

## Polymerase Chain Reaction Localization of Constitutive Nitric Oxide Synthase and Soluble Guanylate Cyclase Messenger RNAs in Microdissected Rat Nephron Segments

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### Abstract

Stimulation of the release of nitric oxide (NO) in the kidney has been shown to result in renal hemodynamic changes and natriuresis. NO is a potent stimulator of soluble guanylate cyclase, leading to an increase of cyclic GMP. The precise localization of NO synthase and soluble guanylate cyclase in the renal structure is not known. In this study, the microlocalization of mRNAs coding for constitutive NO synthase and soluble guanylate cyclase was carried out in the rat kidney, using an assay of reverse transcription and polymerase chain reaction in individual microdissected renal tubule segments along the nephron, glomeruli, vasa recta bundle, and arcuate arteries. A large signal for constitutive NO synthase was detected in inner medullary collecting duct. Small signals were detected in inner medullary thin limb, cortical collecting duct, outer medullary collecting duct, glomerulus, vasa recta, and arcuate artery. Soluble guanylate cyclase mRNA is expressed largely in glomerulus, proximal convoluted tubule, proximal straight tubule, and cortical collecting duct, and in small amounts in medullary thick ascending limb, inner medullary thin limb, outer medullary collecting duct, inner medullary collecting duct, and the vascular system. Our data demonstrate that NO can be produced locally in the kidney, and that soluble guanylate cyclase is widely distributed in glomerulus, renal tubules, and the vascular system. (*J. Clin. Invest.* 1992; 90:659–665.) Key words: cyclic guanosine monophosphate • glomerulus • renal tubule • reverse transcription

### Introduction

Vascular endothelial cells produce several vasoactive mediators that modulate vascular smooth muscle tone in response to a variety of stimuli (1). One such mediator, endothelium-de-

rived relaxing factor (EDRF),<sup>1</sup> has been identified to be at least in part nitric oxide (NO) derived from the guanide nitrogen atom(s) of L-arginine (L-Arg) (2). NO is a potent stimulator of soluble guanylate cyclase, leading to an increase of cyclic guanosine monophosphate (cGMP) (1). However, the importance of NO in renal function remains undefined.

The intrarenal infusion of acetylcholine (ACh), which induces production of EDRF, produces vasodilation accompanied by diuresis and natriuresis (3, 4). ACh infusion induces a dose-dependent increase in urinary cGMP excretion, associated with a marked increase in renal blood flow, but no change in glomerular filtration rate (4). Treatment with *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA), an inhibitor of endogenous NO synthesis, prevents the ACh-induced increase in urinary cGMP excretion as well as the systemic and renal hemodynamic effects of ACh (4). Biondi et al. (5) demonstrated the existence of EDRF in the renal medulla. They showed that cGMP is enhanced in renal medulla slices with ACh, and this effect is abolished by L-NMMA (5). L-NMMA induces a significant reduction in medullary oxygenation, which is almost entirely reversed by L-Arg, suggesting that NO may normally participate in medullary blood flow (6). However little is known about the production and action sites of NO in renal tissue.

Ishii et al. (7) showed that pig kidney epithelial cells, LLC-PK1, produce EDRF and increase the cGMP level via soluble guanylate cyclase. They also reported that endothelin increases the cGMP level in LLC-PK1 cells via the formation of EDRF-like substance (8). L-NMMA inhibits cGMP responses to endothelin (8). These data suggest that renal tubules may have NO synthase and soluble guanylate cyclase. However, the precise localization of NO synthase and soluble guanylate cyclase in renal tubules is not known.

At least two types of NO synthase have been identified. One type is constitutive and is responsible for the release of NO for physiological transduction purposes, whereas the other is induced by cytokines and releases NO as part of the immunologi-

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1. *Abbreviations used in this paper:* ACh, acetylcholine; ANP, atrial natriuretic peptide; CCD, cortical collecting duct; EDRF, endothelium-derived relaxing factor; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; iIMCD and tIMCD, initial and terminal parts, respectively, of inner medullary collecting duct; IMTL, inner medullary thin limb; L-NMMA, *N*<sup>G</sup>-monomethyl-L-arginine; MTAL, medullary thick ascending limb; OMCD, outer medullary collecting duct; PCR, polymerase chain reaction; PCT, proximal convoluted tubule; PST, proximal straight tubule; RT, reverse transcription.

cal response (9). The constitutive NO synthase was found in the brain and vasculature (9). The presence of this enzyme is not known in the kidney. Bredt et al. (10) have successfully cloned constitutive NO synthase from the rat brain. The cDNA reveals recognition sites for NADPH, flavin adenine dinucleotide, flavin mononucleotide, and calmodulin, as well as phosphorylation sites, indicating that the synthase is regulated by many different factors (10). Soluble guanylate cyclase can be activated by NO and it synthesizes cGMP (11). This enzyme exists as a heterodimer of 70- and 82-kD subunits (12). Recently a new form of 70-kD subunit was cloned, which is more abundant in the kidney (13).

Recently, Moriyama et al. (14) and Terada et al. (15) introduced a new method for measurement of relative levels of specific mRNA in single microdissected renal tubules, using the polymerase chain reaction (PCR) coupled to reverse transcription (RT-PCR). By using this technique, relative quantitation of mRNA coding for peptide hormone receptor was performed from only 2-mm lengths of renal tubules (15).

