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# Molecular cloning and characterization of human endothelial nitric oxide synthase

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The constitutive calcium/calmodulin-dependent nitric oxide (NO) synthase expressed in vascular endothelium shares common biochemical and pharmacologic properties with neuronal NO synthase. However, recent cloning and molecular characterization of NO synthase from bovine endothelial cells indicated the existence of a family of constitutive NO synthases. Accordingly, we undertook molecular cloning and sequence analysis of human endothelial NO synthase. Complementary DNA clones predict a protein of 1,203 amino acids sharing 94% identity with the bovine endothelial protein, but only 60% identity with the rat brain NO synthase isoform. Northern blot analysis with an endothelial-derived cDNA identified a 4.6–4.8 kb mRNA transcript in HUVEC and in situ hybridization localized transcripts to vascular endothelium but not neuronal tissue.

Cyclic GMP; Endothelium-derived relaxing factor; Endothelium; Vascular smooth muscle; Vasodilatation

## 1. INTRODUCTION

Synthesis of nitric oxide (NO) by NO synthase derives from one of the two chemically equivalent guanidino nitrogens of L-arginine, producing L-citrulline as a co-product [1,2]. In endothelial cells and neuronal tissue, NO synthase enzymatic activity is constitutively expressed but activation of the calcium/calmodulin signalling pathway is required for maximal activity [3,4]. Synthesis and release of NO is rapid and not dependent upon new protein synthesis. Dynamic control at the cellular level is through activation of specific cell surface receptors by calcium-mobilizing agonists. Constitutive NO synthase contrasts with a pathway for NO synthesis evident in macrophages [5], Kupffer cells, hepatocytes, vascular smooth muscle [6], and mesangial cells [7]. Calcium/calmodulin-independent NO synthase activity is induced in these cell types by cytokines or bacterial wall products over a period of many hours. Induction of NO synthase activity is dependent upon new RNA and protein synthesis. Biological control for this NO synthase pathway is at the level of transcriptional activation [8] and appears to be controlled by the cytokine-response profile of the particular cell type. Sequence comparison of recently isolated molecular clones for rat brain [4] and murine macrophage NO synthase [8,9] indicated that these proteins are the products of separate genes.

The calcium/calmodulin-dependent NO synthases of endothelial cells and neuronal tissue, though similarly regulated and expressing identical cofactor requirements, localize to different subcellular compartments [10] and vary in apparent molecular weight [11]. To determine whether these constitutively expressed NO synthases were encoded by distinct genes we have recently purified bovine cerebellar NO synthase, determined the amino acid composition of tryptic peptides, and used a PCR-based cloning strategy to isolate cDNA probes for bovine neuronal and bovine endothelial NO synthase [12]. Genomic southern analysis using neuronal and endothelial cDNAs identified unique restriction enzyme fragments indicating that these cDNA clones reflected the products of distinct genes. Furthermore, isolation and expression of a full-length cDNA for bovine endothelial NO synthase in eukaryotic transfectants resulted in the acquisition of calcium-stimulated NO synthase activity, as well as NADPH diaphorase activity. Sequence analysis of this full-length clone predicted a protein of 1205 amino acids that differed at numerous residues from the sequence determined for the purified bovine neuronal NO synthase. Moreover, bovine endothelial NO synthase showed only 50 and 60% homology with recently identified murine macrophage and rat brain NO synthases, respectively. These data were taken to indicate that bovine endothelial NO

*Abbreviations:* HUVEC, human umbilical vein endothelial cells; kb, kilobases; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor-alpha.

