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Localization of Erythropoietin mRNA in the Rat Kidney by Polymerase Chain Reaction

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Abstract Erythropoietin (Epo) is a glycoprotein secreted by kidney cells which plays an important role in the regulation of erythropoiesis. Localization of the Epo production by immunohistochemical studies and in situ hybridization has not been definitively established and is still a matter of controversy. Epo and glyceraldehyde 3-dehydrogenase (GAPDH) mRNA levels were determined in total RNA isolated from control and $CoCl_2$-treated rats using a coupled reverse transcriptase/polymerase chain reaction method (RT/PCR). As indicated by the amount of amplification product, Epo mRNA levels were several-fold higher in $CoCl_2$-treated rat kidney. In contrast, GAPDH mRNA levels were similar in control and $CoCl_2$-treated rats. This RT/PCR method was also used to assess the level of Epo and GAPDH mRNA in microdissected nephron segments. All nephron segments tested lacked any detectable levels of Epo mRNA in either control or $CoCl_2$-treated rats. On the other hand, peritubular cells (capillary fraction: afferent/efferent arteriole, vasa recta) were the only cells where the Epo mRNA was detected. Using a specific primer for GAPDH, the RT/PCR method could identify GAPDH mRNA in all microdissected nephron segments where the Epo mRNA was not expressed. Thus, a combination of microdissected nephron segments and RT/PCR enabled us to detect GAPDH mRNA populations in all nephron segments, whereas the failure to detect Epo mRNA in all segments but the capillary fraction, is due to the specific and localized expression of the Epo gene to this fraction.

Key words: gene expression, microdissection, nephron segments, endothelial cells, in situ hybridization, erythropoiesis

Erythropoietin (Epo) is the hormone regulating erythropoiesis in mammals. The major source of Epo in adult mammals has been localized in the kidneys [1]. In adult mice, the kidneys are the source of about 90% of the total Epo production [2]. Since 1957, the nature of the renal Epo-producing cells is a matter of controversy. Studies in whole kidney, cultured glomeruli, and mesangial cells [5] using the immunofluorescence technique demonstrated a glomerular origin for the Epo-producing cells [3–5]. On the other hand, an extraglomerular origin of Epo-producing cells, i.e., tubular or interstitial, is suggested by studies in renal tissue fractions from hypoxic rats [6,7]. Furthermore, using in situ hybridization, we demonstrated that the renal Epo-producing cells are in a peritubular location [8] and are most likely endothelial cells. Using the same technique, these results were confirmed by other investigators [9]. A study by Maxwell et al. [10] using in situ hybridization and immunohistochemistry showed a tubular origin, thus leaving the debate still open. The methods employed in those studies are limited by their sensitivity threshold. As well, Northern blot analysis requires a large amount of tissue which makes it impractical to use for the detection of gene expression in very small samples of nephron segments and single renal cells. Our purpose was to develop a method for detecting several mRNA(s) in a single nephron segment. To achieve such a task, we combined microdissection of the nephron [11,12] with reverse transcription (RT), a technique already used for enzyme studies of mRNA, followed by polymerase chain reaction (PCR) using specific primers of the target cDNA sequence [13–17]. In this method, two oligonucleotide primers that flank the DNA sequence to be amplified were used in repeated cycles of thermal denaturation, annealing to their complementary sequences, and extension of the target fragments. We used a microadaptation of the acid guanidium thiocyanate/phenol-chloroform RNA extraction [18] for microdissected nephron seg-

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ments (1 mm). Thus, total RNA was directly reverse transcribed into cDNA.

In this study, we also amplified glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is constitutively expressed in many tissues. We used it as a control to demonstrate the efficiency of the PCR reaction within the same nephron segments where Epo transcripts were amplified.