In the present study, we employed the RT-PCR technique for the precise localization of constitutive NO synthase and soluble guanylate cyclase mRNAs in microdissected renal tubules, glomeruli, arcuate arteries, and vasa recta bundles.

## Methods

**Renal tubule microdissection.** Male Sprague-Dawley rats weighing 75–100 g were killed by decapitation. The left kidney was perfused initially with 10 ml of ice-cold dissection solution (solution 1) and then with 10 ml of the same solution containing 1 mg/ml collagenase (collagenase solution) (type I, 300 U/mg Sigma Chemical, St. Louis MO) and 1 mg/ml bovine serum albumin (Sigma Chemical Co.). The dissection solution (solution 1) contained the following (in mM): 135 NaCl, 1 Na<sub>2</sub>SO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 5 KCl, 2 CaCl<sub>2</sub>, 5.5 glucose, and 5 HEPES (pH 7.4). The left kidney was removed and a coronal section was made that contained the entire corticopapillary axis. This section was cut into four pieces: cortex, outer medulla, outer 25% of inner medulla, and inner 75% of inner medulla. These pieces were transferred into individual tubes containing 1 ml of the same collagenase solution used to perfuse the kidney. The tubes were incubated for 30 min (cortex and outer medulla) or 40 min (inner medulla) at 37°C in a shaking water bath. The solutions were bubbled with 100% oxygen during these incubations. Then tissues were transferred to the dissection solution containing 10 mM vanadyl ribonucleotide complex (Life Technologies, Inc., Gaithersburg, MD), a potent RNase inhibitor, and they were placed on ice until microdissection.

Following previously described techniques (15), we microdissected the following structures: glomeruli (Glm), proximal convoluted tubule (PCT), proximal straight tubule (PST), cortical collecting duct (CCD), arcuate artery, outer medullary collecting duct (OMCD), outer medullary thick ascending limb (MTAL), inner medullary thin limb (IMTL), initial part of inner medullary collecting duct (iIMCD), terminal part of inner medullary collecting duct (tiMCD), and outer medullary vasa recta bundle. In the dissection of glomeruli, care was taken to remove all attached pieces of tubule, as well as afferent and efferent arterioles.

The lengths of the dissected tubules were measured by using a calibrated eyepiece micrometer. Generally, five glomeruli or 2-mm lengths of the renal tubule segment or arcuate artery were transferred to each assay tube, as indicated. Because it was impossible to separate individual vasa recta in a microdissected vascular bundle, we transferred entire bundles into the assay tubes for the RT-PCR procedure.

Microdissected structures were transferred to separate wash-dishes, which contained 10 ml of the dissection solution. Microdissected tubules, glomeruli, arcuate arteries, and vasa recta bundles were washed free of contaminating debris and vanadyl ribonucleotide complex. These structures were transferred into the appropriate RT-PCR reac-

tion tube, which contained 10  $\mu$ l of ice-cold dissection solution containing > 1 U/ $\mu$ l of human placental RNase inhibitor (Boehringer Mannheim, GmbH, Mannheim, FRG) and 5 mM dithiothreitol (DTT, Cleland's reagent).

**Preparation of RNA from rat tissues.** The brain, kidney, liver, and skeletal muscle were freshly isolated from 75–100-g male Sprague-Dawley rats. RNA was isolated from the tissues by guanidinium thiocyanate followed by centrifugation in cesium chloride solutions (16). Total RNA 1  $\mu$ g was used for RT-PCR procedure.

**RT.** RT was performed using a cDNA synthesis kit (Boehringer Mannheim GmbH). The RNase-inhibitor solution was removed and 9  $\mu$ l of 2% Triton X-100, containing > 1 U/ $\mu$ l RNase-inhibitor, 5 mM DTT, and 3 U/ $\mu$ l RNase-free DNase (Pharmacia Fine Chemicals, Piscataway, NJ) was added to permeabilized the cells, followed by incubation at 37°C for 30 min to digest the genomic DNA. The samples were then heated to 90°C for 5 min to inactivate the DNase. RT components were added to the reaction tubes as described previously (15): 4  $\mu$ l of buffer I, 1  $\mu$ l of RNase inhibitor, 2  $\mu$ l of deoxynucleotide mixture, 2  $\mu$ l of random primer, and 2  $\mu$ l of avian myeloblastoma virus reverse transcriptase. Reaction tubes were incubated at 42°C for 60 min in a Programmed Tempcontrol System (Astec, Tokyo, Japan). At the end of the incubation period, the reaction was stopped by heating at 90°C for 5 min. This heat treatment also denatures RNA-cDNA hybrids and inactivates the reverse transcriptase.

**PCR.** PCR was performed using the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer Cetus, Norwalk, CT), with rat constitutive NO synthase specific primers prepared on a DNA synthesizer (Applied Biosystems, Inc., Tokyo, Japan). We designed specific primers with high calculated  $T_m$  (> 75°C), which allows a stringent annealing temperature in the PCR cycle. Constitutive NO synthase primer 1 (antisense) was defined by bases 3038–3069, and primer 2 (sense) encompassed bases 2456–2487 (10). The sequence of primer 1 was 5'-GCCGGATCCTCCAGGAGGGTGTCCACCGCATG-3', primer 2 was 5'-CCGGAATTGCAATACCAGCCTGATCCATG-GAA-3'. The predominant cDNA amplification product was predicted to be 614 bp in length (the distance between primers plus primer length). A third oligonucleotide was synthesized to serve as an amplification product-specific probe. This oligonucleotide (sense) included bases 2866–2885 of the cDNA, positioned between primer 1 and primer 2. The sequence of this oligonucleotide probe was 5'-GAGGA-GACGCTGTTGAATCG-3'.