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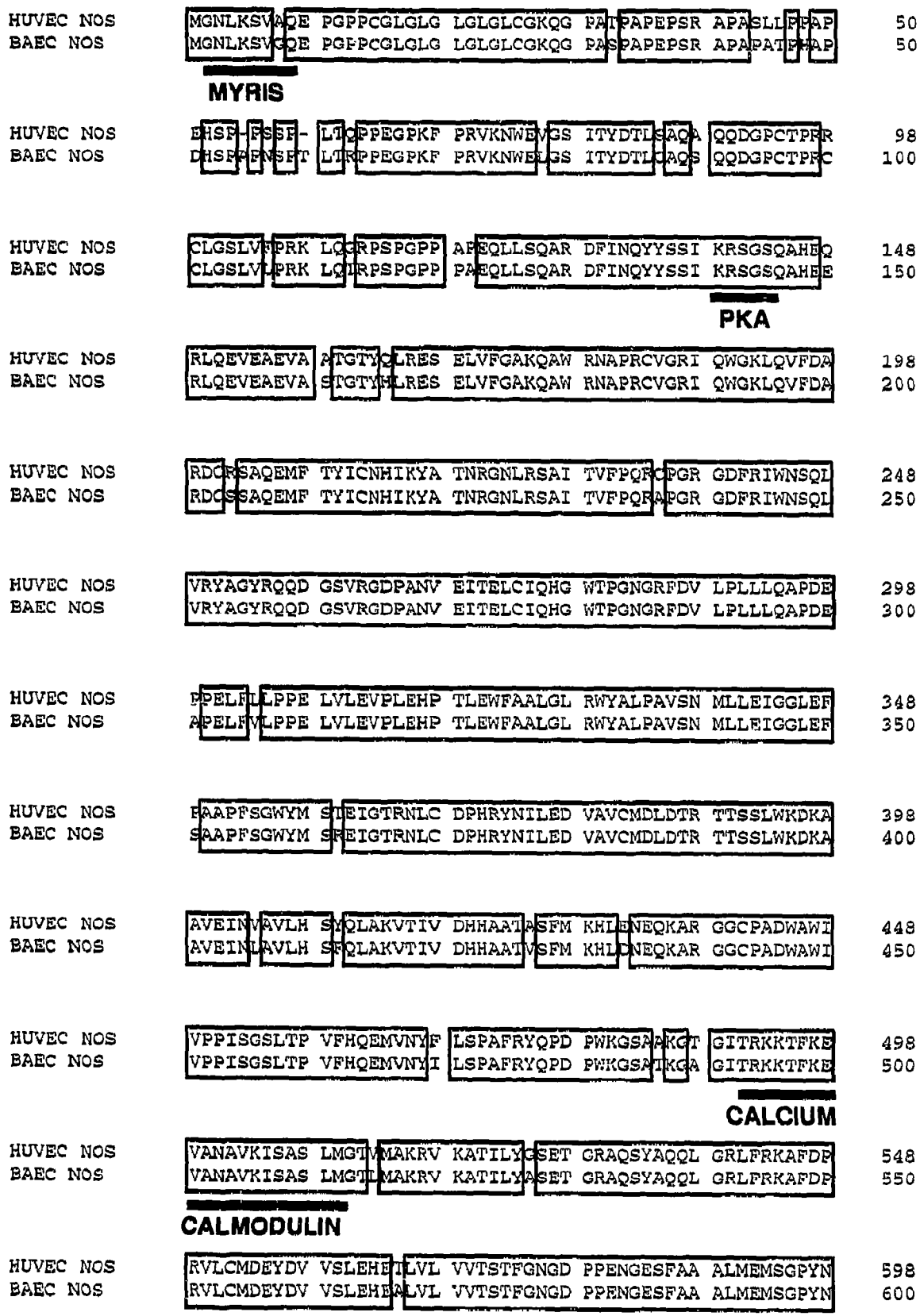
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BAEC NOS MGNLKSVGQE PGPPCGLGLG LGLGLCGKQG PASPAPEPSR APAPATPPAP 50
MYRIS
HUVEC NOS EHSP-PSSP- LTQPPEGPKF PRVKNWEVGS ITYDTLSAQA QQDGPCTPRR 98
BAEC NOS DHSPAPNSPT LTRPPEGPKF PRVKNWELGS ITYDTLCAQS QQDGPCTPRC 100
HUVEC NOS CLGSLVPPRK LQGRPSPGPP APEQLLSQAR DFINQYYSSI KRSGSQAHEQ 148
BAEC NOS CLGSLVLPRK LQIRPSPGPP PAEQLLSQAR DFINQYYSSI KRSGSQAHEE 150
PKA
HUVEC NOS RLQEVEAEVA ATGTYQLRES ELVFGAKQAW RNAPRCVGRI QWGKLQVFDA 198
BAEC NOS RLQEVEAEVA STGTYHLRES ELVFGAKQAW RNAPRCVGRI QWGKLQVFDA 200
HUVEC NOS RDCRSAQEMF TYICNHIKYA TNRGNLRSAI TVFPQRCPGR GDFRIWNSQL 248
BAEC NOS RDCSSAQEMF TYICNHIKYA TNRGNLRSAI TVFPQRAPGR GDFRIWNSQL 250
HUVEC NOS VRYAGYRQQD GSVRGDPANV EITELCIQHG WTPGNGRFDV LPLLLQAPDE 298
BAEC NOS VRYAGYRQQD GSVRGDPANV EITELCIQHG WTPGNGRFDV LPLLLQAPDE 300
