intron boundaries were determined on both strands. Intron sizes were determined with restriction enzyme mapping and PCR amplification with Eukaryotic Life Technologies (46). 5′-Flanking regions of the two testis nNOS exon 1 examples were sequenced on both strands and potential cis-acting DNA sequences identified (Eukaryotic Transcription Factor Data Base release 7, Genetics Computer Group sequence analysis software package, Madison, WI).

**RT-PCR**

Semiquantitative RT-PCR was utilized to assess the tissue specificity of novel nNOS mRNA transcripts. First strand cDNA was synthesized with total cellular RNA (5 μg) derived from varied normal human tissues (CLONTECH) using random primers and SuperScript II reverse transcriptase (Life Technologies, Inc.). The following primers were used in PCR amplifications for testis and full-length nNOS (see Fig. 3; testis exon 1 sense 5′-TAG GTG GGG GTT GAT AAG TG-3′; testis exon 1b sense 5′-AGA GGC TGG GTG GAA ACT-3′; exon 2 sense 5′-ACC AGA GTC AGC CTC CAA-3′; and exon 4 antisense 5′-CAA TGG CCT AAT GGT G-3′. PCR amplifications were performed under well-characterized semiquantitative conditions in a total volume of 100 μl for 30 cycles. An 880-bp fragment of human glyceraldehyde-3-phosphate dehydrogenase was amplified as a quantitative control (sense primer, 5′-ATC AGG ACA ACG GCT ATT GCA CAC AGG ACA GAC GAG AGA CAC GAT GG-3′/antisense primer, 5′-GCT GAC GAT AAG GGC CTT TGT GGT GCA AGA AGA GTC AGC CTC CAA-3′). PCR products were size-fractionated by agarose gel electrophoresis and transferred to GeneScreen Plus membranes (DuPont). Southern blots were hybridized with 1[γ-32P]ATP-labeled oligonucleotides positioned internally to flanking PCR primers.

**Heterologous Eukaryotic Expression of Testis nNOS**

Eukaryotic expression vectors containing either the testis or the full-length nNOS cDNAs were constructed as follows. For the testis nNOS, the 5′- and EcoRI/SphI fragment of the 7.5-kb full-length human nNOS cDNA (45) was replaced with a 365-bp EcoRI/SphI fragment representing the major 5′-end of the testis nNOS cDNA. This 5247-bp testis nNOS cDNA containing the full 5′-UTR, coding region, and 1615 bp of the 3′-UTR was then inserted into the eukaryotic expression vector pcDNA3 (Invitrogen). For the full-length nNOS, an XbaI/EcoRI fragment of the 7.5-kb human nNOS cDNA that contained 533 bp of the 5′-UTR coding region, and 6130 bp of total cellular RNA derived from human nNOS cDNA was subcloned into pcDNA3. CHO-K1 cells (ATCC, Rockville, MD) grown in Ham’s F-12 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) were transfected with TnNOS, full-length nNOS, or empty vector using electroporation. Multiple independent G418-resistant clones were selected 72 h later in 800 μg/ml G418 (Life Technologies, Inc.).

**Western Blot Analysis**

Stably transfected CHO-K1 cell monolayers were lysed with boiling sample buffer (62.5 mM Tris-HCl (pH 7.4); 2% SDS, 10% glycerol, and 5% β-mercaptoethanol). Cell lysates were boiled for 5 min and centrifuged at 100,000 χ g for 30 min. The cellular supernatant was collected and protein concentration measured using the Bio-Rad protein assay kit II. Human testis protein extracts were obtained from CLONTECH. Rat cerebellum homogenates were prepared with a Polytron PT3000
(Brinkmann Instruments). Protein samples were electrophoresed in 6% polyacrylamide-SDS gels and transferred by electroblotting onto nitrocellulose membranes. Blots were incubated with anti-human nNOS monoclonal antibody (1 μg/ml) (Transduction Laboratories, Lexington, KY) and subsequently with horseradish peroxidase-conjugated sheep anti-mouse secondary antibody (1:20,000 dilution) (Amersham Corp.). Signal detection was facilitated with ECL (Amersham Corp.).

