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# Human, rat, and mouse kidney cells express functional erythropoietin receptors

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**Cells of human, rat, and mouse kidney express functional erythropoietin receptors.**

*Background.* Erythropoietin (EPO), secreted by fibroblast-like cells in the renal interstitium, controls erythropoiesis by regulating the survival, proliferation, and differentiation of erythroid progenitor cells. We examined whether renal cells that are exposed to EPO express EPO receptors (EPO-R) through which analogous cytokine responses might be elicited.

*Methods.* Normal human and rat kidney tissue and defined cell lines of human, rat, and mouse kidney were screened, using reverse transcription-polymerase chain reaction, nucleotide sequencing, ligand binding, and Western blotting, for the expression of EPO-R. EPO's effects on DNA synthesis and cell proliferation were also examined.

*Results.* EPO-R transcripts were readily detected in cortex, medulla, and papilla of human and rat kidney, in mesangial (human, rat), proximal tubular (human, mouse), and medullary collecting duct cells (human). Nucleotide sequences of EPO-R cDNAs from renal cells were identical to those of erythroid precursor cells. Specific $^{125}$I-EPO binding revealed a single class of high- to intermediate-affinity EPO-Rs in each tested cell line (kD 96 pM to 1.4 nM; $B_{max}$ 0.3 to 7.0 fmol/mg protein). Western blots of murine proximal tubular cell membranes revealed an EPO-R protein of approximately 68 kDa. EPO stimulated DNA synthesis and cell proliferation dose dependently.

*Conclusion.* This is the first direct demonstration, to our knowledge, that renal cells possess EPO-Rs through which EPO stimulates mitogenesis. This suggests currently unrecognized cytokine functions for EPO in the kidney, which may prove beneficial in the repair of an injured kidney while being potentially detrimental in renal malignancies.

Erythropoietin (EPO) is a 34 kDa glycoprotein hormone that controls erythropoiesis by receptor-mediated regulation of survival, proliferation, and differentiation of erythroid progenitor cells [1]. In the adult, EPO is secreted primarily by fibroblast-like interstitial cells of the renal cortical labyrinth and, to a smaller degree, by the liver [1–5]. Renal EPO is first secreted into peritubular capillary blood that also contains residual EPO [1, 6]. From here, renal veins deliver the hormone into the systemic circulation [1, 6].

Intriguingly, EPO-producing cells are also in direct contact with the basal aspects of proximal and distal tubular cells [1–5, 7]. We reasoned that this anatomical relationship between EPO-secreting cells, the intrarenal capillary network, and tubular and other renal cells could facilitate endocrine and paracrine actions of EPO within the kidney itself. This would require that potential targets, such as renal tubular cells, possess functional EPO receptors (EPO-Rs) on their surface [8]. This has, however, not been systematically investigated to date, and as a consequence, endocrine or paracrine actions of EPO within the kidney have not been considered. This is primarily due to the assumption, until recently, that EPO-Rs existed only on erythroid progenitor cells and that, therefore, EPO's effects were restricted to these cells [8].

Recently, however, Anagnostou et al showed that vascular endothelial cells possess EPO-Rs and proliferate and migrate in response to EPO [9, 10]. Others have demonstrated that EPO-Rs are present in rodent placenta [11], rat brain and neuronal PC12 and SN6 cells [12, 13], testicular Leydig cells [14], and gastric epithelial cells [15]. In Leydig cells, EPO stimulates testosterone synthesis, and in gastric mucosal cells, it stimulates mitogenesis. In the brain of gerbils, EPO was shown to ameliorate the injury caused by ischemia [16], whereas in glomerular mesangial cells, it was found to activate calcium channels, potentially resulting in cellular contraction [17]. Taken together, these observations provide growing evidence in support of the notion that EPO may possess important physiological functions in various nonhemopoietic cells.

To explore EPO's nonhemopoietic functions further, we conducted a systematic search for EPO-R in human and rat kidney tissue and in various lines of cultured renal cells. We detected authentic EPO-R transcript and protein expression and specific binding of EPO to cell

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surface receptors. Receptor activation stimulated mitogenesis *in vitro*, demonstrating that EPO may act through its receptors in the kidney as a currently unrecognized cytokine.

## METHODS

### Human kidney

Normal tissue from two different nephrectomy specimens was obtained with our Institutional Review Board (IRB) approval. Tissues were immediately placed into chilled phosphate-buffered saline (PBS), rinsed free of blood, frozen in liquid nitrogen, and dissected into cortex, outer medulla, and papilla. The samples were stored at −70°C until RNA was extracted. mRNA from normal human fetal liver (week 19) served as a positive control.

### Rat kidney

For all studies (approved by the Institutional Animal Care and Use Committee), normal, adult, male Sprague-Dawley rats weighing 300 to 350 g were used. Animals had free access to food and water. Before sacrifice, rats were anesthetized (ketamine, 1 mg, and acepromazine, 0.1 mg/100 g body wt i.p.), and hearts and kidneys were rapidly removed, placed in chilled PBS, rinsed free of blood, frozen in liquid nitrogen, and stored at −70°C until RNA was extracted. Kidneys were dissected into cortex, outer medulla, and papilla.

Because anemia causes the accumulation of EPO-R–expressing erythroid progenitor cells, it was used to obtain negative and positive controls [8]. Accordingly, using a modification of a previously reported method [18], adult male Balb C mice ($N = 3$), weighing 40 to 50 g, were made anemic with a 5 mg/ml solution of phenylhydrazine in PBS administered once a day for three days (50 mg/kg body wt i.p.). Sham animals ($N = 3$) were treated with an equal volume of PBS. After two additional days, hematocrits in phenylhydrazine-treated animals had decreased from 46 ± 2% to 31 ± 3% ($P < 0.05$), whereas they remained stable in sham animals (45 ± 2% and 46 ± 2%). Animals were then sacrificed under nembutal anesthesia (40 mg/kg body wt i.p.), and spleens were harvested, immediately frozen in liquid nitrogen, and stored at −70°C. Subsequently, RNA was extracted, and cell membranes were prepared for Western blotting, as described later here.

### Cell lines

Defined, normal human proximal tubular cells (HCTs; Clonetics Corp., San Diego, CA, USA) at passage 2 and normal human medullary collecting duct cells (HMCDs) at passages 2 and 3 were grown to high subconfluence in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and F12 containing 1 × insulin, transferrin, selenium (Sigma, St. Louis, MO, USA), epithelial growth factor (10 ng/ml), T3 (4 pg/ml), and 10% newborn calf serum (NCS; Hyclone, Logan, UT, USA) at 37°C in 5% $CO_2$/air [19]. Defined, normal mesangial cells obtained from adult human (HMCs) and rat (RMCs) kidney at passages 2 to 3 were grown to high subconfluence in DMEM with 10% NCS at 37°C in 5% $CO_2$/air. Transformed murine proximal tubular cells (MCTs) are a well-defined cell line that retains many characteristics of native proximal tubular cells [20]. They were grown in DMEM with 10% NCS at 37°C in 5% $CO_2$/air. A human erythroleukemia cell line that expresses high numbers of EPO-Rs, OCIM 1 cells [21], served as positive controls. They were grown at 37°C in 5% $CO_2$/air in suspension culture, using Iscove's modified Dulbecco's media, containing 10% heat-inactivated fetal calf serum (FCS) and $5 \times 10^{-5}$ M β-mercaptoethanol. HeLa cells (ATTC, Rockville, MD, USA) served as negative human controls. They were grown in MEM and 10% NCS. HCD 57, an EPO-dependent murine erythroleukemia cell line with high level expression of EPO-Rs [22], served as the positive control. They were grown in suspension culture media as described for OCIM 1 cells. In addition, 1 U/ml of human EPO was added to the culture media. Mouse NIH 3T3 cells, embryonic mouse fibroblasts, served as negative controls (ATTC). They were grown in DMEM with 10% NCS at 37°C in 5% $CO_2$/air.