HUVEC NOS PPELPLLPPE LVLEVPLEHP TLEWFAALGL RWYALPAVSN MLLEIGGLEF 348
BAEC NOS APELPVLPPE LVLEVPLEHP TLEWFAALGL RWYALPAVSN MLLEIGGLEF 350
HUVEC NOS PAAPFSGWYM STEIGTRNLC DPHRYNILED VAVCMDLDTR TTSSLWKDKA 398
BAEC NOS SAAPFSGWYM SREIGTRNLC DPHRYNILED VAVCMDLDTR TTSSLWKDKA 400
HUVEC NOS AVEINVAVLH SYQLAKVTIV DHHAATASFM KHLENEQKAR GGCPADWAWI 448
BAEC NOS AVEINLAVLH SFQLAKVTIV DHHAATVSFM KHLDNEQKAR GGCPADWAWI 450
HUVEC NOS VPPISGSLTP VFHQEMVNYF LSPAFRYQPD PWKGSAAKGT GITRKKTFKE 498
BAEC NOS VPPISGSLTP VFHQEMVNYI LSPAFRYQPD PWKGSATKGA GITRKKTFKE 500
CALCIUM
HUVEC NOS VANAVKISAS LMGTVMAKRV KATILYGSET GRAQSYAQQL GRLFRKAFDP 548
BAEC NOS VANAVKISAS LMGTLMAKRV KATILYASET GRAQSYAQQL GRLFRKAFDP 550
CALMODULIN
HUVEC NOS RVLCMDEYDV VSLEHETLVL VVTSTFGNGD PPENGESFAA ALMEMSGPYN 598
BAEC NOS RVLCMDEYDV VSLEHEALVL VVTSTFGNGD PPENGESFAA ALMEMSGPYN 600