**Measurement of NOS Activity**

**Determination of l-[14C]Citrulline Formation (47)**—Stably transfected CHO-K1 cells were cultured in six-well plates (Costar, Cambridge, MA) to confluence and allowed to equilibrate in 1 ml of a physiological salt solution composed of (in mM) 130 NaCl, 5 KCl, 1.5 CaCl₂, 1 MgCl₂, 25 HEPES (pH 7.4), and 10 d-glucose for 30 min at 37 °C. After being labeled with l-[14C]arginine (5 × 10⁶ cpm/ml) (Amersham Corp., specific activity ~300 mCi/mmol) for 30 min, cells were treated with either ionomycin (5 μM) (Calbiochem) or vehicle for 20 min. The reaction was stopped by washing the cell monolayers with 4 ml of physiological salt solution composed of (in mM) 130 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 1 NaH₂PO₄, 10 HEPES, and 5.5 K₃HPO₄ for 30 min at 4 °C. After neutralization, the extract was loaded onto a 1-ml wet bed volume of Dowex AG 50WX-8 cation-exchange resin (Bio-Rad, Na⁺ form, 100–200 mesh) followed by 4 ml of water. l-[14C]Citrulline in the 5-ml column effluent was quantitated by scintillation counting. To validate the product of cation-exchange chromatography, fractions of the column effluent were resolved using TLC silica gel (Alltech Associates, Inc., Deerfield, IL). Results indicated that l-[14C]citrulline was the only detectable product.

**Release of NO**—Direct measurement of NO in cell culture was performed using an NO-sensitive electrode as described previously (48). Briefly, cells grown in 35-mm dishes (Costar) were preincubated in Krebs-Ringer buffer (pH 7.4) of the following composition (in mM): 136 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 1 NaH₂PO₄, 10 HEPES, and 5.5 glucose, supplemented with 7.5 units/ml superoxide dismutase (Sigma). NO release was monitored with an NO-selective microprobe (Inter Medical Co., Nagoya, Japan). The working electrode made of platinum/iridium alloy was coated with a film containing KC1, NO-selective nitrocellulose resin (pyroxyline lacquer), and gas-permeable silicon membrane (49). A counter electrode was made of carbon fiber. The redox current was detected with a current-voltage converter circuit and continuously recorded. The probe tip (diameter, 25 μm) was enclosed in a Faraday’s chamber and positioned 3–5 μm above the cell surface. Calibration of the electrochemical sensor was performed using varied concentrations of a nitrosothiol donor, S-nitroso-N-acetyl-DL-penicillamine (Sigma) (48).

**RESULTS**

**cDNA Sequences**—Screening of approximately 2 × 10⁶ plaques from two independent testis cDNA libraries yielded one positive clone. Restriction mapping and sequence analysis indicated that the 2.3-kb random primed cDNA insert represented exons 9–23 of human nNOS. No insertion or deletion of exons was detected.

5'-RACE was utilized to define 5'-termini of nNOS transcripts in human testis. This PCR-based approach allowed isolation of human nNOS cDNA sequences upstream of exon 6. Primers were located in exons 12 (P1), 8 (P2), and 7 (P3) (Fig. 1A). Thirty-three cDNA clones were sequenced. Nine of these clones were identical in nucleotide composition to nNOS sequences defined in human brain (45). Twelve independent clones featured a novel 5'-terminus. All of these 12 clones exhibited 155 bp of new sequence upstream of nNOS exon 4 (Fig. 1). This novel nNOS mRNA transcript, hereafter referred to as TnNOS, appeared to be expressed at a level comparable to that of the full-length nNOS in the testis as judged by the number of clones isolated in the 5'-RACE experiment and assessment of semiquantitative RT-PCR signals (see below). Subsequent studies (Fig. 2, see below) indicated that this 5'-terminus was encoded by two novel exons (Tex 1 and Tex 2) spliced to exon 4 of human nNOS. Characterization and analysis of the TnNOS mRNA transcript indicated an open reading frame of 3294 nt, which predicted a protein of 1098 amino acids with a calculated molecular mass of 125,017 Da. Translation initiation was predicted to occur within exon 5 at the nucleotide sequence TCTGCAATGG, which was consistent with a Kozak consensus sequence for initiation of translation in eukaryotes (50). 5'-UTR sequences of 311 nt showed stop codons in all three translation reading frames. Although lacking 336 amino acids at the NH₂ terminus of the full-length nNOS protein, the novel TnNOS protein contained all of the functional modular domains perceived to be necessary for NOS enzymatic activity, specifically those for binding of heme, tetrahydrobiopterin, calmodulin, FMN, FAD, and NADPH. Further analysis indicated that this novel human nNOS protein is an analogue of the mouse nNOSγ. A third testis nNOS cDNA variant was also isolated. This minor species (3 clones of the 33 isolated), designated as TnNOSb, differed from TnNOS at sequences 5'- of Tex 2, possessing a unique 95-bp exon 1 termed as Tex 1b (Fig. 1B). The structure of this cDNA predicted a protein that is identical to nNOSγ. Nine of the 33 cDNA clones isolated from testis mRNA sources revealed an interesting cassette insertion of Tex 2 between exons 3 and 4 of the full-length nNOS (Fig. 3B). A 56-bp insertion in the coding region of the full-length nNOS resulted in a frameshift and introduced a stop codon (TGA) 371 nt downstream of the splice junction within exon 6. The 1221-nt open reading frame encoded a COOH-terminal truncated novel protein of 407 amino acids with a calculated molecular mass of 43,788 Da (Fig. 1C).