MATERIALS AND METHODS

Microdissection of Nephron Segments

Methods for tissue preparation and microdissection of tubular segments have been described in detail by Morel et al. [11] and Omata et al. [19], and will be presented only briefly here. Male WKY purchased from Charles River (Wilmington, PA) and maintained on food and tap water ad libitum were used at 7–9 weeks of age. Following pentobarbital (40 mg/kg, i.p.) anesthesia, the abdominal cavity was opened and kidneys perfused in situ via the aorta with 25 ml of cold Eagle MEM medium containing 0.075% collagenase, 0.1% hyaluronidase, and 0.1% bovine serum albumin; removed; and sliced with a razor blade along the corticomedullary axis. These strips of renal tissue were incubated in aerated Eagle MEM (GIBCO Labs, Grand Island, NY) medium containing collagenase, hyaluronidase, and bovine serum albumin for 45–55 min at 35°C. Subsequently, the strips were transferred to petri dishes containing Hanks' solution. All remaining procedures were performed at 0°C–4°C.

Single nephrons were microdissected under a stereomicroscope and divided into the following ten segments according to the criteria previously described [19]: 1) glomerulus (G1); 2) early portion of the proximal convoluted tubule (S_1); 3) late portion of the proximal convoluted tubule (S_2); 4) early proximal straight tubule (S_3); 5) medullary portion of the ascending limb of Henle's loop (MAL); 6) cortical portion of the thick ascending limb of Henle's loop (CAL); 7) distal convoluted tubule (DCT); 8) connecting tubule (CNT); this preparation also contains DCT; 9) cortical collecting tubule (CCT); and 10) outer medullary collecting tubule (MCT). Besides these ten fragments, interlobulary arteries, afferent and efferent arterioles were dissected using the same preparation (the fraction of pooled afferent and efferent arterioles may contain vasa recta). Approximately 20–50 of each nephron segment were dissected, pooled and stored at −70°C until used. Figure 1 illustrates the intrarenal localization of the microdissected tubular segments. The duration of the microdissection period was less than 90 min.

We also studied the effect of an inducing agent of Epo transcripts ($CoCl_2$) on localization of Epo transcripts within the nephron segments. Rats were treated with $CoCl_2$ (5 mM/kg body wt) for 4 h, sacrificed and nephron segments were microdissected as described above. This time point was chosen since studies by Abraham et al. [20] and Beru et al. [26] demonstrated that the maximum accumulation of Epo mRNA is achieved between 3–4 h after $CoCl_2$ injection.

Isolation of Total RNA From Rat Kidney Nephron Segments

RNA from nephron segments or total kidney was extracted by the technique described by Chomczynski and Sacchi [18]. Briefly, tissues were homogenized in a solution containing guanidium thiocyanate, 4 M; sodium citrate, 25 mM, pH 7; sarcosyl, 0.5%; and 2-mercaptoetha-

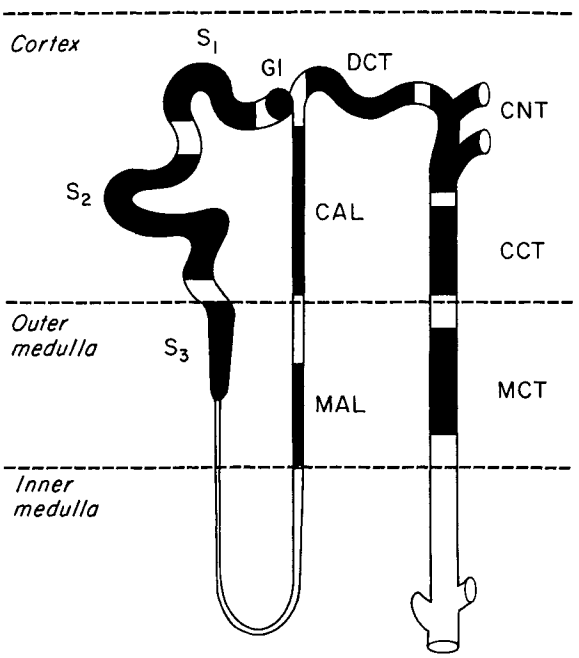

Fig. 1. Schematic representation of the rat nephron. The dark area gives the localization of the microdissected tubular segments. G1, glomerulus; S1, early portion of the proximal convoluted tubule; S2, middle portion of the proximal convoluted tubule; S3, late portion of the proximal convoluted tubule; MAL, medullary thick ascending limb of Henle's loop; CAL, cortical thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CNT, connecting tubule; CCT, cortical collecting tubule; MCT, medullary collecting tubule.