Soluble guanylate cyclase primer 1 (antisense) was defined by bases 1542–1564, and primer 2 (sense) encompassed bases 1235–1258 (13). The sequence of primer 1 was 5'-GCATGGCTTTCAACGGG-TACTGG-3', primer 2 was 5'-GCGTGTCTTTCCAATCACT-GGC-3'. The predominant cDNA amplification product was predicted to be 330 bp in length. A third oligonucleotide was synthesized to serve as an amplification product-specific probe. This oligonucleotide (sense) included bases 1347–1366 of the cDNA, positioned between primer 1 and primer 2. The sequence of this oligonucleotide probe was 5'-GCAGCAGAATTTGAAACATG-3'.

RT and PCR of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) served as a positive control. The primers were defined by the following cDNA base sequence (17): primer 1 (antisense), bases 795–814, sequence, 5'-AGATCCACAACGGATACATT-3'; primer 2 (sense), bases 506–525, sequence, 5'-TCCCTCAAGATTGTGAC-CAA-3'. The predominant cDNA amplification product was predicted to be 309 bp in length. When we used GAPDH as an internal control primers, after reverse transcription, we divided 20- $\mu$ l samples into 15  $\mu$ l for constitutive NO synthase or soluble guanylate cyclase and 5  $\mu$ l for GAPDH. The volume was adjusted to 20  $\mu$ l with sterile water. Then we ran parallel PCR reactions with each set of primers.

To carry out the PCR, 80  $\mu$ l of a PCR Master mix was added to each tube directly. 50 pmol of each of primers 1 and 2 was used per reaction. Deoxynucleotides were added to a final concentration of 0.20 mM each. Reaction buffer (10 $\times$ ) was diluted (1/10) to have a final composition of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.001% (wt/vol) gelatin, and 2.5 U of Taq DNA polymerase.

100  $\mu$ l of mineral oil was overlaid to prevent evaporation during the high temperature incubations. The tubes were placed in a Programmed Tempcontrol System programmed as follows. First, incubation at 94°C for 3 min (initial melt). Then, 30 cycles of the following sequential steps: 94°C for 1 min (melt); 65°C for 1 min (anneal) for NO synthase, 60°C for 1 min (anneal) for soluble guanylate cyclase; 72°C for 3 min (extend). Last, incubation at 72°C for 7 min (final extension).

**PCR product analysis.** 90  $\mu$ l of the total reaction volume was ethanol precipitated (16). The PCR products were size-fractionated by agarose gel electrophoresis. After electrophoresis and ethidium bromide staining, DNA bands were visualized with an ultraviolet transilluminator (Funakoshi, Tokyo, Japan).

For Southern blot analysis, gels were denatured, neutralized, and blotted onto a nitrocellulose filter (Funakoshi) essentially as described by Maniatis et al. (16). Blots were baked at 80°C for 4 h to fix the DNA. The synthetic oligonucleotide probes were end labeled with  $^{32}$ P as described previously (15). Prehybridization/hybridization washes were also the same as previously described (15).

To confirm that the PCR products were really constitutive NO synthase and soluble guanylate cyclase cDNAs, the PCR products were sequenced. PCR products of constitutive NO synthase from tIMCD, and soluble guanylate cyclase from PCT, were separated by gel electrophoresis. PCR products of constitutive NO synthase and soluble guanylate cyclase were subcloned into pGEM-3Zf(–) vector (Promega Corp., Madison, WI), then sequenced using the dideoxynucleotide-chain termination reaction of Sanger et al. (18).

**Relative quantitation of mRNA levels from autoradiographs.** The PCR products of constitutive NO synthase were detectable from several nephron segments. The relative amount of PCR products was determined by densitometer scanning of autoradiographs using a laser densitometer (Hoefer Scientific Instruments, Inc., San Francisco, CA). The slit width of densitometer scanning was 4 mm, and the area under the curve was calculated for each scanning. Each RT-PCR included a tIMCD sample. For relative quantitation, we used the densitometry value from 2-mm tIMCD sample as an internal standard (100%), and calculated the percentage of the tIMCD value for each segment.

To test the relationship between the quantity of starting material and that of amplification product as reflected by densitometry values, we compared amplification products from several lengths of tIMCD (0.2–2.0 mm,  $n = 12$ ).

The PCR products of soluble guanylate cyclase were also detectable from several nephron segments. Each RT-PCR included a PCT sample. For relative quantitation, we used the densitometry value from 2-mm PCT samples as an internal standard (100%), and calculated the percentage of the PCT value for each segment.