**Genomic Organization and 5'-Flanking Regions**—Nucleotide sequences reported in this study have been submitted to the GenBankTM/EMBL Data Bank with accession numbers U66360–U66362. DNA sequence analysis and restriction enzyme mapping of genomic DNA fragments indicated that the novel 155-bp sequence at the 5'-end of the TnNOS cDNAs was encoded by two unique exons (Tex 1, 99 nt; Tex 2, 56 nt) that were spliced to exon 4 of the full-length nNOS (Fig. 2A). The minor variant TnNOSb started with another unique exon (Tex 1b, 95 nt) that was spliced to Tex 2. Tex 1, Tex 1b, Tex 2, and corresponding 5'-flanking regions were localized to intron 3 of the full-length nNOS (Fig. 2A). Intron/exon boundaries confirmed to the GT/AG donor/acceptor site rule, maintaining the consensus sequences described by Mount (51) (Table I).

Analysis of 1800 nt of 5'-flanking regions of Tex 1 revealed a GC content of approximately 50%. The dinucleotide CpG was underrepresented (45 observed, 109 predicted), consistent with the balance of mammalian genomic DNA. Two independent rounds of 5'-RACE, using different primers, indicated a common putative transcription start site. We take these data to indicate that these genomic regions represent sites for transcription initiation and that these 5'-flanking regions likely represent promoter regions active in testis tissue. Future analyses with primer extension, S1 nuclease, and/or RNase protection will be necessary to confirm this transcription initiation site. Although lacking a classical TATA box, sequence analysis of these 5'-flanking regions indicated the presence of numerous binding sites for ubiquitous transcription factors.
Fig. 1. Human TnNOS cDNA sequences. A, 5'-RACE strategy. Numbered boxes represent exons of the human nNOS gene. The gene-specific antisense primers used in the two 5'-RACE experiments are depicted (not drawn to scale). B, sequence alignment of the 5'-ends of the two TnNOS cDNA variants. Unboxed regions are coded by independent exon 1 examples (Tex 1 and Tex 1b), whereas boxed regions indicate shared sequence starting from Tex 2. Note that Tex 2 is spliced to exon 4 of the full-length nNOS. The inverted triangle depicts the ATG translation initiation site for TnNOS. Nucleotide sequences reported in this study have been submitted to the GenBank/EMBL Data Bank with accession numbers U66360–U66362. C, deduced amino acid sequence of the novel nNOS-related protein with Tex 2 insertion between exons 3 and 4. A GLGF motif is boxed. The inverted triangle indicates the starting of the novel sequence.