nol, 0.1 M. RNA was then extracted first with phenol-chloroform, followed by an additional extraction with chloroform and precipitated with isopropanol. Total RNA was vacuum dried, dissolved in water treated with diethyl pyrocarbonate. The RNA extract was precipitated with 2.5 M LiCl and desalted with 70% ethanol. The final RNA precipitate was resuspended in water. Concentration and purity of RNA ($OD_{260\ nm}$/$OD_{280\ nm}$ > 1.7 in all samples) was then determined.

Primer Design and Probe for RT and Amplification

We applied a computer program for PCR primer design to the Epo cDNA sequence [21] to select sense and antisense primers (Table I). These primers were chosen in two different exons (2 and 5) in the mouse genomic Epo DNA sequence to prevent amplification of any contaminating Epo genomic DNA from interfering with the 419 bp length Epo mRNA signal. The probe for Epo used in this study was the 243 bp Pst I-Xho II restriction fragment encompassing the second exon of the mouse Epo gene that was inserted at the Pst I and Bam HI sites of a pUC 18 vector. A 265 bp Epo-insert/pUC 18 PstI-Eco RI purified fragment was derived from this construct [8]. The probe for GAPDH used in this study was a 19 mer described by Sharefkin et al. to detect the PCR product. The DNA probes were labeled with ^{32}P-dCTP using the Amersham multiprime DNA labelling system (Amersham Corp., Arlington Heights, IL) or the Terminal transferase tail elongation (Boehringer Mannheim).

Reverse Transcription and Polymerase Chain Reaction (RT/PCR)

200 ng of total RNA extracted from various segments were reverse transcribed into cDNA as previously described in 15 μl reaction mixture containing 60 U of Moloney-murine leukemia virus reverse transcriptase) (M-MLV/RT) (BRL, Rockville, MD), 0.1 μg of Epo antisense primer (Epo or GAPDH), 1 mM of each of four deoxynucleotide triphosphates (dNTPs), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 20 U RNasin (an inhibitor of RNase) and 0.001% gelatin [17]. Before adding the mixture containing the different electrolytes and the enzyme, the RNA was dissolved in H_2O to adjust the reaction to the final volume of 15 μl. The RNA in water was heated at 65°C for 5 min to denature secondary structures and quickly chilled on ice for 5 min to prevent renaturation before adding the RT enzyme. After the mixture was added, the reaction was carried out at 42°C for 60 min. The reaction was stopped by heating at 95°C for 5 min. The cDNA obtained from this step was amplified in the same tube by PCR by adding 80 μl of the same buffer containing 0.1 μg of the sense primer (Epo or GAPDH) and an additional 0.05 μg of the antisense primer, 0.15 mM of each dNTPs, 2.5 U Taq DNA polymerase (Stratagene, La Jolla, CA) [17,20] and H_2O for volume adjustment. After layering with 50 μl of mineral oil to prevent evaporation and 30 s centrifugation at 14,000 rpm, 30 to 40 PCR cycles were run (30 cycles were used in the semiquantitative experiment). The PCR cycle was the following: for Epo, 90 s at 94°C to denature, 2 min at 57°C to anneal and 2 min at 72°C for the polymerization step; for GAPDH, 90 s at 94°C, 2 min at 50°C and 2 min at 72°C. A final elongation at 72°C for 10 min was done after the last cycle in both cases. As positive controls, we performed a PCR reaction with total RNA (50 ng) from anemic mouse kidney and total RNA (100 ng) from whole rat kidney. The negative control was a reaction without RNA.