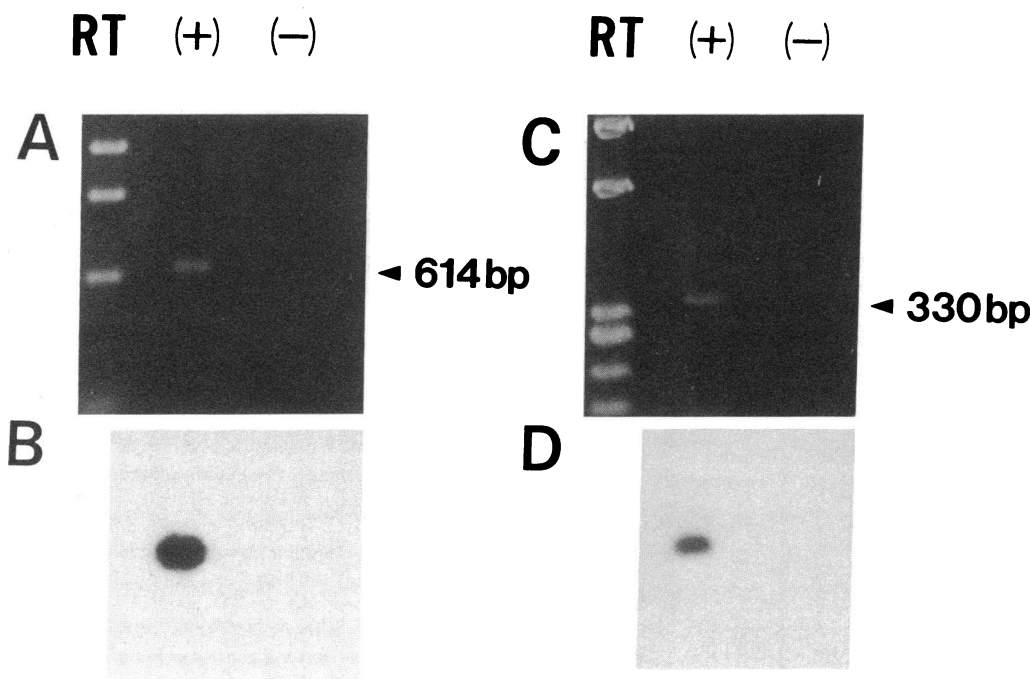
To test the relationship between the quantity of starting material and that of amplification product as reflected by densitometry values, we compared amplification products from several lengths of PCT (0.2–2.0 mm,  $n = 12$ ).

**Statistics.** The results were given as means  $\pm$  SEM. The differences were tested using analysis of variance.  $P < 0.05$  was considered significant.

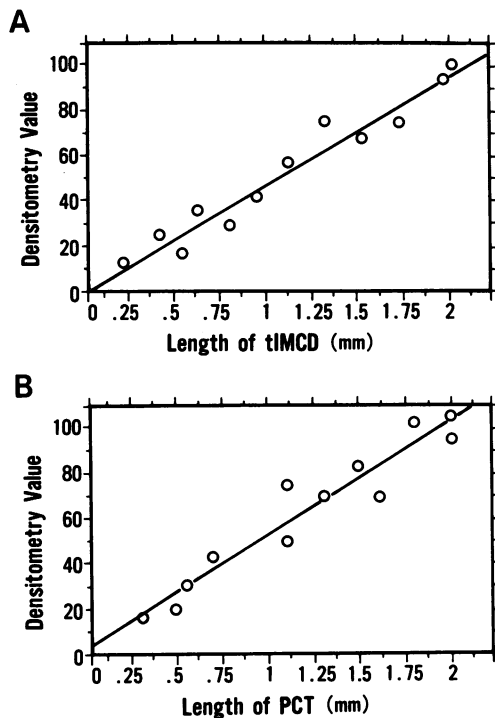
## Results

**Effect of reverse transcription on constitutive NO synthase and soluble guanylate cyclase mRNAs amplification (Fig. 1).** With reverse transcription, we detected a clear single band, which was the predicted size of 614 bp for constitutive NO synthase from tIMCD (Fig. 1, A and B), and we also detected a clear single band, which was the predicted size of 330 bp for soluble guanylate cyclase from PCT (Fig. 1, C and D). When the PCR procedure was carried out in the absence of reverse transcriptase, the 614-bp band and the 330-bp band were not seen and there was no other recognizable band. This indicated that the 614- and the 330-bp bands originated from mRNA, not from genomic DNA, which was presumably digested by DNase treatment. The Southern blots of the gels demonstrated that constitutive NO synthase specific probe binds to the 614-bp product, and soluble guanylate cyclase specific probe binds to the 330-bp product, confirming their identity.

**Relationship between the quantity of starting material and that of amplification product for constitutive NO synthase and soluble guanylate cyclase mRNAs (Fig. 2).** We compared amplification products from several lengths of tIMCD for constitutive NO synthase and PCT for soluble guanylate cyclase, assigning the 2-mm tIMCD or PCT sample as an arbitrary unit of



**Figure 1.** Effect of RT on constitutive NO synthase and soluble guanylate cyclase mRNAs amplification. (A) Ethidium bromide-stained agarose gels for constitutive NO synthase. (B) Autoradiographs of corresponding Southern blots. The blots were probed with a  $^{32}$ P-labeled oligonucleotide that localized between the constitutive NO synthase primers. (C) Ethidium bromide-stained agarose gels for soluble guanylate cyclase. (D) Autoradiographs of corresponding Southern blots. The blots were probed with a  $^{32}$ P-labeled oligonucleotide that localized between the soluble guanylate cyclase primers. In each panel, the left lane is the PCR carried out with RT; the right lane is the PCR carried out without RT.