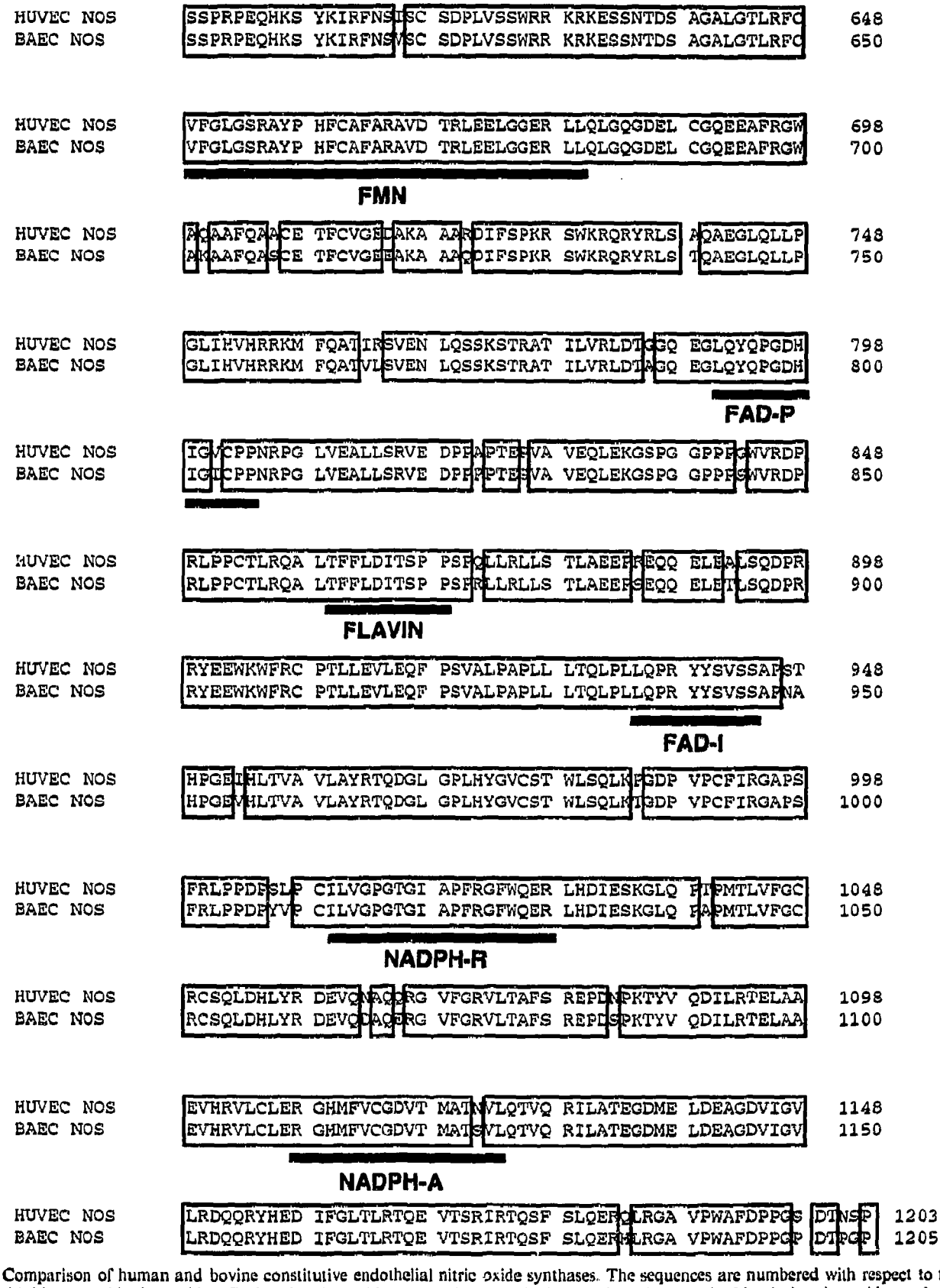


Fig. 1. Comparison of human and bovine constitutive endothelial nitric oxide synthases. The sequences are numbered with respect to the first potential initiator methionine residue. Deduced amino acid sequence is denoted with the single letter code. Identical amino acids are framed. A consensus sequence for N-terminal myristoylation is noted (**MYRIS**). A potential phosphorylation site for cyclic AMP-dependent protein kinase (**PKA**) and a putative binding site for calmodulin (**CALCIUM-CALMODULIN**) is indicated. Cofactor consensus sites include; flavin mononucleotide binding (**FMN**), flavin adenine dinucleotide pyrophosphate (**FAD-P**), flavin-flavin electron transport (**FLAVIN**), FAD-isoalloxazine (**FAD-I**), nicotine adenine dinucleotide phosphate ribose (**NADPH-R**) and NADPH adenine (**NADPH-A**).

synthase represented a novel member of a family of constitutive calcium/dependent NO synthases [12].

Endothelium-dependent vasorelaxation is suggested to be impaired in important diseases of the human vascular wall; atherosclerosis, diabetes, and hypertension, among others. The ability to dissect the pathophysiologic basis of these defects at the molecular level has been hampered by the lack of molecular clones for the human constitutive endothelial NO synthase. The isolation of cDNA clones encoding the human constitutive endothelial NO synthase is reported herein.

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Cell culture media were from Gibco, Grand Island, NY; cell culture plates from Costar, Cambridge, MA; human recombinant tumor necrosis factor-alpha (rHuTNFα, spec. act. $9.8 \times 10^6$ U/mg) was a gift of Knoll Pharmaceuticals, Whippany, NJ; DNA restriction and modifying enzymes from Pharmacia LKB, Baie d'Urfe, Quebec; Taq DNA polymerase from Perkin-Elmer Cetus, Emeryville, CA; all other reagents were as described [13].