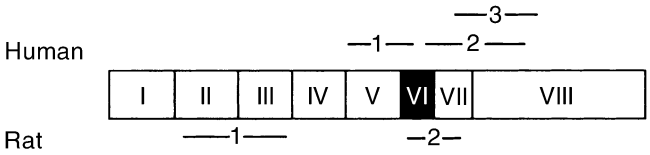


**Fig. 1. Schema of the mRNA that encodes the erythropoietin receptor [24, 25].** It is composed of eight exons; exons I through V represent the exoplasmic domain (N-terminus), exon VI, the single transmembrane domain, and exons VII and VIII, the cytoplasmic domain (C-terminus). Regions of the human EPO-R transcript that are bound by the three PCR primers (1 through 3) are shown above, and PCR primers (1 and 2) utilized for rat and mouse EPO-R transcripts, below the diagram (not to scale).

### mRNA isolation, RT-PCR, and gel electrophoresis

Total RNA was isolated from tissues and cultured cells by an acid guanidinium-thiocyanate-phenol-chloroform method [23], using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). For the reverse transcription-polymerase chain reactions (RT-PCR) 1 μg of total mRNA was reverse transcribed (M-MLV reverse transcriptase; GIBCO, Grand Island, NY, USA), and the resulting cDNAs were subjected to 35 cycles of the PCR. The amplification of the examined cDNAs was linear up to 40 PCR cycles for all primer sets used in this study. For human samples, three sets of PCR primers were chosen, each specific to a different domain of human EPO-R cDNA (Fig. 1). Their individual sequences

and respective positions within the coding sequence are as follows [24]: (1) sense, 5′-CCTGGTCGGAGCCTGT GT-3′, antisense, 5′-CACGACGACTGGCACGAG-3′, yielding a 104 bp PCR product of nucleotides 704–807 located on exons V and VI, spanning extracellular and transmembrane domains; (2) sense, 5′-TCGTGGTCAT CCTGGTGCTGCTGA-3′, antisense, 5′-ACCTTCAG GAGAGTCTCGCGACGA-3′, yielding a 240 bp PCR product of nucleotides 776–1015, located on exons VI and VIII, representing transmembrane and cytoplasmic domains; and (3) sense, 5′-CCCGAGCCCAGAGAGC GAGTT-3′, antisense, 5′-TAGGAGGACGAGTAGA CGAAA-3′, yielding a 372 bp PCR product of nucleotides 885–1229 on exons VII and VIII, representing cytoplasmic domains of the human EPO-R.

For rat and mouse EPO-R cDNAs, two sets of primers were used for PCR amplification of homologous sequences (Fig. 1) [12, 25]: (1) sense, 5′-TCTGGGAGGC GGCGAACT-3′, antisense, 5′-GAGGAGCGATGGTG GCGTAGT-3′, yielding a 219 bp PCR product of nucleotides 188–406 on exons II and III, representing an extracellular domain; and (2) sense, 5′-CGCTGTCTCTCAT TCTCGTC-3′, antisense, 5′-GGTTCGGGTCTCTTAC TCAA-3′, yielding a 118 bp PCR product of nucleotides 758–875 on exons VI and VII, representing transmembrane to cytoplasmic domains of the rat/mouse EPO-R.

All primer pairs were chosen to span introns (Fig. 1), thus facilitating control for contamination by genomic DNA. All cell and tissue extracts were subjected to the PCR reaction once with and once without an initial reverse transcription.

As internal controls, human and mouse/rat β-actin mRNAs were simultaneously reverse transcribed and PCR amplified. The primers used for human β-actin cDNA had the following sequence: sense, 5′-TGTCC ACCTTCCAGCAGATGT-3′, antisense, 5′-CACCTT CACCGTTCCAGTTTT-3′, yielding a 249 bp product. The primers used for rat/mouse β-actin cDNA had the following sequence: sense, 5′-AGAGGGAAATCGTG CGTGACA-3′, antisense, 5′-CACTGTGTTGGCATA GAGGTC-3′, yielding a 279 bp product.

All PCR products were size fractionated on 2.5% NuSieve agarose and 1% agarose gel (FMC Bioproducts, Rockland, ME, USA), stained with ethidium bromide, and band locations were recorded by photographing them under ultraviolet light. Size standards of DNA were run in parallel (pBR322 DNA MSP I digest; New England Biolabs, Beverly, MA, USA).

### Nucleotide sequencing

Besides the use of appropriate negative and positive controls, unequivocal proof for the authenticity of renal EPO-R transcripts was obtained by subcloning and sequencing of the respective PCR products. Accordingly, PCR products generated with the species-specific first and second EPO-R primer sets (Fig. 1) from HCT and MCT mRNAs were subcloned (pCR 3.1 vector; Original TA Cloning Kit; Invitrogen, San Diego, CA, USA). The obtained subclones then underwent automated fluorescent DNA sequencing at the University of Utah Core Sequencing Facility.

### Erythropoietin binding to cells

Cells were grown to subconfluence, as described earlier here, and were mechanically harvested. They were then washed at 4°C for three minutes with 0.5 M NaCl and 0.25 M acetic acid (pH 2.5) in order to remove EPO bound to the cell surface [12] and were washed twice more with PBS (pH 7.3). Binding assays were carried out by placing approximately $1 \times 10^6$ cells in an incubation mixture, containing PBS with 0.1% bovine serum albumin, 0.1% $NaN_3$, to inhibit internalization, and a 200-fold excess of recombinant human erythropoietin (rHuEPO) in a total volume of 170 μl. $^{125}$I-rHuEPO (specific activity 948 Ci/mmol; Amersham, Arlington Heights, IL, USA) was added to cell aliquots in increasing concentrations (40 to 620 pM) and was incubated for three hours at 15°C. The cells were then pelleted and washed three times with 200 μl of ice-cold PBS, and cell-associated radioactivity was measured with a gamma counter. By measuring the difference in radioactivity when cells were incubated either in the presence or in the absence of 200-fold excess unlabeled EPO (recombinant human EPO; Amgen, Royal Oaks, CA, USA), the amount of specific EPO binding was determined. Assay conditions were designed to keep total EPO binding (specific plus nonspecific) below 10% of the total amount of radioligand added. To obtain $K_d$ and $B_{max}$, both linear Scatchard [26] and nonlinear (GraphPad Software, Inc., San Diego, CA, USA) regression analyses of the equilibrium-binding data were performed. The time course of ligand binding was determined by incubating $^{125}$I-rHuEPO at a concentration of 620 pM with approximately $1 \times 10^6$ cells at 15°C for four hours. To test whether specific EPO binding has a linear correlation with the number of cells used, increasing numbers of cells, from 0 to $9 \times 10^6$, were incubated with $^{125}$I-rHuEPO (620 pM) for three hours at 15°C both in the absence and the presence of 200-fold excess unlabeled EPO.

### Immunological detection of erythropoietin receptor protein

Murine proximal tubular cells (approximately $5 \times 10^6$), grown in 75 $cm^2$ flasks, were washed twice with ice-cold PBS and were collected by scraping and centrifuging. The pellet was frozen at −70°C. Spleens from anemic and sham mice were obtained, rinsed free of blood, and frozen at −70°C. Cell membranes from MCTs and spleens were prepared as previously reported [17]. In brief, approximately 50 mg of spleen tissue or $5 \times 10^6$ MCTs were homogenized in a polytron in 1 ml hypotonic

buffer (50 mM Tris-HCl, pH 7.4, 2 mM $Na_3VO_4$, 50 mM NaF, 10 mM $Na_2P_2O_7$, 1 mM phenylmethylsulfonyl fluoride) and incubated for 30 minutes at 4°C. The sample was centrifuged at 4°C for 20 minutes at 6000 × g. Membrane proteins in the pellet were extracted over 60 minutes at 4°C with lysis buffer (PBS containing 5 mM ethylenediaminetetraacetic acid, pH 7.4, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride). Following centrifugation for 20 minutes at 6000 × g at 4°C, the supernatant, containing cell membrane protein, was collected.