**Figure 2.** Relationship between the quantity of starting material (tubular length) and the resulting amplification products for constitutive NO synthase and soluble guanylate cyclase mRNAs. (A) An arbitrary value of 100 was assigned to the sample representing 2-mm tIMCD for constitutive NO synthase. (B) An arbitrary value of 100 was assigned to the sample representing 2-mm PCT for soluble guanylate cyclase.

100, respectively. Linear regression analysis showed high correlations between densitometry values and the length of each tubule for constitutive NO synthase ( $r = 0.95$ ) and soluble guanylate cyclase ( $r = 0.93$ ).

**Expression of constitutive NO synthase and soluble guanylate cyclase mRNA in rat tissues (Fig. 3).** To evaluate the expression of the constitutive NO synthase and soluble guanylate cyclase mRNA. We analyzed the brain, kidney, liver, and skeletal muscle by RT-PCR (30 cycle). Large PCR signal for constitutive NO synthase was observed in the brain, and small signal was detectable in the kidney (Fig. 3, A and B). We did not detect NO synthase mRNA from liver or skeletal muscle. PCR signals for soluble guanylate cyclase were detected from the brain, kidney, and liver (Fig. 3, C and D). We did not detect soluble guanylate cyclase mRNA from skeletal muscle.

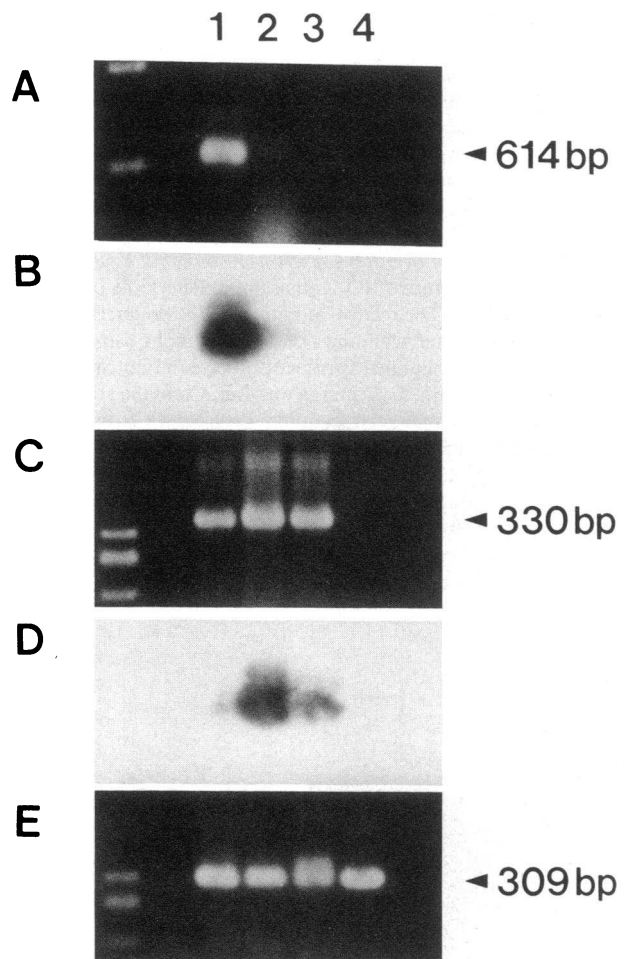
**Distribution of constitutive NO synthase mRNA in micro-dissected structures (Fig. 4).** Each reaction was performed using either 2 mm of tubule length, five glomeruli, a single vasa recta bundle, or a 2-mm length of arcuate artery. A single band of predicted size (614 bp) was consistently found from glomeruli, IMTL, CCD, OMCD, iIMCD, tIMCD, vasa recta bundle, and arcuate artery for constitutive NO synthase (Fig. 4 A). The Southern blots of the gels demonstrated specific binding of the oligonucleotide probe to the 614-bp product (Fig. 4 B). We confirmed that the PCR product is constitutive NO synthase cDNA by sequencing of subcloned PCR product.

Large signals for constitutive NO synthase were detected in iIMCD and tIMCD. Small signals were also found in IMTL, CCD, OMCD, glomerulus, vasa recta, and arcuate artery. Am-

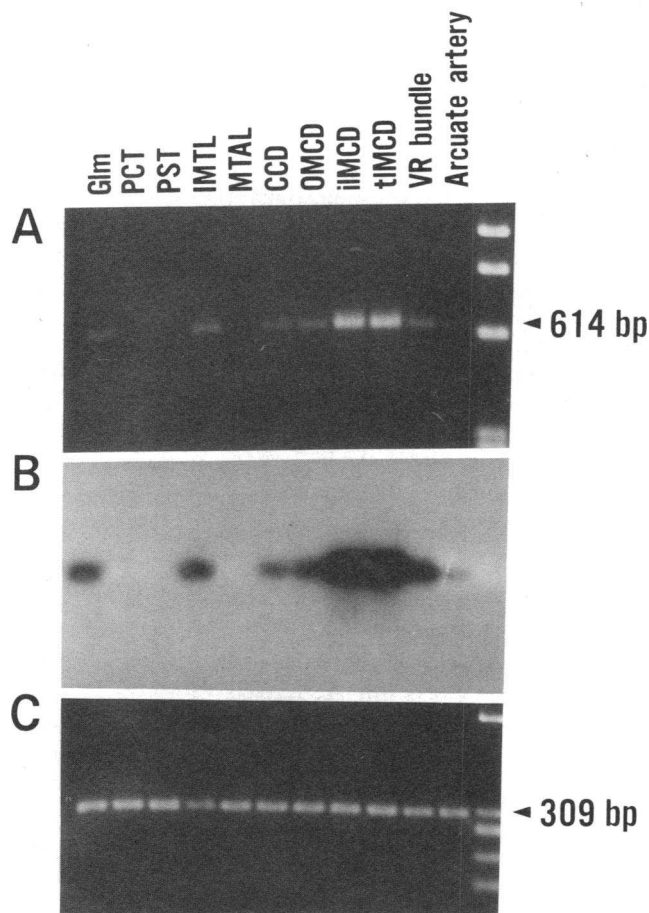
plification product of GAPDH was detected from all renal structures at the predicted size (309 bp) and served as a positive control for the RT-PCR reaction (Fig. 4 C).