### 2.2. *cDNA clones*

Recombinant bacteriophage clones were isolated by plaque hybridization from a random-primed HUVEC λgt11 cDNA library with cDNA probes labelled with [α-$^{32}$P]dCTP (New England Nuclear, Wilmington, DE, spec. act. 3000 Ci/mmol) by the random-primer method. Initial cDNA clones were isolated using a 400 bp bovine endothelial cDNA encoding amino acids 516 to 654 (bovine mid-region probe) of bovine endothelial NO synthase [12]. A total of 5 cross-hybridizing phage clones were isolated from $5.0 \times 10^5$ plaques screened. To isolate flanking sequences a 360 bp HUVEC cDNA encoding amino acids 1105 to 1203 and 3′ non-coding sequence (3′ probe), and a 360 bp HUVEC cDNA encoding amino acids 13 to 134 (5′ probe) of human endothelial NO synthase were utilized. In total, 10 and 15 cross-hybridizing phage clones were isolated from screens of $5.0 \times 10^5$ plaques, respectively. Cross-hybridizing clones were plaque-purified, isolated by preparative agarose gel electrophoresis, subcloned into the *Eco*RI multiple cloning site of pBluescript I SK(−) (Stratagene, San Diego, CA), and subjected to dideoxy chain-termination sequence analysis with Sequenase 2.0 (US Biochemical Corp., Cleveland, OH). Oligonucleotide primers were synthesized on a Pharmacia LKB Gene Assembler Plus. Deduced nucleotide sequences were confirmed on both strands of multiple overlapping phage clones.

### 2.3. *Rapid amplification of 5′ cDNA end (RACE protocol)*

Modifications of the RACE protocol were utilized to isolate 50 nucleotides of 5′ coding sequence [14]. Briefly, human umbilical vein endothelial cells (HUVEC) total cellular RNA (5 μg) was reverse transcribed with an anti-sense gene-specific primer (5′- GCG GGG AAC TCC AGG CCC -3′) by murine moloney leukemia virus reverse transcriptase (Gibco BRL/Life Technologies). RNase H (BRL) was utilized to remove mRNA [15]. First stand cDNA product was tailed with dATP by terminal deoxynucleotide transferase (BRL). First round PCR amplification was performed in a total volume of 50 μl for 35 cycles at 45°C (Perkin-Elmer Cetus 480 thermocycler) (sense primers 5′- GAC TCG AGT CGA CGA ATT CAA $T_{(17)}$ -3′, 2 pmol; 5′- GAC TCG AGT CGA CGA ATT CAA -3′, 25 pmol; anti-sense gene-specific primer 5′- AAT TTC CAG CAG CAT GTT -3′, 25 pmol). A second round of amplification was performed for 35 cycles at 55°C in a total volume of 50 μl (sense primer 5′- GAC TCG AGT CGC CGA ATT CAA -3′, 25 pmol; antisense gene-specific primer 5′- TTC CCC CAC TGG ATC CGG CCC ACG CAG -3′, 25 pmol). Products of the desired size were isolated with preparative gel electrophoresis, digested with *Eco*RI/*Bam*HI, and subcloned into pBluescript I SK(−). A library of $10^4$ RACE-PCR products, transformed into DH10β electrocompetent cells (BRL), were screened with in situ colony lift hybridization using the above mentioned human 5′ cDNA probe. Multiple positive colonies were subjected to DNA sequence analysis to exclude PCR-associated nucleotide incorporation errors.

### 2.4. *RNA isolation, northern blotting and hybridization*

HUVEC were isolated and maintained as described [16]. Northern analysis was performed as previously published [13]. A 790 bp human endothelial cDNA corresponding to amino acids 380 to 643 (human mid-region probe) was labelled with the random-primer method ($5 \times 10^6$ cpm/ml). Hybridization and washes were performed under conditions of high stringency. Blots were rehybridized with a human β tubulin cDNA under conditions of high stringency (0.75 kb *Eco*RI insert) (ATCC 77044) to control for the amount of RNA loaded per lane. A 1.0 kb *Eco*RI/*Pst*I restriction fragment, corresponding to 3′ coding and non-coding regions of human cytochrome P450 reductase, was also used in Northern analysis. This cDNA was isolated from an *Eco*RI/*Xho*I dT-primed λZAP II human vascular smooth muscle cDNA library. $4.0 \times 10^5$ plaques were screened with the human endothelial 3′ cDNA described above. VCS M13 helper phage allowed in-vitro phagemid rescue following manufacturer's suggestions (Stratagene).