(Fig. 2B) including an inverted Sp1 site (CCCGCC) (52), two ATF/CRE-like elements (TGACGTCA) (53), a high affinity site for NF1 (TGGCNNNNNNCCA) (54), a consensus sequence for NFκB binding (GGGRHTYYHC) (55), an AP-1 site (TGASTCA) (56), four AP-2 sites (GSSWGSCC)/(YCSCCMNSSS), and three inverted AP-2 sites (57, 58). In addition, a variety of cis-regulatory elements implicated in testis-specific transcription were evident, including three consensus GATA sites (WGATAR) (59) and five GATA-like sites, a putative p53 half-element (RRRC-WWGGY) (60), an Ets family protein binding site (SMAGGWGY) (61), three related Pu box sequences (GAGGA) (62) and three PEA3 sequences (AGGAAR) (61), an MEF-2 motif (YTAWAAATAR) (61), and an insulin response element (IRE) site (CCCGCCTC) (63) overlapping the Sp1 site, among others.

Three copies of the Alu family of short interspersed repetitive sequences were found within 1.8 kb of 5'-flanking region sequences (Fig. 2). Comparison with Alu consensus sequences revealed that these three Alu elements belonged to different Alu subfamilies. A proximal element (−419 to −245 nt) on the bottom strand belonged to the Alu-Sb subfamily (89% identity with the Alu-Sb consensus sequence), whereas the other two Alu elements (−1748 to −1579 nt and −1039 to −759 nt) on the top strand belonged to the Alu-Sx subfamily (87 and 83% identity with the Alu-Sx consensus sequence, respectively). Alu repetitive sequence appears approximately every 4–8 kb in human genomic DNA (64). Therefore, the frequent appearance of this repetitive element in the 5'-flanking region of human TnNOS is atypical.

Testis exon 1b (Tex 1b) of TnNOSb and its 5'-flanking region were located farther 5' of Tex 1 (Fig. 2A). Putative cis-regula-
FIG. 2. Genomic organization of the 5′-ends of TnNOS variants and their flanking regions. A, restriction map and exon/intron location. The top line shows the structure of the human nNOS gene, where numbered boxes represent exons and horizontal lines represent introns. The two plasmid clones used to characterize these regions are depicted. Bm, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; S, Sall; X, XbaI. Alu,
Table I

| Exon | Exon Sequence | Intron | Intron size | 5' Donor | 3' Acceptor | Exon | Exon Sequence |
|------|---------------|--------|-------------|----------|-------------|------|---------------|
| Tex 1 | GCAG          | Tex 1/Tex 2 | 2.6kb       | GTAGA    | GTTCATTTTACAGg | Tex 2 | ATGA         |
| Tex 1b | TGGS         | Tex 1b/Tex 2 | 6.8kb       | GTAGGG   | GTTCATTTTACAGg | Tex 2 | ATGA         |
| Tex 2 | ACCA          | Tex 2/4   | 10.5kb      | GATAAGT  | CATCTCTCTGAGC | 4    | CCC |

*Indicates that they are identical because they splice to the same exon (Tex 2).
FIG. 3. A, tissue-specific expression of TnNOS. i, strategy for RT-PCR and Southern probing. Horizontal arrows depict PCR primers and vertical arrows depict primers used for hybridization (not drawn to scale). The ATG codon for translation initiation in TnNOS is marked. In addition to variant exon 1 examples, unique mRNA transcripts may also arise from alternative splicing. ii, RT-PCR between Tex 1 and exon 4 probed with a Tex 1 primer. TnNOS is specifically expressed in testis. The major band at 282 bp represents the type A mRNA shown in i, whereas the minor band at 226 bp represents the type C mRNA in which Tex 2 is spliced out. T, testis; B, brain; S, skeletal muscle; H, heart; L, lung; K, kidney; A, adrenal gland. iii, RT-PCR between Tex 1 and exon 4 probed with a Tex 2 primer. Only the type A mRNA is detected with a Tex 2 primer. B, ubiquitous expression of the full-length nNOS and evidence for alternative splicing. i, strategy for RT-PCR and Southern probing (not drawn to scale). ii, RT-PCR between exons 2 and 4 probed with an exon 3 primer (2-h exposure). The full-length nNOS is ubiquitously expressed in all the tissues. The band at 408 bp corresponds to the type A mRNA in i. A weak signal could be detected at 464 bp with prolonged radiograph exposure. iii, RT-PCR between exons 2 and 4 probed with a Tex 2 primer (30-h exposure). The band at 464 bp represents the type B mRNA in i in which Tex 2 is inserted between exons 3 and 4. Note the particularly low frequency of this event in the brain compared with that shown in ii on this prolonged exposure. As predicted, when these RT-PCR products are probed with a Tex 1 primer, no signal was detected even on long exposure (data not shown). Representative results from three independent experiments are shown.
shown in Fig. 5B, stable TnNOS transfectants produced comparable amounts of NO as full-length nNOS transfected cell lines following treatment with calcium ionophore. Taken together, these data indicate that the protein derived from the TnNOS mRNA transcript is a calcium-dependent nitric-oxide synthase.