**Relative levels of the constitutive NO synthase amplification products among the nephron segments (Fig. 5).** The densitometer values are presented as percentages of the 2-mm tIMCD value measured in the same experiment. In glomerulus, the signal was  $22.1 \pm 5.1\%$  (vs. tIMCD,  $n = 5$ ). In IMTL, the signal was  $12.9 \pm 3.9\%$ . Among collecting ducts segments, the values were: CCD,  $9.1 \pm 2.8\%$ ; OMCD,  $20.6 \pm 7.0\%$ ; and iIMCD,  $95.3 \pm 14.3\%$ .

**Distribution of soluble guanylate cyclase mRNA in micro-dissected structures (Fig. 6).** Each reaction was performed using either 2 mm of tubule length, five glomeruli, a single vasa



**Figure 3.** Expression of constitutive NO synthase and soluble guanylate cyclase mRNA in rat tissues. RT-PCR analysis of total RNA (1  $\mu$ g) from brain (lane 1), kidney (lane 2), liver (lane 3), and skeletal muscle (lane 4). (A) Ethidium bromide-stained agarose gels for constitutive NO synthase. The arrow indicates expected PCR product size (614 bp). (B) Autoradiograms of corresponding Southern blots. The blots were probed with a  $^{32}$ P-labeled oligonucleotide that localized between the PCR primers. (C) Ethidium bromide-stained agarose gels for soluble guanylate cyclase. The arrow indicates expected PCR product size (330 bp). (D) Autoradiograms of corresponding Southern blots. The blots were probed with a  $^{32}$ P-labeled oligonucleotide that localized between the PCR primers. (E) Ethidium bromide-stained agarose gel of GAPDH as positive control primers. The arrow indicates expected PCR product size (309 bp).



**Figure 4.** Detection of constitutive NO synthase mRNA in microdissected renal structures by RT-PCR. (A) Ethidium bromide-stained agarose gels for constitutive NO synthase. The arrow indicates expected PCR product size (614 bp). (B) Autoradiograms of corresponding Southern blots. The blots were probed with a  $^{32}\text{P}$ -labeled oligonucleotide that localized between the PCR primers. (C) Ethidium bromide-stained agarose gel of GAPDH as positive control primers. The arrow indicates expected PCR product size (309 bp). Abbreviations are as defined in footnote 1 and the following: Glm, glomerulus; VR bundle, vasa recta bundle.

recta bundle, or a 2-mm length of arcuate artery. A single band of predicted size (330 bp) was consistently found from glomeruli, PCT, PST, IMTL, MTAL, CCD, OMCD, iIMCD, tIMCD, vasa recta bundle, and arcuate artery (Fig. 6 A). The Southern blots of the gels demonstrated specific binding of the oligonucleotide probe to the 330 bp product (Fig. 6 B). We confirmed that the PCR product is soluble guanylate cyclase cDNA by sequencing of subcloned PCR product. A large signal was consistently found in PCT, PST, glomerulus, and CCD. Small but detectable bands were observed from IMTL, MTAL, OMCD, iIMCD, tIMCD, vasa recta bundle, and arcuate artery. The amplification product of GAPDH was detected from all renal structures at the predicted size (309 bp) and served as a positive control for the RT-PCR reaction (Fig. 6 C).

*Relative levels of the soluble guanylate cyclase amplification products among the nephron segments (Fig. 7).* The densitometer values are presented as percentages of the 2-mm PCT value measured in the same experiment. In glomerulus, the

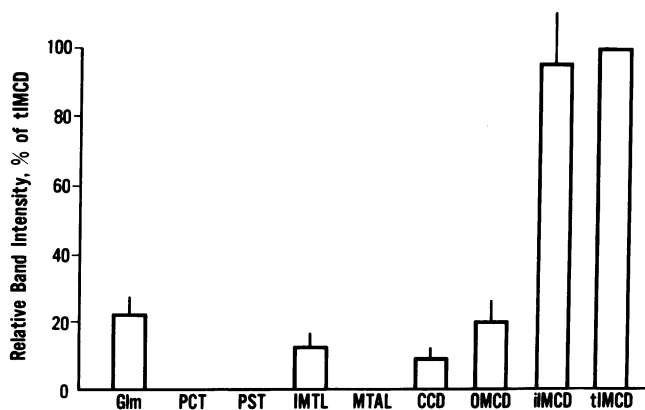
signal was  $65.7 \pm 12.1\%$  (vs. PCT,  $n = 5$ ). In proximal straight tubules, the signal was  $89.2 \pm 15.6\%$ . The value in IMTL was  $2.5 \pm 0.9\%$ . The value in MTAL was  $1.9 \pm 0.5\%$ . Among collecting ducts segments, the values were the following: CCD,  $44.9 \pm 11.0\%$ ; OMCD,  $35.2 \pm 8.1\%$ ; iIMCD,  $18.4 \pm 3.2\%$ ; and tIMCD,  $13.6 \pm 4.0\%$ .