### 2.5. *In situ hybridization*

Tissues were collected at necropsy from an adult human male and adult baboon and fixed in 4% paraformaldehyde with 0.1 M $NaPO_4$ (pH 7.4) for 3–4 h at 4°C, cytoprotected in 15% sucrose-PBS overnight, embedded in OCT, frozen in liquid nitrogen, and stored at −70°C. Cryosections (7–10 μm) were thaw-mounted onto Vectabond-(Vector Laboratories, Burlingame, CA) coated-slides, refrozen, and stored at −70°C with desiccant until use. The above-mentioned human mid-region probe was transcribed with T3 RNA polymerase (Promega) using [α-$^{35}$S]UTP (Amersham, Arlington Heights, IL, spec. act. 1200 Ci/mmol) to produce full-length anti-sense transcripts. Experiments were controlled by hybridizing sections with the same cDNA probe transcribed in the sense orientation with T7 RNA polymerase (Promega). Studies were performed as previously described [17]. Briefly, cryosections were pretreated with paraformaldehyde, proteinase K (Sigma Chemical, St. Louis, MO), and prehybridized in 100 μl hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.02% polyvinylpyrrolidine, 0.02% ficoll, 0.02% bovine serum albumin, 10% dextran sulfate, and 10 mM dithiothreitol) at 42°C. Serial sections were hybridized with $6.0 \times 10^5$ cpm [$^{35}$S]riboprobe/slide at 55°C. After hybridization, the sections were washed with 2 ×SSC (1 × SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0) with 10 mM β-mercaptoethanol and 1 mM EDTA, treated with RNase A (Sigma), again washed in the same buffer, followed by a high stringency wash in 0.1 × SSC with 10 mM β-mercaptoethanol and 1 mM EDTA, at 55°C. Slides were then washed in 0.5 × SSC and dehydrated in graded alcohols containing 0.3 M $NH_4Ac$. Sections were dried, coated with NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY), and exposed in the dark at 4°C for 4 to 10 weeks. After development, the sections were counterstained with hematoxylin and eosin to aid in cell identification.

## 3. RESULTS AND DISCUSSION

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) M95296. Nucleotide coding sequences are 90% identical comparing human and bovine endothelial constitutive NO synthases. The methionine-encoding sequences near the 5′ end of the open reading frame have neighbouring nucleotide sequences consistent with eukaryotic translational start

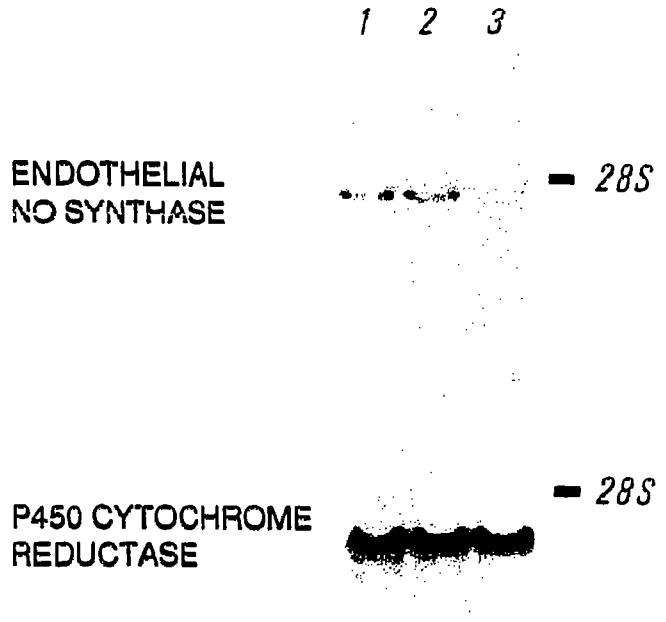


Fig. 2. Northern blot analysis of constitutive human endothelial nitric oxide synthase; effect of TNF-α. In the upper panel total cellular RNA (10 μg/lane) isolated from HUVEC; control (lane 1), TNF-α (100 ng/ml, 4 h) (lane 2), TNF-α (100 ng/ml, 24 h) (lane 3). Nylon membranes were probed with a $^{32}$P-labelled constitutive human endothelial NO synthase cDNA. In the lower panel membranes were reprobed with a $^{32}$P-labelled human cytochrome P450 reductase cDNA.

sites AGTAAC(ATG)G [18]. The deduced amino acid sequence of human endothelial NO synthase, comprising 1203 amino acids, predicts a protein with a mw of 133 kDa. This is in close agreement with the predicted molecular mass of bovine endothelial NO synthase (1205 amino acids) [12] as well as the molecular weight of 135 kDa suggested from purification of the enzyme from bovine endothelial cells [11]. Indicated in Fig. 1 is an amino acid sequence comparison of the human and bovine endothelial NO synthases. The sequence is numbered with respect to the first potential initiator methionine codon.