Agarose Gel Electrophoresis and Southern Hybridization

After the PCR reaction, the oil was extracted with 100 μl of chloroform. PCR products were precipitated with 3 M sodium acetate and etha-

TABLE I. Oligonucleotide Primers and Probes Used for RT/PCR of Total Cellular RNA and Southern Blotting of Reaction Products for Epo and GAPDH

Gene	Oligonucleotide	Sequence	Position
Epo	Sense primer (upstream)	5′CTCTGGGCCTCCCAGTC 3′	56–72
	Antisense primer (downstream)	5′TGTTCGGAGTGGAGCAG 3′	474–458
GAPDH	Sense primer	5′CCATGGAGAAGGCTGGGG 3′	386–403
	Antisense primer	5′CAAAGTTGTCATGGATGACC 3′	580–561
	Probe	5′CTAAGCAGTTGGTGGTGCA 3′	527–547

nol 100% (1/10;V/2V) overnight at −20°C. After centrifugation, the DNA was resuspended in 10 μl of water and 2 μl of dye buffer. PCR products were electrophoresed on a 3% agarose gel (FMC Corp., Rockland, ME) using DNA molecular weight marker VI (Boehringer Mannheim, Indianapolis, IN) as a size marker. Gels were stained with ethidium bromide, photographed under ultraviolet light, and treated as follows: 15 min in HCl 0.25 N; 30 min in NaCl 1.5 M, NaOH 0.5 N; and 30 min in NaCl 1.5 M/Tris-HCl 0.2 M, pH 7.5. The gels were blotted overnight to a nylon membrane which was ultraviolet light crosslinked for 2 min and baked for 2 h at 80°C in a vacuum oven. The filters were prehybridized for 6 h in 5 × SSPE (sodium chloride-sodium phosphate-EDTA), 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml of denatured salmon sperm DNA; and hybridized overnight by adding 300 μl of ^{32}P-labeled Epo or GAPDH probes. After washing, the filters were dried and exposed to X-ray film (Kodak X-OMAT, Sigma, St. Louis, MO) at −70°C with intensifying screens for varying periods of time.

In Situ Hybridization

Sections (5 μM thick) of unfixed frozen kidneys from five anemic and two control mice were prepared. The anemia in the mouse was induced according to the protocol of Lacombe et al. [8] which resulted in an increased level of Epo by about 300-fold. They were fixed in 4% formaldehyde in 0.1 M phosphate buffer saline (pH 7.4) and dehydrated. The procedures for in situ hybridization have been previously described [8,22,23]. Briefly, tissue sections were immersed in 0.2 n HCl for 10 min. They were then incubated in 15 μg/ml proteinase K (Protease XI; Sigma Chemical Co.) in 20 mM Tris-HCl, pH 7.4, and 2 mM calcium chloride at 37°C for 15 min. Tissue sections were then hybridized under a sealed coverslip for 24 h at 37°C in 15 μl of a solution containing 50% deionized formamide, 10 mM polyvinylpyrrolidone, 0.02% bovine serum albumin, 10% dextran sulfate, 2 mg/ml yeast transfer RNA (Sigma Chemical Co.), 400 μg/ml salmon sperm DNA (Sigma Chemical Co.), 10 mM dithiothreitol, and 0.2 μg/ml of the radiolabeled probe denaturated at 100)C for 2 min. The probe for Epo was ^{35}S-labeled using the multiprime DNA labeling system (Amersham Corp., Arlington Heights, IL), specific activity, 2.10^8 cpm/μg DNA). The slides were then washed at room temperature with gentle agitation successively in 50% formamide-4 × SSC for 1 h, followed by two washes in 2 × SSC for one-half hour, and finally in 2 × SSC overnight. Sections were then dehydrated in ethanol and covered with Kodak NTB-2 emulsion for autoradiography. After 10 to 12 days of exposure, the slides were developed in Kodak D19, fixed with Kodak A44, and stained with hematoxylin and phloxin. Three control experiments carried out in parallel and included: (a) treatment of tissue sections with 50 μg/ml ribonuclease A (type III, Sigma Chemical Co.) for 30 min at 37°C, (b) hybridization with a ^{35}S-labeled pUC18 vector without the Epo probe, (c) hybridization of nonanemic tissue kidney sections with the specific Epo probe.