## Discussion

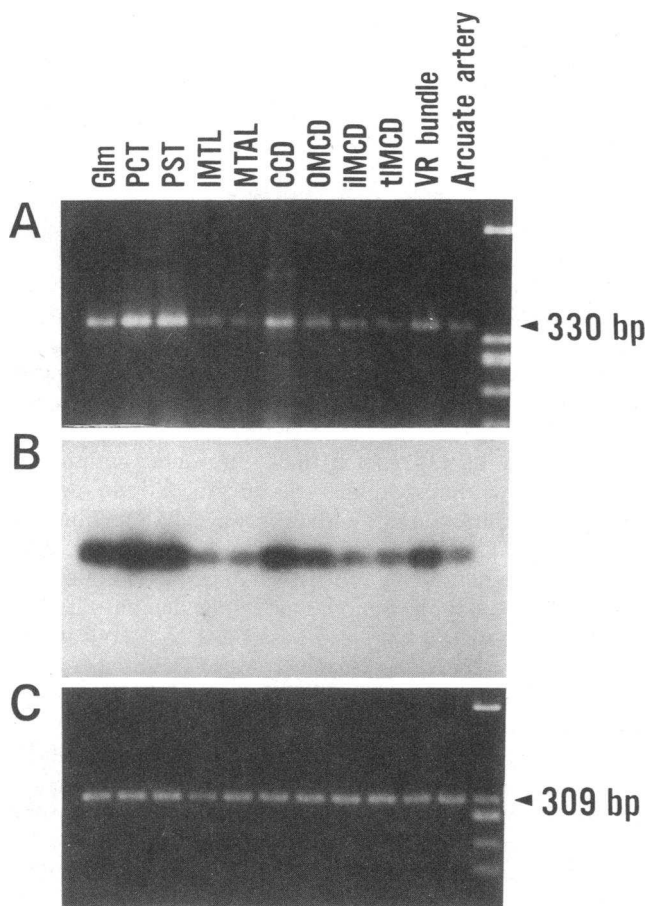
Our study documents that constitutive NO synthase mRNA is present in IMCD, IMTL, OMCD, CCD, glomerulus, and the vascular system, and that soluble guanylate cyclase mRNA is expressed largely in PCT, PST, glomerulus, and CCD, and in small amounts in IMTL, MTAL, OMCD, and the vascular system.

Grandes et al. (3) reported that intravenous infusion of ACh induces natriuresis, diuresis accompanied by an increase in glomerular filtration rate and urinary cGMP excretion. Tolins et al. (4) also demonstrated increased renal plasma flow and renal vasodilation, but no change in glomerular filtration rate, after ACh administration. Our results demonstrate that glomerulus, arcuate artery, and vasa recta have constitutive NO synthase and soluble guanylate cyclase. These data suggest that NO synthesis and activation of soluble guanylate cyclase influence renal hemodynamics.

Our data are the first report to show clearly that constitutive NO synthase and soluble guanylate cyclase are present in glomerulus. An increase of cGMP in glomerulus after administration of sodium nitroprusside was reported by an immunocytochemical study (19) and an isolated glomerulus study (20). Shultz et al. (21) reported that cultured rat mesangial cells alone do not increase the cGMP level in the presence of bradykinin, but when they are coincubated with endothelial cells, cGMP levels in mesangial cells increase after bradykinin administration. This study suggests that endothelial cells can produce NO and that mesangial cells have soluble guanylate cyclase. Marsden et al. (22) reported that tumor necrosis factor- $\alpha$  induces expression of NO synthase in mesangial cells and that NO acts in an autocrine and paracrine fashion to activate soluble guanylate cyclase in mesangial cells. Our data indicate that



**Figure 5.** Relative quantitation of constitutive NO synthase mRNA in renal nephron segments. The values for each segment are expressed as a percentage of the value from 2-mm tIMCD obtained in the same amplification run. The total number of each segment is five experiments from five rats.



**Figure 6.** Detection of soluble guanylate cyclase mRNA in microdissected renal structures by RT-PCR. (A) Ethidium bromide-stained agarose gels for soluble guanylate cyclase. The arrow indicates expected PCR product size (330 bp). (B) Autoradiograms of corresponding Southern blots. The blots were probed with a  $^{32}\text{P}$ -labeled oligonucleotide that localized between the PCR primers. (C) Ethidium bromide-stained agarose gel of GAPDH as positive control primers. The arrow indicates expected PCR product size (309 bp).