DISCUSSION

The human nNOS gene is a highly complex locus. In the current study we have demonstrated that in the normal testis transcription can initiate from downstream regions within intron 3. Depending on alternative splicing, one or two novel exons are produced that in turn are spliced to exon 4 of the full-length nNOS mRNA (Fig. 3A). Translation of this novel nNOS transcript initiates in exon 5, resulting in a 125-kDa protein that is an analogue of the mouse nNOSγ. In comparison with the 160-kDa full-length nNOS, whose translation initiates in exon 2, this protein lacks the NH2-terminal 336 amino acids but possesses NOS enzymatic activity comparable to that of its full-length counterpart and is therefore a functional nitric-oxide synthase. This novel nNOS transcript is testis-specific and is designated as TnNOS in contrast to the full-length nNOS.

Putative transcription initiation sites for TnNOS were mapped with 5'-RACE methods. 5'-RACE has been used for the mapping of transcription start sites of a number of other uncommon mRNA transcripts, producing results consistent with those obtained with conventional methods such as primer extension, RNase protection, and 5' nucleotide protection assays (45). 5'-RACE is robust compared with other cloning approaches and is therefore a valuable alternative in structural characterization of infrequent mRNA transcripts. TnNOS cDNA clones isolated in the first 5'-RACE analysis were identical in 5'-UTR length to results obtained from a further 5'-RACE analysis using a different cloning strategy and multiple testis RNA sources. However, further confirmation with other methods is warranted.

Translation initiation for this novel TnNOS transcript within exon 5 was confirmed by functional heterologous eukaryotic expression in stable cell lines and assessment of protein size with immunoblot analysis (Fig. 4). Enzymatic activity was assessed in multiple independent stable transfectants using two independent methods. Measurements of whole cell calcium-activated L-[14C]arginine to L-[14C]citrulline conversion (9, 67). It has been demonstrated that in skeletal
Muscle nNOS is associated with the sarcolemma through interaction of the NH₂-terminal PDZ/GLGF motif with the dystrophin complex (9). In neurons, synaptic association of nNOS has been shown to be mediated by the binding of this NH₂-terminal PDZ/GLGF motif to proteins such as postsynaptic density 95 (PSD-95) and the related novel protein PSD-93 (10). Without the PDZ/GLGF motif, nNOS in nNOS mutant mice has been reported to be restricted to soluble fractions of skeletal muscle (10). It follows that the human protein derived from TnNOS would be similarly distributed in testis. However, in stable CHO-K1 transfectants, Western analysis using proteins derived from subcellular fractionization (67) demonstrated that the human protein derived from TnNOS as well as the full-length nNOS was distributed in both cytosol and particulate fractions (data not shown). Because the full-length nNOS is not restricted to membrane fractions in CHO-K1 transfectants, we were unable to directly determine whether the truncated protein varies in subcellular localization in this specific cell type. It is likely that unique cell-specific docking mechanisms exist in varied cell types.