RESULTS

Detection of Epo mRNA by In Situ Hybridization

The histological structure of the hypoxic kidneys did not differ from the normal structure of control kidneys. On the sections of anemic kidneys hybridized with the Epo probes, significant clusters of silver grains were observed on cells located in the cortex and the outer medulla. Glomerular and tubular epithelial cells were negative (Fig. 2A). All the positive cells appeared to be in a peritubular location, along the capillary lumen with their nuclei protruding into the lumen. Kidney sections from anemic mice either treated with RNase before hybridization or hybridized with a ^{35}S-labeled pUC18 vector without the Epo insert were completely negative (Fig. 2B). Furthermore, no specific labeling could be observed in kidney sections from two normal mice (data not shown).

Detection of Epo mRNA in Rat Kidney by RT/PCR

To investigate the effect of $CoCl_2$, a known inducer of Epo, on the levels of Epo mRNA in this RT/PCR method, rats were treated with $CoCl_2$ for 4 h and total RNA was used for RT/PCR. Southern blots probed for the amplified Epo product showed a several-fold increase in Epo mRNA (Fig. 3, upper half). When RNA from the same rat kidney was used in similar amplification experiments using GAPDH primers to study the effect of $CoCl_2$ on transcript levels (Fig. 3, lower half), no increase in transcript levels such as that seen for Epo was noted for GAPDH. Therefore, this RT/PCR method can be used to study the localization of Epo mRNA. Furthermore, this constancy of the