These cell membrane protein preparations from spleens and MCTs were then immunoprecipitated by adding 10 μl of an affinity-purified antibody directed against the C-terminus of the murine EPO-R (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This mixture was incubated overnight at 4°C. Protein A/G Plus-Agarose (Santa Cruz Biotechnology) was then added, and the mixture was incubated for another three hours at 4°C. After four washes in lysis buffer, the pelleted agarose antibody complex was suspended in 40 μl 2 × Laemmli's sample buffer and heated at 100°C for four minutes prior to electrophoresis on 9% sodium dodecyl sulfate-polyacrylamide gel, as previously reported [27, 28]. The separated protein bands were then electrophoretically transferred to Hybond-P membrane (Amersham). These were incubated for two hours at room temperature in a mixture of blocking buffer (TBS-T: 10 mM Tris-buffered saline, pH 8.0, 0.1% Tween 20) and 5% nonfat powdered milk in order to prevent binding to nonspecific sites. A 1:1000 dilution of the primary antibody in blocking buffer was then added to the membranes, and they were incubated at room temperature for 45 minutes. After three washes in TBS-T, the second antibody was added (1:1000 dilution of a horseradish peroxidase-conjugated antirabbit IgG), followed by a one-hour incubation at room temperature. After four washes in TBS-T, the protein bands were made visible, using a chemiluminescence detection system (ECL, Amersham) and recorded on radiographic film as previously reported [27].

The specificity of anti-EPO-R antibody binding by MCT protein extracts was further assessed by reacting the primary blots, prepared as described earlier here, with a 1:1000 dilution of primary antibody that had been neutralized (17 hours at 4°C) with a 10-fold (by mass) excess of EPO-R antigen (C-terminal peptide; Santa Cruz Biotechnology). The remaining steps of the immunodetection protocol were then performed as described earlier here.

### DNA synthesis

Cells were grown in 24-well plates. Once attached and subconfluent, the cells were serum deprived for 24 hours. The effect of EPO on DNA synthesis, determined by [$^3$H]-thymidine incorporation, was assessed by incubating the cells for 20 additional hours with varying concentrations of EPO or 10% NCS. The cells were then pulsed with 0.5 μCi [$^3$H]-thymidine (specific activity 70 to 90 Ci/mmol; New England Nuclear, Boston, MA, USA) and incubated for four more hours before being washed with 750 μl cold PBS and again with cold 5% trichloroacetic acid. Next, 750 μl of 0.25 M NaOH in 0.1% sodium dodecyl sulfate were added for 30 minutes in order to solubilize proteins. Radioactivity was determined by scintillation counting, with counts expressed as cpm per microgram of protein. Protein was measured by the BCA Protein Assay (Pierce, Rockford, IL, USA).

### Cell proliferation

All tested cells were seeded at $1 \times 10^4$ per well in 96-well plates and allowed to attach overnight in appropriate serum-containing media. Cells were made quiescent by incubation in serum-free media for 24 hours. Newborn calf serum (10%) or EPO, at incremental concentrations, was then added, and the incubation was continued for 48 hours. At this time, media were removed and replaced with fresh media (without phenol red and serum) containing 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thiazolyl blue; Sigma] and cells were incubated at 37°C for an additional three hours. After this, the media were removed, and 100 μl of 0.04 N HCl in isopropanol were added to each well in order to solubilize the blue MTT reduction product formed in viable cells. The absorbance was read on a microplate reader at 570 nm with background subtraction at 650 nm. Media D-glucose concentration at the time of the MTT assay was well maintained, thus preventing an underestimation of cell numbers [29]. In addition, cell proliferation was assessed by hemocytometer counting of trypsinized cells. There was an excellent linear correlation ($r = 0.99$) between cell counts and results obtained by the MTT assay.

### Data reporting and statistical analysis

All data on rat and human tissues and individual cell lines are representative of at least three to six replications or independent experiments, respectively. Data are reported as means ± SE. Differences between data means were analyzed for statistical significance ($P < 0.05$) using Student's *t*-test for paired and independent populations, as well as analysis of variance [30]. Apparent $K_d$ and $B_{max}$ values for $^{125}$I-EPO binding were derived by both linear Scatchard [26] and nonlinear (GraphPad Software) regression analyses of the equilibrium-binding data.

## RESULTS

### Erythropoietin receptor transcripts in human and rat kidney

Figure 2 shows ethidium bromide-stained agarose gels of EPO-R PCR products obtained from normal human

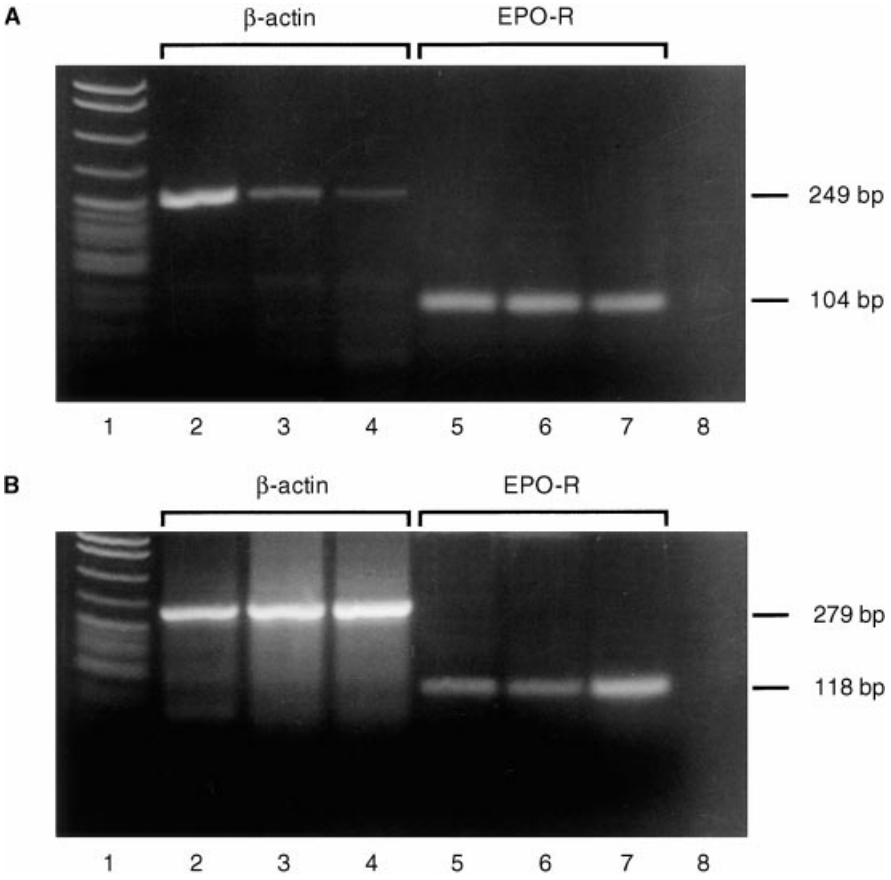


**Fig. 2. Erythropoietin receptor (EPO-R) transcripts in human and rat kidney.** (*A*) Ethidium bromide-stained gel shows the expected 104 bp EPO-R PCR products (first primer set in Fig. 1) from human renal cortex (lane 5), medulla (lane 6), and papilla (lane 7). The first lane shows a DNA ladder. Lanes 2 through 4 show the corresponding 249 bp β-actin PCR products, and lane 8 demonstrates that deletion of the RT reaction prevents subsequent formation of PCR products (tested on papilla, lane 7). (*B*) Ethidium bromide-stained gel shows the expected 118 bp EPO-R PCR products (first primer set in Fig. 1) from rat renal cortex (lane 5), medulla (lane 6), and papilla (lane 7). Other lane explanations as in (A).