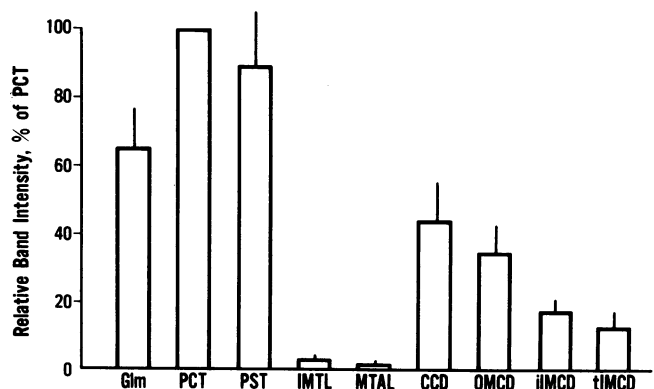
NO can be synthesized in glomerulus and that NO stimulates soluble guanylate cyclase to modulate glomerular function.

Our data demonstrate that mRNAs for constitutive NO synthase and soluble guanylate cyclase are detected in arcuate artery. This suggests that NO can be synthesized and that NO activates soluble guanylate cyclase in arcuate artery. L-NMMA infusion inhibits basal and ACh-stimulated release of NO in renal cortical vasculature and decreases renal cortical blood flow (23). Thus, endogenous NO and cGMP could play an important role in the local regulation of renal cortical blood flow.

As indicated in Fig. 4, a large amount of constitutive NO synthase mRNA is present in IMCD, and a small amount is detected in thin limbs and vasa recta in inner medulla. Soluble guanylate cyclase mRNA distributes largely in vasa recta and in small amounts in IMCD and IMTL, as shown in Fig. 6. The function of NO synthase and soluble guanylate cyclase in medulla was reported to increase medullary blood flow and maintain medullary  $\text{Po}_2$  (6). L-NMMA induces a significant reduction in medullary blood flow, which is almost entirely reversed

by L-Arg, suggesting that NO may normally participate in medullary hemodynamics (6). Our data show that a large amount of constitutive NO synthase mRNA is detected in IMCD. With the presence of constitutive NO synthase, NO can be produced in IMCD and might have some action in IMCD, IMTL, and vasa recta via an autocrine or paracrine fashion. Vasa recta possess a large amount of soluble guanylate cyclase mRNA. NO in medulla could increase the cGMP level in vasa recta and control medullary blood flow. Descending vasa recta are enveloped by smooth muscle cells in their proximal portions, and more distally are encircled by pericytes capable of contractile function (24). This abundance of smooth muscle cells along the medullary vasculature, flanked by a vast surface of endothelial cells within the vasa recta, could represent an important site for NO release and action (6). In IMCD, a soluble guanylate cyclase mRNA level is detectable, but relatively low. IMCD is known as the target site of atrial natriuretic peptide (ANP). ANP receptor/guanylate cyclase mRNA was reported to be present in IMCD, and ANP administration increases the cGMP level in IMCD (15). A microperfusion study in IMCD showed that cGMP administration inhibits water permeability (25). Both pathways of cGMP production, i.e., soluble guanylate cyclase and ANP receptor/guanylate cyclase, could influence water transport in IMCD.

Among renal tubule segments, constitutive NO synthase mRNA is detected mainly in IMCD, and in small amounts in IMTL, CCD and OMCD. Large signals of soluble guanylate cyclase are detected in PCT, PST, and CCD, small signals are found in MTAL, IMTL, OMCD, and IMCD. The functions of NO synthase and soluble guanylate cyclase are poorly understood in renal tubules. EDRF was reported to inhibit transport in CCD via a cGMP-dependent mechanism (26). Ishii et al. (7) reported that oxytocin and bradykinin increase the cGMP level through activation of soluble guanylate cyclase in LLC-PK1 cells. Reduction of the cGMP level by L-NMMA indicates that this reaction is mediated by NO (7). They speculated that NO is produced in LLC-PK1 cells and acts as a paracrine or autacoid to regulate cGMP formation (7). Although we could not detect constitutive NO synthase mRNA in proximal tubule, other types of NO synthase might be expressed in these segments (27). NO produced in glomerulus or the vascular system might



**Figure 7.** Relative quantitation of soluble guanylate cyclase mRNA in renal nephron segments. The values for each segment are expressed as a percentage of the value from 2-mm PCT obtained in the same amplification run. The total number of each segment is five experiments from five rats.



stimulate soluble guanylate cyclase in proximal tubules. In a microperfusion study, cGMP was reported to inhibit water transport in proximal straight tubule in the presence of angiotensin II (28). Thus, cGMP synthesized via not only ANP receptor/guanylate cyclase but also soluble guanylate cyclase could control proximal tubular function.

We chose a simple approach to relative quantitation of PCR products rather than any of the complicated alternatives, such as competitive PCR (29) and PCR-aided transcript titration assay (30). We believe that our method gives a relative measure of the amounts of constitutive NO synthase and soluble guanylate cyclase mRNAs initially present in the cells. This conclusion is based on the observation that there is an approximately linear relationship between the length of tubule used for the assay (i.e., the amount of starting material) and the value obtained from densitometry of the amplification product band.

In summary, large signals for constitutive NO synthase are detected in IMCD. Small signals are seen in IMTL, CCD, OMCD, glomerulus, vasa recta, and arcuate artery. Soluble guanylate cyclase mRNA is expressed largely in glomerulus, PCT, PST, CCD, and a small amount in MTAL, IMTL, OMCD, IMCD, and the vascular system. Our data indicate that NO can be produced locally in the kidney, and NO activates soluble guanylate cyclase, which is widely distributed in the kidney, in autocrine and paracrine fashions.

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