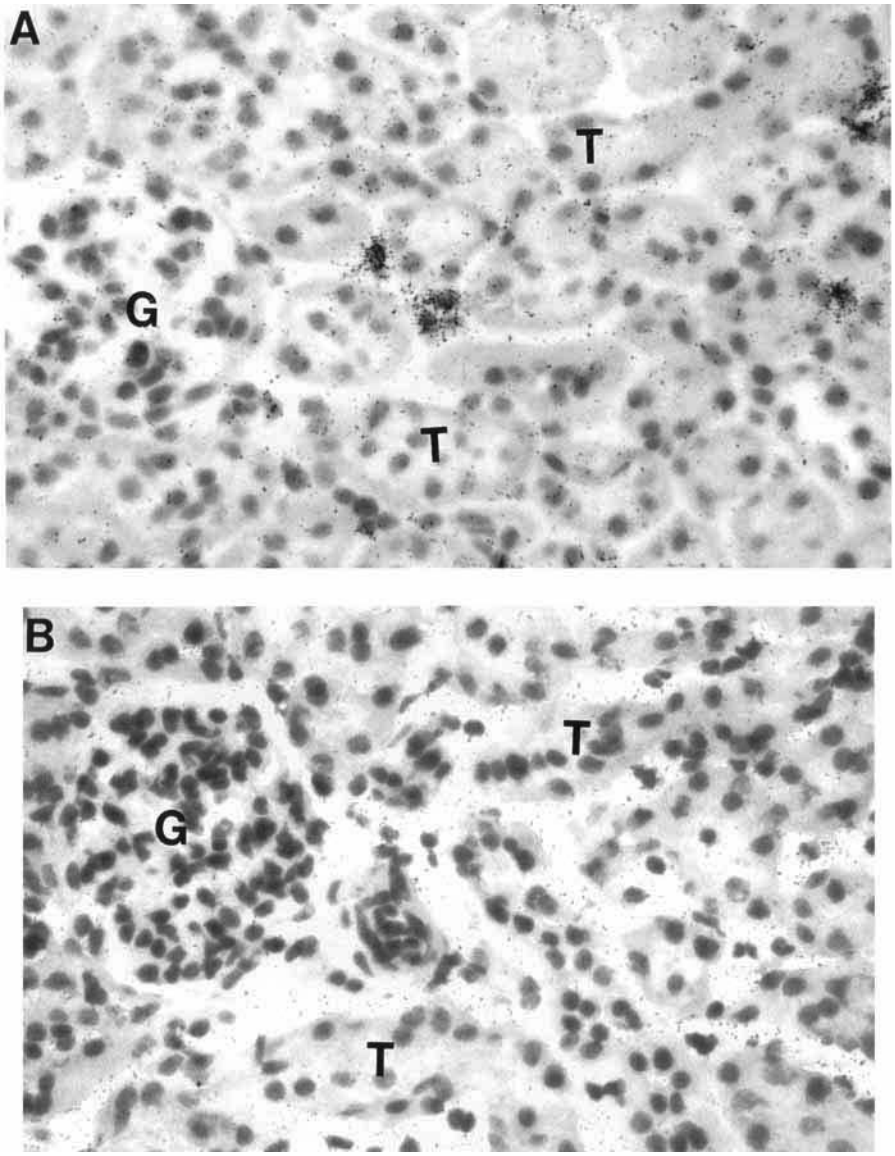

Fig. 2. **A:** In situ hybridization of a kidney section with the specific probe for Epo. The positive cells strongly labeled by a cluster of silver grains appear clearly in a peritubular location (arrows). **B:** In situ hybridization with a pUC18 probe used as a non-specific probe; no signal is observed; G, glomerulus; T, tubules.

GAPDH transcript level confirmed that the observed marked variation seen in Epo transcript levels was not an artifact of the amplification levels.

Detection of Epo mRNA in Nephron Segments by RT/PCR

Since in situ hybridization does not conclusively prove the nature of the Epo mRNA producing cells, we performed the RT/PCR method used above to analyze the level of Epo transcripts in microdissected nephron segments. Analysis of amplification of RT/PCR for Epo mRNA in nephron segments obtained from normal rats and in kidneys obtained from anemic mice is seen in Figure 4A. Total RNA from the whole kidney and different nephron segments (Fig. 1) were reverse transcribed/PCR and loaded separately in each lane. A predominant amplification product of the predicted size (419 bp) was evident in the autoradiography of the Southern blot of total kidney of anemic mice (Fig. 4A, lane 14). In parallel, the control reaction performed in the absence of RNA did not result in amplification of the product (lane 15). The basal level of

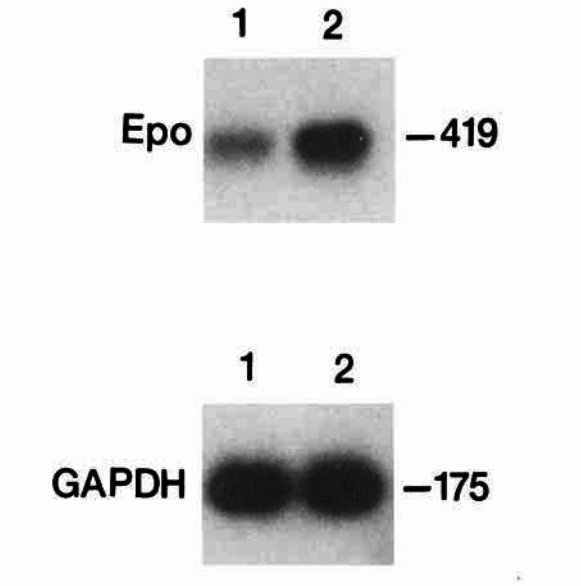

Fig. 3. Effect of $CoCl_2$ on Epo and GAPDH mRNA levels in rat kidney. Total RNA isolated from control and $CoCl_2$-treated rats for 4 h were reverse transcribed with Epo and GAPDH antisense primers and then amplified for 30 cycles using PCR with primer sets designed for Epo cDNA and GAPDH cDNA. Amplification products run on 2% agarose gels were blotted onto nylon membrane and hybridized with ^{32}P-labeled probes for Epo (upper half) and GAPDH (lower half), respectively, **lane 1,** normal rat; **lane 2,** rat + $CoCl_2$.

the Epo transcript in WKY total rat kidney RNA was detected (lane 1). As seen in Figure 4A, no signal was detected in any lane corresponding to the different nephron segments (lanes 2 to 11), with the exception of lane 12 which corresponded to the amplification of RNA extracted from the capillary fraction containing a pool of afferent/efferent arterioles and vasa recta. In addition, no signal was detected in the interlobulary artery fraction (lane 13).