(Fig. 2A) and rat (Fig. 2B) kidney. The bands in lanes 5 (cortex), 6 (medulla), and 7 (papilla) of Figure 2A represent the expected 104 bp PCR products of the human EPO-R cDNA. The first lane shows DNA size standards, and lane 8 demonstrates that deletion of the reverse transcription step yields no detectable PCR product in papillary tissue (lane 7). The bands in lanes 2 through 4 represent the 249 bp β-actin PCR products from cortex, medulla, and papilla, respectively. Bands of comparable intensity were obtained with the other two primer sets, yielding expected PCR products of 240 bp and 372 bp. Human fetal liver served as the positive control; it yielded identical PCR products (104, 240, and 372 bp) as shown in Figure 3A.

Figure 2B depicts, in lanes 5 (cortex), 6 (medulla), and 7 (papilla), the expected 118 bp PCR products of the rat EPO-R cDNA, and lanes 2 through 4 show the corresponding 279 bp β-actin PCR product from renal cortex, medulla, and papilla, respectively. The first lane shows DNA size standards, and lane 8 demonstrates that deletion of the reverse transcription step yields no detectable PCR product in papillary tissue (lane 7). Bands of similar intensity were obtained with the other primer set, yielding the expected PCR product of 219 bp. Spleen from anemic mice served as positive control (Fig. 3B); it yielded PCR products of predicted sizes (118 and 219 bp for EPO-R; 279 bp for β-actin), whereas rat myocardium showed no EPO-R message (Fig. 3C).

### Erythropoietin receptor transcripts in renal cells

Because the renal circulation may contain erythroid progenitor cells that express EPO-Rs and because the kidney is composed of vascular, interstitial, glomerular, and distinct tubular cells, we next screened for the presence of EPO-R message in defined renal cell lines grown in culture. Figure 4A shows the data from HCTs and HMCs. Bands in lanes 4, 6, and 8 depict the expected 104, 240, and 372 bp products of the human EPO-R in HCT cells. Bands in lanes 5, 7, and 9 show the corresponding signals from HMCs. In lane 1 DNA size standards and in lanes 2 and 3, the respective 249 bp β-actin signals are depicted. Figure 4B shows the data from HMCDs. Lane 2 shows the β-actin signal, and lanes 3 through 6 show the EPO-R PCR products of expected sizes (372 bp, 240 bp, and 104 bp). Figure 5A confirms that EPO-R transcripts from human erythroleukemia cells (OCIM 1) are comparable to those obtained in renal cells (positive control: bands in lanes 3, 4, and 5 represent EPO-R PCR products, lane 1 DNA size standards, and lane 2 β-actin signal, respectively), whereas no such mes-

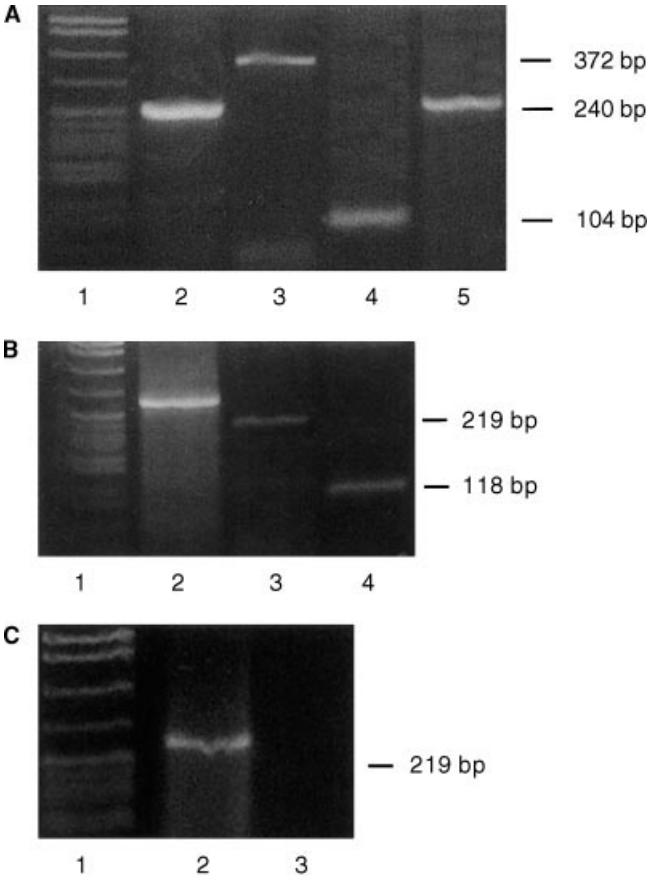


**Fig. 3. EPO-R transcripts in human, mouse, and rat control tissues.** (*A*) Fetal human liver served as a positive control. Gel shows the EPO-R PCR products obtained with the three primer sets (Fig. 1): 372 bp (lane 3), 104 bp (lane 4) and 240 bp (lane 5). Lane 1 depicts a DNA ladder and lane 2 the 249 bp β-actin signal. (*B*) Anemic mouse spleen (positive control). Gel shows the EPO-R PCR products obtained with the two mouse/rat primer sets (Fig. 1): 219 bp (lane 3) and 118 bp (lane 4). Lane 1 depicts a DNA ladder, and lane 2 shows the 279 bp β-actin signal. (*C*) Rat myocardium (negative control). EPO-R expression (219 bp PCR product) was not detected (lane 3). Lane 1 depicts a DNA ladder and lane 2 the 279 bp β-actin signal.

sage is detected in HeLa cells (negative control: lane 7 shows no 372 bp EPO-R PCR product; lane 6 is the β-actin signal). Together, these data demonstrate that HCTs, collecting duct, as well as glomerular mesangial cells express EPO-R mRNA.

Figure 4C depicts corresponding data from MCTs and RMCs. Bands in lanes 4 and 6 represent the expected 118 bp and 219 bp PCR products of the mouse EPO-R in MCTs. Bands in lanes 5 and 7 represent those from RMCs. Bands in lanes 2 and 3 represent the respective 279 β-actin signals, and lane 1 shows DNA size standards. Figure 5B confirms that EPO-R transcripts from murine erythroleukemia cells (HCD 57, positive control) are comparable to those obtained in renal cells. Lanes 3 and 4 show 118 bp and 219 bp EPO-R PCR products, lane 1 DNA size standards, lane 2 β-actin signal), whereas no EPO-R message is detected in NIH 3T3 cells (negative control: lanes 6 and 7 correspond to lanes 3 and 4, and lane 5 represents β-actin). Collectively, these data demonstrate that murine proximal tubular and rat glomerular mesangial cells express EPO-R mRNA.

### Sequencing of EPO-R cDNA

To directly show that the PCR products derived from renal cells represent authentic EPO-R message, generated cDNAs were subcloned, and their sequences were analyzed. Thus, sequencing of EPO-R cDNAs generated from HCT mRNA, using both set 1 (104 bp, exons V and VI) and set 2 (240 bp, exons VII and VIII) of PCR primers (Fig. 1; **Methods** section), yielded results identical to published EPO-R DNA sequences [19]. The sequences of cDNAs generated from MCTs and rat kidney cortex by set 1 (219 bp, exons II and III; Fig. 1; **Methods** section) and set 2 (118 bp) of primer pairs specific to rat/mouse EPO-R were also identical to published EPO-R DNA sequences [20]. These data prove unequivocally that HCTs and MCTs as well as rat kidney cortex express authentic EPO-R transcripts.