Cellular fractionation has indicated that endothelial NO synthase may be localized to the particulate fraction of endothelial cell preparations [10]. This contrasts with the cytosolic localization of constitutive neuronal and inducible macrophage NO synthases. A consensus motif for N-terminal myristoylation is noted in both human and bovine endothelial sequences [19]. Such a motif is not present in neuronal and macrophage N termini. Binding sites for cofactors and nucleotides are highly conserved between human and bovine endothelial NO synthases, indeed overall amino acid sequence identity is 94%. This contrasts with a predicted amino acid identity of 50 and 60% with murine macrophage and rat neuronal NO synthase, respectively. Constitutive human endothelial NO synthase reveals no significant sequence similarity over the initial 226 residues of neuronal NO synthase [4].

Northern blot analysis (Fig. 2, upper panel) indicated the most prominent mRNA transcript to be 4.6 to 4.8 kb in size. We and others have previously demonstrated that proinflammatory cytokines, such as TNF-α, induce a pathway for NO synthesis in endothelial cells [20,21]. Addition of human recombinant TNF-α (100 ng/ml) for 4 and 24 h failed to increase levels of endothelial NO synthase mRNA transcripts. In fact, such treatment decreased levels of constitutive endothelial NO synthase mRNA when assessed at 24 h ($n$ = 3). NO synthases show a high degree of homology to cytochrome P450 reductase [4]. This is especially evident at the COOH termini of the molecules, regions of putative nucleotide and cofactor interactions. A human cytochrome P450 reductase cDNA was utilized to control for the amount of RNA loaded per lane (Fig. 2, lower panel). Similar results were obtained when blots were reprobed with a human β-tubulin cDNA (results not shown). Therefore, the increase in endothelial NO synthase activity induced by prolonged treatment with TNF-α is likely to involve post-transcriptional modification of NO synthase, induction of co-regulatory processes, or the induction of a distinct endothelial NO synthase isoform.

Anti-sense cRNA probes were utilized under conditions of high stringency for in situ hybridization studies. These studies were performed to assess the cellular expression of endothelial constitutive NO synthase. Shown in Fig. 3A is localization of NO synthase mRNA to vascular endothelium within a human intercostal artery. In Fig. 3B constitutive endothelial NO synthase mRNA was also localized in representative sections of baboon cerebellum solely to endothelial cells of blood vessels and not to neurons. In contrast to findings obtained with anti-sense riboprobes no hybridization signal was detected using a sense riboprobe (data not shown). Endothelial cell identity was confirmed by *Ulex europaeus* lectin immunohistochemistry [17] and in-situ hybridization using a von Willebrand factor cRNA probe on adjacent serial sections [22]. Such studies demonstrated specific hybridization to endothelial NO synthase mRNA and not neuronal NO synthase mRNA.

There are striking areas of regional amino acid homology among the NO synthase family. Functional assessment of putative regulatory and functional domains are under active investigation. For instance, whether N-terminal myristoylation of endothelial NO synthase targets the protein to membranes remains to be determined. Functional expression of a full-length cDNA for human endothelial NO synthase will be an important component of such studies. Bovine endothelial NO synthase cDNAs clearly confer calcium-activated NO synthase activity in a heterologous system [12]. We believe that the marked sequence identity as well as equivalent size and tissue distribution of mRNA transcripts, all