The effect of $CoCl_2$ on rat kidney Epo mRNA levels was also studied by RT/PCR. Rats were treated with $CoCl_2$ for 4 h as described in Methods, and nephron segments were prepared as for the control rats. The results were identical and confirm the localization to the capillary fraction. These experiments were repeated three times with different samples, and each time the same sample was tested for the presence of Epo at least two times. For GAPDH, one test per sample was performed. All these experiments show the same results (data not shown).

Detection of GAPDH in Nephron Segments by RT/PCR

We made an RT/PCR using the GAPDH primer using the same RNA extracted from vari-

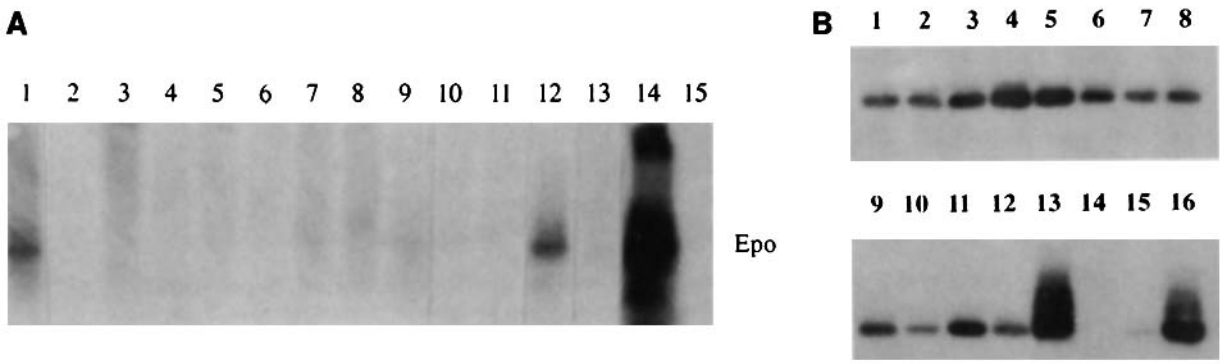

Fig. 4. **A:** Southern blot analysis of RT/PCR products obtained by amplification of RNA of the different nephron segments from normal rats using an Epo probe. **Lanes 2–11:** Segments in the order they appear in Figure 1, starting from the glomerulus; no signal is visible. **Lane 12:** Amplification of the RNA extracted from the capillary fraction (afferent/efferent arteriole, vasa recta) one band appears which corresponds to Epo mRNA. **Lane 13:** Amplification of the RNA extracted from the interlobulary arteries; no signal is detectable. The positive controls are in lane 1—amplification of total RNA of total rat kidney—and **lane 14**—amplification of the RNA extracted from anemic mouse kidney. The negative control is in lane 15 (H_2O instead of RNA). **B:** Southern blot analysis of RT/PCR products obtained by amplification of RNA of the different nephron segments from normal rats using a GAPDH probe. **Lanes 1–12:** Segments in the following order: G1, glomerulus; S1, early portion of the proximal convoluted tubule; S2, middle portion of the proximal convoluted tubule; S3, late portion of the proximal convoluted tubule; CAL, cortical thick ascending limb of Henle's loop; MAL, medulary portion of the ascending limb of Henle's loop; DCT, distal convoluted tubule; CNT, connecting tubule; CCT, cortical collecting tubule; MCT, outer medullary collecting tubule; interlobulary fraction and capillary fraction (afferent/efferent arteriole, vasa recta). A signal is observed in all lanes corresponding to the hybridization with the GAPDH probe. **Lane 13** is the amplification of total RNA of whole rat kidney, a strong signal is detected. The negative control is in **lane 14** (no RNA added in the RT/PCR reaction). **Lane 16:** Amplification of RNA from anemic mouse kidney. **Lane 15:** Amplification of human poly A+ RNA from kidney, another positive control of size (we used 1 μg of RNA for the RT/PCR).