### Specific erythropoietin binding to cell surface and immunologic detection of erythropoietin receptor protein

In MCTs and HCTs, and in HMCDs, specific binding of $^{125}$I-EPO to cell surface EPO-R was examined. Figure 6A shows equilibrium binding data in MCTs. Specific binding of $^{125}$I-EPO in these cells occurred to a single class of high-affinity receptors ($K_d$ 96.1 ± 6.1 pM; $B_{max}$ 0.3 ± 0.07 fmol/mg protein; $N = 3$), and binding became saturated over time (Fig. 6B). Nonlinear and Scatchard analyses yielded comparable results. There was excellent linear correlation ($r = 0.99$) between specific ligand binding in tested cell lines and cell numbers. The apparent $K_d$ in HCT cells was 1.1 ± 0.1 nM and $B_{max}$ 1.6 ± 0.2 fmol/mg ($N = 3$), and in HMCDs ($N = 3$) the apparent $K_d$ was 1.3 ± 0.2 nM and $B_{max}$ 7.0 ± 0.8 fmol/mg protein.

Western blots for EPO-R protein (Fig. 7) were generated after initial immunoprecipitation of MCT and mouse spleen cell membrane protein with anti-EPO-R antibody. Lane 1 on Figure 7A shows a representative Western blot from MCTs. Two immunoreactive protein bands of approximate molecular mass of 68 and 90 kDa, respectively, are identified. When anti-EPO-R antibodies were first neutralized with an excess of specific antigen (C-terminal EPO-R peptide; **Methods** section), no EPO-R–specific immunoreactivity was detected (lane 2). Lane 3 shows molecular weight standards. These data imply that the detected bands possess EPO-R–specific antigenicity. As positive control, spleen cell membrane proteins from anemic mice were utilized. In these, immunoreactive protein bands of comparable molecular mass

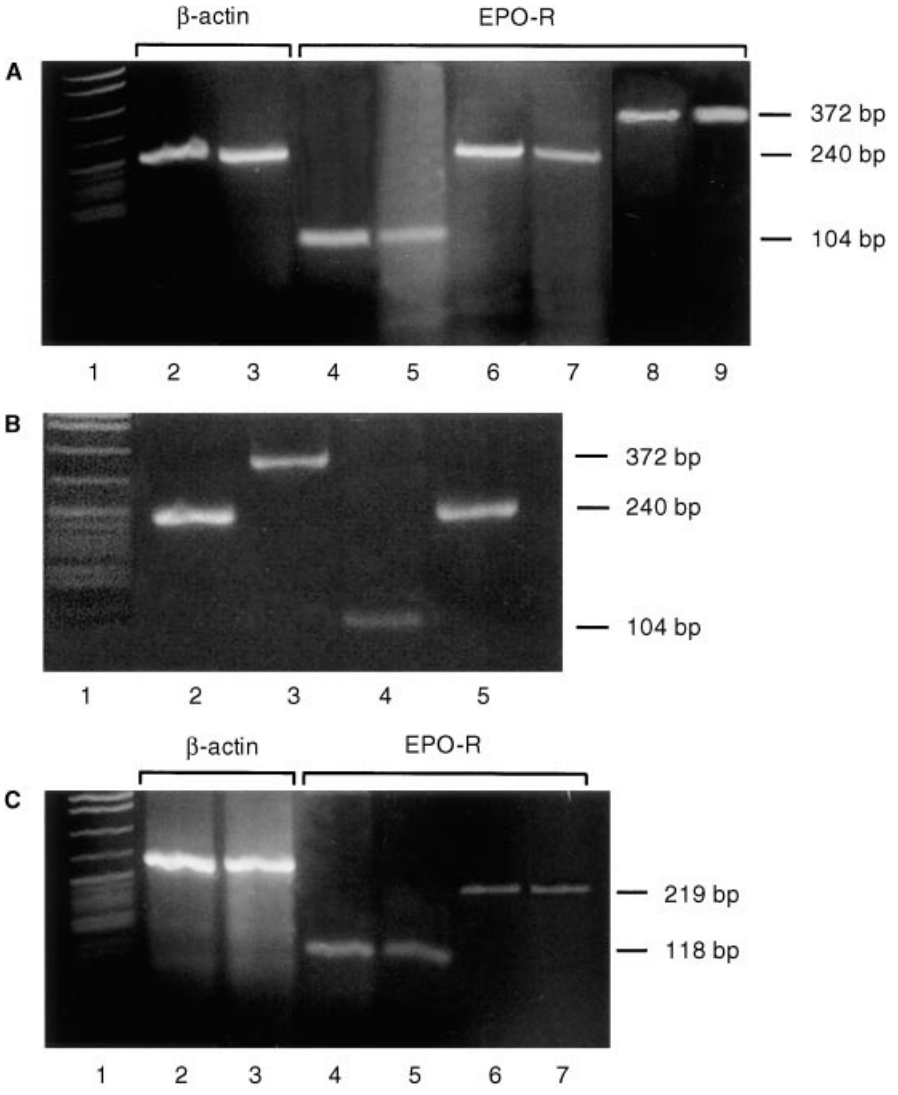


**Fig. 4. EPO-R transcripts in renal cells.** (*A*) Human proximal tubular and mesangial cells. Gel shows in lanes 4, 6, and 8 the expected 104, 240, and 372 bp PCR products of the human EPO-R in proximal tubular cells (HCT), and in lanes 5 and 9 those in mesangial cells (HMC). The first lane shows a DNA ladder, lanes 2 and 3, the 249 bp β-actin signals from HCT and HMC, respectively. (*B*) Human medullary collecting duct cells. Gel shows all three EPO-R PCR products (lanes 3 through 5, 372, 104, 240 bp), a DNA ladder (lane 1) and β-actin signal (lane 2). (*C*) Mouse proximal tubular and rat mesangial cells. Lanes 4 and 6 depict the expected 118 and 219 bp PCR products of the EPO-R in murine proximal tubular (MCTs), and lanes 5 and 7 those in at mesangial cells (RMCs), respectively. Lane 1 shows a DNA ladder and lanes 2 and 3 the corresponding 279 bp β-actin signals for each cell line.

(68 and 90 kDa) were identified (lane 2 on Fig. 7B); however, the 68 kDa protein was not detected in normal spleen (lane 1), whereas the 90 kDa band was detectable both in normal and anemic spleens (lanes 1 and 2). This pattern suggests that the 68 kDa protein likely represents the EPO-R, because anemia stimulates the accumulation of erythroid progenitor cells in the spleen, that is, cells that express the EPO-R [8]. Lane 3 shows molecular weight standards.

### Mitogenic action of erythropoietin in MCTs

Figure 8A shows that EPO dose dependently stimulates DNA synthesis in MCTs. Figure 8B shows that 48 hours after EPO was added, MCT proliferation was stimulated significantly and dose dependently. This proliferative response was smaller but also evident 24 hours after EPO addition. The addition of 10% NCS to quiescent MCTs served as the positive control.

## DISCUSSION

The unique anatomical relationship between EPO-producing interstitial fibroblasts and adjacent tubular cells [1, 6] prompted us to ask whether renal cells possess biologically active EPO-Rs that would enable them to respond to EPO. In our examination of human and rat kidney as well as various renal cell lines, we detected authentic, species-specific EPO-R expression at both the transcriptional and the translational level, a single class of moderate- to high-affinity EPO-Rs, and specific, dose-dependent, mitogenic activity of EPO *in vitro*. These findings, when considered as a whole, make this the first study, to our knowledge, to demonstrate conclusively that renal cells in culture possess functional EPO-Rs through which EPO can elicit mitogenic and possibly other cytokine actions. We are currently investigating how these initial *in vitro* observations may apply to the biology of the normal and diseased kidney.