The 5'-flanking region of TnNOS may contain the promoter that directs testis-specific expression of the nNOS gene. The presence within this region of multiple binding sites for both ubiquitous and testis-specific transcription factors is consistent with this proposal. The proximal Sp1 site at −184 may represent a core element in the transcriptional activation of this TATA-less transcription unit (68). An unusual feature of this region is the presence of three clustered Alu elements within 1.8 kb of the putative transcription initiation site (Fig. 2, A and B). Originating from 7SL RNA, Alu sequences are short, interspersed repetitive DNA elements, appearing approximately every 4–8 kb in human genomic DNA and representing about 5–6% of the total human genome (64). Most Alu sequences have been considered to be functionally inert. However, when located in promoter regions, Alu elements have been shown to exert transcriptional repressor effects (69), transcriptional enhancer effects (70, 71), or both (72). Alu sequences may be involved in cell-specific expression of the erythropoietin receptor (73) and the y chain of Fe and T-cell receptors (72). Multiple Alu elements clustering within the promoter/5'-flanking region have been reported in a number of human genes including the poly(ADP-ribose) polymerase gene (74), lysozyme gene (75), prostatic acid phosphatase ACPP gene (76), and erythropoietin receptor gene (73). An interesting functional feature of clustered Alu elements in promoter regions is that those located on opposite strands may be involved in the formation of DNA tertiary structures implicated in juxtaposing distal cis-regulatory elements to the proximal general transcription factors (74). This feature may be of functional relevance in the transcriptional regulation of TnNOS in that the most upstream and most downstream Alu elements of the 5'-flanking region are on the opposite strands of the helix.

The functional relevance of this complex putative TnNOS promoter was not addressed in the current study. Future studies using promoter-reporter constructs in vitro and in vivo are required for the functional characterization of this transcriptional regulatory region. Based on the above findings, however, it is likely that multiple positive and negative cis-regulatory elements may cooperate in the tissue-cell-specific and developmental regulation of TnNOS expression. Given its downstream localization, the TnNOS promoter is likely to be functional in the nNOS-deficient mice that were reported recently (25). Whether mRNA expression from this downstream promoter is modulated secondary to disruption of upstream regulatory regions remains to be assessed.

Recent studies suggest that NO/NOS play fundamental roles in maintaining reproductive endocrine function (24, 77–80). In particular, nNOS has been implicated in penile erection (24) and testicular function (78). However, the methods used in these prior studies did not allow a distinction to be made between the biological contribution of the proteins derived from full-length nNOS and TnNOS mRNA transcripts. We posit that TnNOS mRNA transcripts may exist in other sexually dimorphic organs of the male and female. The current study indicates that both mRNA transcripts are expressed in the testis in approximately equivalent amounts. Effort was made in this study to identify the cell types in testis that express the TnNOS mRNA transcripts using in situ hybridization. Although hybridization signals were detected in testis tissues of an adult human male and an adult baboon collected at necropsy, the low level of mRNA expression and/or detection precluded definition of cell-type specificity. The biological significance of this transcript warrants study. Jaffrey and Snyder (11) have recently cloned and characterized a full-length nNOS-inhibiting protein (PIN) that inhibits full-length nNOS enzymatic activity by protein-protein interaction with the NH₂-terminal region (amino acids 163–245) of the full-length nNOS. The binding of PIN apparently prevents the dimerization of the enzyme by interfering with a unique dimerization domain located within the first 165 amino acids of the full-length rat nNOS. Interestingly, PIN mRNA transcription expression is most abundant in the testis (11). This suggests that PIN would inhibit full-length nNOS function in this organ. Given that the NH₂-terminal truncated protein does not possess the PIN-binding domain and thus would not be inhibited by PIN, it is intriguing to postulate that this novel variant of nNOS derived from a unique downstream promoter sustains biological function of nNOS in the testis in the presence of PIN.

Cassette deletions of exons 9/10 or 10 in full-length human nNOS have been reported (29). An additional finding in the current study is that the testis exon 2 (Tex 2) originally isolated from testis was subsequently found to be alternatively inserted between exons 3 and 4 of the full-length nNOS in a variety of human tissues. Although occurring as minor events in comparison with the normal full-length nNOS transcript, the biological consequences of these alternative splicing events may be important. It is unclear what the determinant of these alternative splicing events is or how these events are regulated. Nor is it known whether these alternative mRNA transcripts are translated in vivo and, if they are, what the biological significance is. Given that the NOS proteins all function as dimers in the enzymatic synthesis of NO, fruitful areas for future study would be whether a cell expresses both the full-length and shorter mRNA transcripts, whether these shorter mRNA transcripts are translated in vivo, and whether alternate proteins participate as heterodimers in the catalytic synthesis of NO.

Acknowledgments—We thank Dr. Yu Hu for his technical assistance in NO measurement and Eddy Wong for his assistance in DNA sequence analysis.

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