ous nephron segments to assess the quality and reliability of our RNA preparation and the efficiency of our RT/PCR. Figure 4B represents the analysis of amplified products after RT/PCR for GAPDH mRNA. Southern blot analysis demonstrates specific binding of RT/PCR 175 cDNA products to the GAPDH 19 mr designed from human probes [24], confirming its identity as the GAPDH mRNA similar to that described above. The specific primers used were able to identify GAPDH mRNA in all nephron segments tested, indicating that nephron segments are able to generate GAPDH cDNA products. Thus, the failure to detect Epo mRNA in all nephron segments is not due to RNA preparation, but to the lack of expression in nephron tubules. This conclusion is consistent with our data that describe the presence of GAPDH mRNA in all nephron segments studied including those which fail to express Epo mRNA.

DISCUSSION

Our previous data obtained by in situ hybridization [8] showing an Epo production by peritubular cells of the cortex and the outer medulla (Fig. 2) already suggested that the cells that produce Epo may be the endothelial cells of the renal peritubular capillaries [8]. These results were confirmed by another group [9]. Because of the low resolution of in situ hybridization and its rather high threshold, we could not exclude the possibility of an Epo production by the tubular cells. In order to resolve this problem, we prepared single nephron segments by microdissection of tubules and applied an extremely sensitive technique, i.e., to the RT/PCR to detect possible mRNA for Epo. We first studied the presence of Epo mRNA in nephron segments microdissected from normal rats. Our results clearly indicated that there is no Epo mRNA in tubular epithelium. Several reports have demonstrated that $CoCl_2$ is a potent inducer of Epo [1,25], and it increases Epo levels in the circulation in vivo [1,25]. The increase of Epo by $CoCl_2$ induction was a result of the accumulation of Epo mRNA [26] and an increased rate of transcription of the Epo gene [27]. Therefore, we used $CoCl_2$ to increase Epo mRNA levels. $CoCl_2$ treatment failed to demonstrate any signal in nephron tubular fraction, although a marked increase of several-fold of Epo mRNA in whole kidney was observed. However, RT/PCR, a very sensitive technique, showed a positive signal (Fig. 4A) in the capillary fraction isolated from the rat kidney (afferent/efferent arteriole, vasa recta).

Several reports have indicated that Northern blot analysis failed to detect Epo mRNA [8,20] in whole normal kidneys. The current RT/PCR method developed in the present study accurately allowed us to detect the Epo mRNA in control or induced rat kidneys. The results demonstrate that Epo RNA is solely found in the peritubular capillary fraction (afferent/efferent arterioles, vasa recta). Our RT/PCR procedure could identify the mRNA of GAPDH (Fig. 4B) in all nephron segments tested. Although GAPDH mRNA is expressed in all nephron segments tested, the levels of mRNA may vary between various segments. Thus, our ability to demonstrate by the RT/PCR technique the presence of GAPDH mRNA in different segments, and the absence of the Epo mRNA in all of the tubular fractions, demonstrates the specificity of our technique, as well as the selectivity of the localization of different mRNA along the nephron.

Our results are in agreement with those of Eckardt et al. [28] and others [8,9], who have shown that the Epo producer cells in polycystic human kidney [28] or murine [8,9] are nonepithelial cells. This contradicts those claims that the Epo producer cells are tubular epithelial cells of the cortex [10]. Our report suggests that localization of Epo producing cells are found preferentially in the peritubular cells and not in the nephron segments. One explanation for this pattern of distribution may be the result of blood flow patterns within the kidney and the renal oxygen gradient. Changes in O_2 content perceived by the peritubular capillary endothelial cells may serve as the stimulus for Epo mRNA production.

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