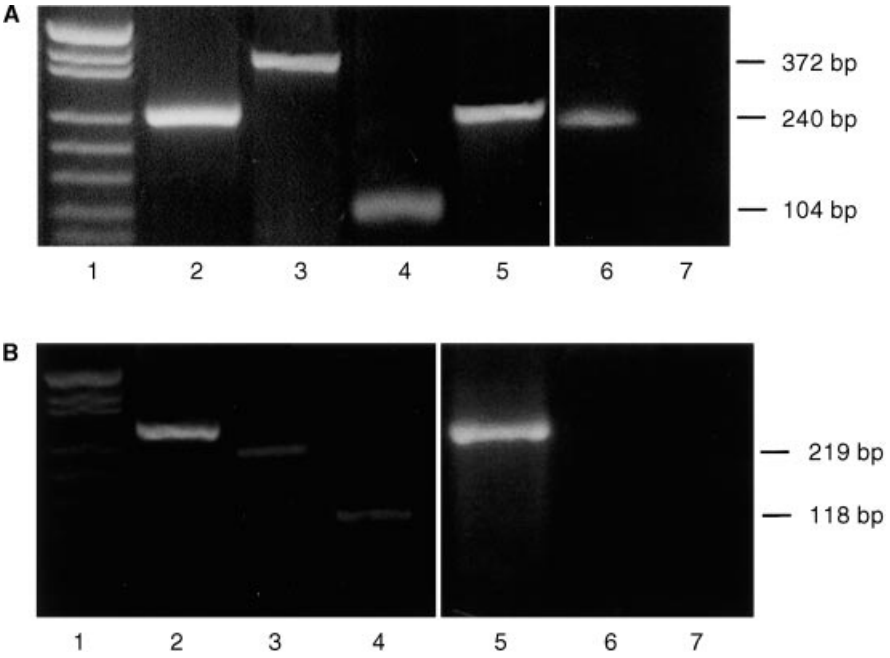


**Fig. 5. EPO-R transcripts in control cells.** (*A*) Human cells. In OCIM 1 cells (positive control), all three EPO-R PCR products are detected (lanes 3 through 5, 372, 104, 240 bp). Lane 1 shows a DNA ladder and lane 2 the β-actin signal. In HeLa cells (negative control), no EPO-R message (lane 7, 240 bp) is detected. Lane 6 represents β-actin message. (*B*) Mouse cells. In HCD 57 cells (positive control), two EPO-R PCR products are shown (lanes 3 and 4, 219 bp, 118 bp). In NIH 3T3 cells (negative control), corresponding EPO-R message is not detected (lanes 6 and 7). Lane 1 shows DNA ladder, and lanes 2 and 5 show β-actin from respective cell lines.

Given our findings, one would expect that clinically occurring or experimentally induced alterations in renal EPO production *per se* would result in detectable changes in renal structure and function. Chronic anemias that are not caused by sickle cell disease or other hemolytic disorders have not been reported to cause significant anatomical or functional changes in the kidney [31]. This may indicate that elevated EPO levels cause down-regulation of EPO-R expression in the kidney or that the trophic effects of EPO are suppressed by counteracting factors or that renal histology and function in such conditions have not been specifically examined. Severe, more acute anemia induced in rats was shown to lead to significant injury in S1 segments of proximal tubular cells and additional morphological changes in the interstitium of the cortical labyrinth [32]. Whether the associated sharp increase in EPO production influenced the observed histologic picture or renal function in this study is not known.

Acquired renal cysts may be another condition in which EPO's renotrophic effects could be significant. Although cyst fluid is often found to contain increased levels of EPO [33], it is unknown whether cyst growth is, at least in part, the result of EPO's growth-promoting capability or whether the latter contributes to the increased incidence of renal cell carcinomas noted in these patients. Similarly, a significant percentage of patients with autosomal dominant polycystic kidney disease and end-stage renal failure has relatively normal EPO and hematocrit levels [34], and renal cyst fluid has also been found to be rich in EPO [35]. Again, it is unexplored whether EPO's proliferative actions do, in fact, stimulate cystogenesis in this condition. Finally, a small percentage of patients with renal cell carcinoma produces increased amounts of EPO [36]. Preliminary data from our laboratory demonstrate that EPO stimulates mitogenesis in human and rat renal carcinoma cell lines, raising the concern that the administration of EPO for the treatment of anemia could conceivably hasten the growth of some renal malignancies.

Only one study published to date has shown that EPO treatment of rats with *cis*-platinum–induced acute renal failure enhances functional recovery [37]. Because most forms of acute renal failure caused by acute tubular necrosis are associated with a transient depression of renal EPO synthesis [38–40], EPO replacement may thus serve to hasten the repair of the injured kidney.

Recent work on the biological functions of EPO in nonerythroid cells has demonstrated that vascular endothelial cells express EPO-Rs and that EPO acts in these as a mitogen and a motogen [9, 10], as vasoconstrictor and activator of angiogenesis, in part via induction of endothelin-1 [41, 42]. We reported that incubation of vascular endothelial cells with EPO causes significant changes in the expression of immediate early response genes (c-*jun*, c-*myc*) and the genes of several vasoactive peptides (atrial, brain, and C-type natriuretic peptide, and endothelin-1) [43]. These findings reconfirm the ability of vascular endothelial EPO-Rs to transmit mitogenic and other transactivating signals. It was recently reported that EPO increases $Ca^{2+}$ influx in glomerular mesangial cells [17], a response previously observed in vascular