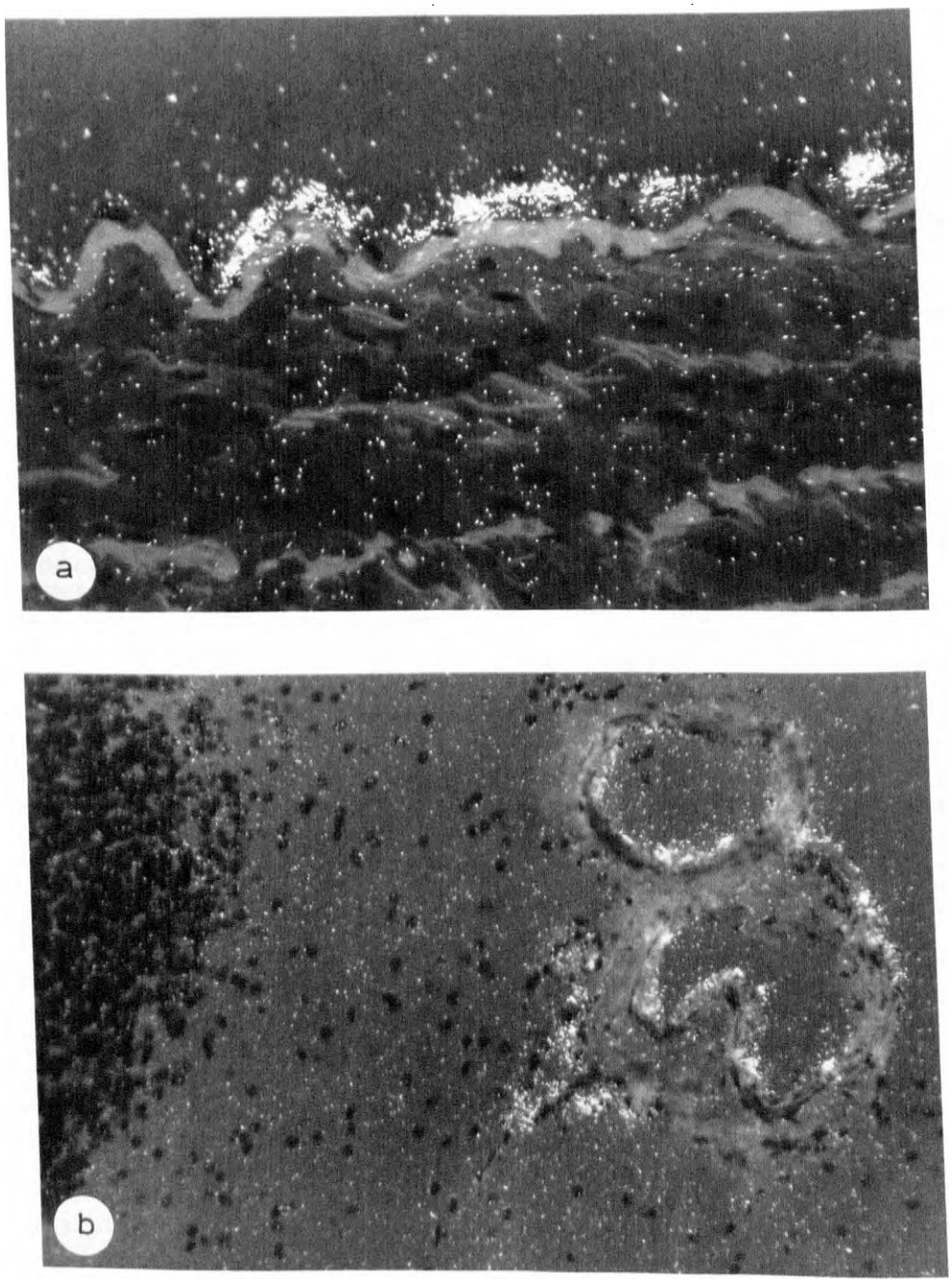


Fig. 3. Representative sections of in situ localization of constitutive endothelial NO synthase mRNA within a human intercostal artery (A) and baboon cerebellum (B). (A) NO synthase mRNA was detected in the endothelial cells of a human intercostal artery by in situ hybridization using an endothelial NO synthase [α-$^{35}$S]UTP-labelled cRNA probe. (B) NO synthase expression was localized within baboon cerebellum solely to endothelial cells of blood vessels and not neuronal tissue. Similar results were obtained on serial sections hybridized to a human von Willebrand factor [α-$^{35}$S]UTP-labelled cRNA probe. Serial sections hybridized with a corresponding sense human endothelial NO synthase [α-$^{35}$S]UTP-labelled cRNA probe did not show hybridization. Autoradiographs were photographed using a combination of bright field illumination and polarized epi-illumination. A, 1250×; B, 416×.

suggest that these cDNA clones encode the human and bovine constitutive endothelial isoforms. Indeed, the current study suggests that isoforms may be highly conserved across species. The isolation of human sequences may now allow direct assessment of endothelial NO synthase mRNA expression in important vascular diseases of man and permit studies of genomic organization and transcriptional regulation.

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