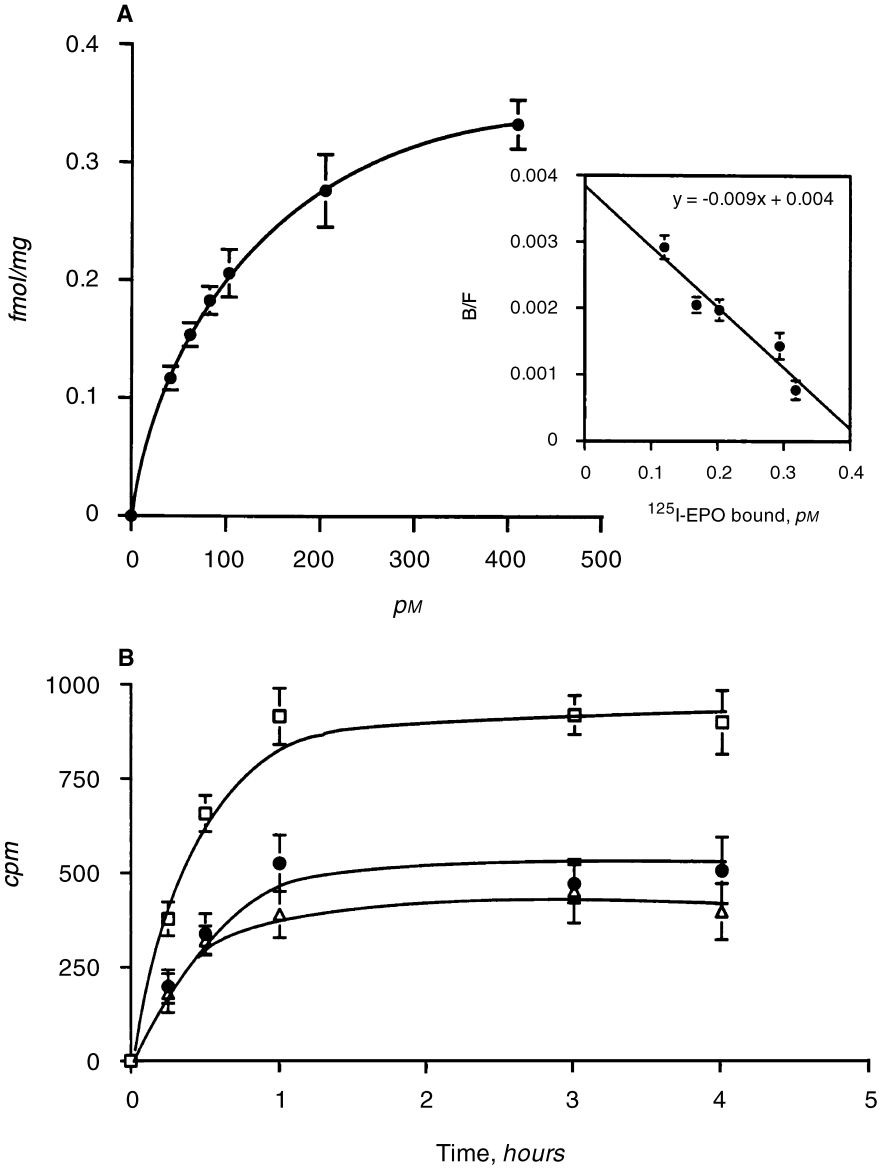


**Fig. 6. Specific $^{125}$I-EPO binding in murine proximal tubular cells (MCTs).** (*A*) Equilibrium binding curve and Scatchard plot (insert) of $^{125}$I-rhEPO binding in MCTs (means ± SE). Apparent $K_d$, derived by Scatchard or nonlinear analysis, was 96.1 ± 6.1 pM ($N$ = 3), and $B_{max}$ was 0.3 ± 0.07 fmol/mg protein; there was only a single class of high-affinity receptors. (*B*) Time dependence of total (□), nonspecific (△), and specific $^{125}$I-EPO binding (●) to MCTs (means ± SE). Binding, assessed at 0.25, 0.5, 1, 2, 3, and 4 hours, reached a plateau at approximately 2 hours.

smooth muscle cells [44]. This response occurs via an EPO-R–operated $Ca^{2+}$ channel, which, in turn, is activated by tyrosine phosphorylation of phospholipase C-γ1. This mechanism may be an additional explanation for the ability of EPO to generate clinical hypertension.

In neuronal cells, EPO-Rs have been located and were found to increase intracellular calcium and protect against glutamate neurotoxicity [12, 13, 16, 45]. It appears that some of the *in vitro* functions of EPO in Leydig cells, placental endothelial cells, and gastric epithelial cells are also found *in vivo* [11, 14, 15].

Specific binding of EPO to cultured renal cells occurred to a single class of receptors and with relatively low $B_{max}$ values, a pattern also found in erythroid progenitor and other nonerythroid cells [8]. Binding affinity was highest in MCTs ($K_d$ approximately 96 pM), intermediate in HCTs ($K_d$ approximately 1.1 nM), and low in HMCDs ($K_d$ approximately 1.3 nM). Serum levels of EPO under nonanemic and nonhypoxic conditions are between 5 and 35 mU/ml, that is, in the low picomolar range [1]. These concentrations are well below the $K_d$ values we identified in cultured renal cells. Although not known, it appears conceivable that EPO concentrations in the interstitium of the renal cortical labyrinth, that is, in the immediate vicinity of EPO secreting and adjacent tubular cells exceed those seen in the periphery. These higher local concentrations of EPO may, in turn, be sufficient to activate EPO-Rs *in vivo*. Furthermore, ambient EPO concentrations in the kidney can be dramatically increased by tissue hypoxia or the administration

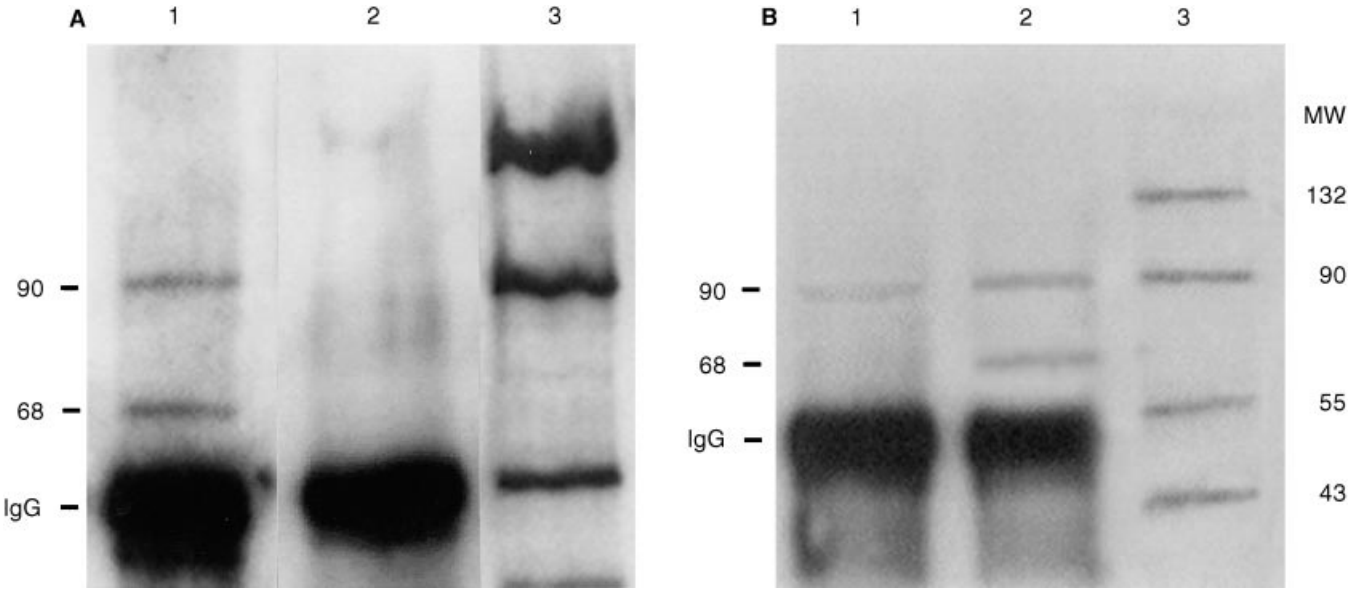


**Fig. 7. Immunodetection of EPO-R protein in MCTs**. (*A*) Western blots of immunoprecipitated MCT membrane protein. Lane 1 shows two EPO-R immunoreactive bands of approximate molecular mass of 68 and 90 kDa, respectively. Lane 2 demonstrates that preincubation of anti-EPO-R antibody with excess EPO-R antigen prevents the detection of EPO-R proteins, confirming immunospecificity of the anti-EPO-R antibody. Lane 3 shows molecular weight markers. (*B*) Western blots of immunoprecipitated mouse spleen cell membrane protein. Lane 2 depicts two immunoreactive bands of approximate molecular mass of 68 and 90 kDa, respectively. These were obtained from anemic animals. Lane 1 shows corresponding data from nonanemic mouse spleen. In these, no 68 kDa protein was detected, whereas the 90 kDa band remained detectable, thus suggesting that the smaller protein represents the EPO-R. Lane 3 shows molecular weight markers.

of recombinant EPO [1]. Under these circumstances, even if EPO-R–binding affinities and surface expression were as low as those found in this *in vitro* study, EPO-R activation could occur. An additional factor in this regard may be the surface distribution of EPO-Rs. Renal tubular cells, distinct from erythroid progenitor cells, grow in polarized monolayers. A large number of hormone and cytokine receptors in tubular cells are concentrated on their basolateral side [46]. If EPO-Rs in tubular cells were likewise preferentially expressed in a basolateral distribution, their endocrine or paracrine stimulation *in vivo* would be facilitated, whereas *in vitro* activation may be difficult. Under the latter culture conditions, apically added EPO may not reach basolaterally located EPO-Rs in concentrations sufficient to initiate a physiologic response. These possibilities await investigation. Finally, the as yet undefined expression pattern and ligand-binding kinetics of the EPO-R in the kidney, whether regulated, constitutive, or altered by pathologic conditions, are expected to modify biological responses to EPO further.

We detected in murine proximal tubular cells two EPO-R immunoreactive proteins of approximately 68 and 90 kDa, respectively (Fig. 7). The cloned murine EPO-R cDNA encodes a 55 kDa polypeptide, and the EPO-R is post-translationally modified by N-linked glycosylation and phosphorylation, increasing its mass to 78 kDa and above [47, 48]. The 68 kDa protein that we consistently detected in MCTs and in anemic mouse spleen was also found in HCD 57 cells (not shown). This moiety of EPO-R has been previously described in the latter cells [22], strongly suggesting that MCTs express this receptor protein as well. The fact that experimental anemia induced the expression of the 68 kDa protein, whereas it only modestly affected that of the 90 kDa protein, supports the notion that the former is likely the EPO-R. The exact nature of the 90 kDa protein requires additional investigation.

The mitogenic response elicited by EPO was dose dependent and specific. It was, not unexpectedly, less in magnitude than that elicited by 10% NCS (Fig. 8) and became most prominent after 48 hours of incubation, a pattern also observed when MCTs are stimulated with other growth factors [20]. Although it remains to be proven, it is conceivable that EPO stimulates mitogenesis in renal cells not only through its own receptor but also via as yet unidentified mediators or pathways. Such indirect action has recently been reported in vascular endothelial cells, in which EPO stimulates angiogenesis, at least in part, via induction of endothelin-1 synthesis [41, 42].

It is of note that cells of the neuronal type express EPO-Rs on their surface but fail to undergo mitosis when exposed to EPO [12]. Vascular endothelial and gastric mucosal cells, by contrast, proliferate when exposed to EPO through mitogenic signals transmitted by its receptor, as well as through induction of endothelin-1 synthesis

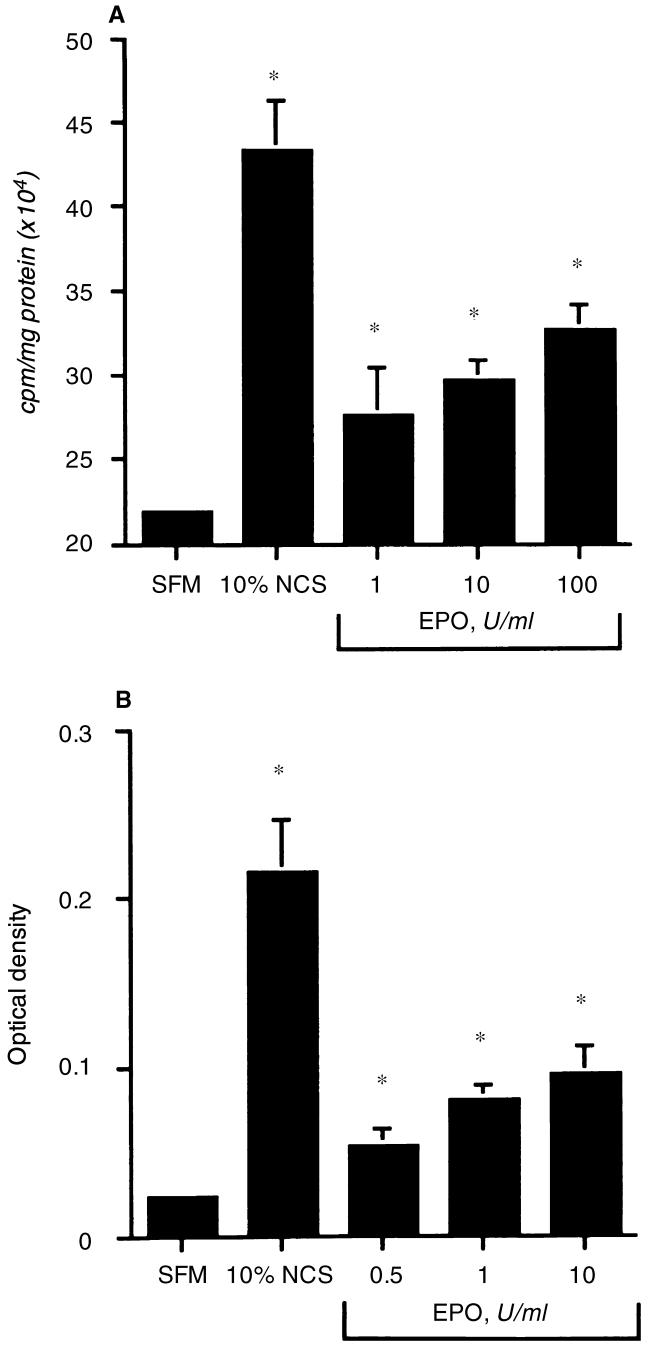


**Fig. 8. DNA synthesis and cell proliferation in response to EPO in MCTs.** (*A*) DNA synthesis in quiescent MCTs, assessed by [$^3$H]thymidine incorporation, was stimulated by EPO dose dependently and significantly. Ten percent NCS served as positive control. $*P < 0.05$ compared with quiescent control cells (first column). (*B*) Cell proliferation in response to EPO in quiescent MCTs. EPO increased cell numbers dose dependently and significantly (after 48 hours of incubation). Ten percent NCS served as positive control. Abbreviations are: MCTs, murine proximal tubule cells; EPO, erythropoietin; NCS, newborn calf serum; SFM, serum free media; $*P < 0.05$ compared with quiescent control cells (first column).

in the former [9, 10, 15, 40, 41]. Although we show that EPO acts as a mitogen in renal cells, it is very unlikely that renal cells depend exclusively on EPO for their *in vitro* or *in vivo* growth or survival. Erythroid progenitor cells, at certain stages, depend for growth and survival on EPO and in addition on interleukin-3, insulin-like growth factor-I, stem cell factor, and other cytokines [1, 8, 49]. Some of the same growth factors, for example, insulin-like growth factor-I, but also epidermal growth factor and hepatocyte growth factor, act as mitogens and survival factors when the kidney is injured as in acute tubular necrosis [50]. Following toxic or ischemic tubular injury, adaptive increases in cytokine or cytokine receptor expression or the administration of renotrophic growth factors appear to aid the tubular repair process by stimulating cell proliferation and inhibiting apoptosis [50]. It is conceivable that EPO elicits analogous effects in acute tubular necrosis, as was suggested by Vaziri, Zhou and Liao [37].

In summary, we have shown that cortex, medulla, and papilla of both human and rat kidney express species-specific EPO-R mRNAs. More specifically, authentic EPO-R transcripts are expressed in HCTs and MCTs, HMCs, RMCs, and HMCDs. EPO binding occurs to a single class of receptors. EPO-R protein has been identified in mouse proximal tubular cells, and in these, EPO stimulates DNA synthesis and cell proliferation. In conclusion, our data show that EPO acts *in vitro* as a renotropic mitogen and that the expression of authentic EPO-R, a member of the cytokine receptor superfamily [51], in renal tubular and mesangial cells and in kidney cortex, medulla, and papilla has allowed us to identify the critical system through which endocrine and paracrine actions of EPO may be elicited. Although its functions *in vivo* remain to be identified, EPO may play a role in renal development, cell survival, cell proliferation, cell migration, and differentiation.

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## APPENDIX

Abbreviations used in this article are: EGF, epithelial growth factor; EPO, erythropoietin; EPO-R, erythropoietin receptor; HCT, human proximal tubule cells; HMC, human mesangial cells; HMCD, human medullary collecting duct cells; IGF, insulin-like growth factor; MCT, murine tubular cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); NCS, newborn calf serum; OCIM, human erythroleukemia cell line; PBS, phosphate buffered saline; rHuEPO, recombinant human erythropoietin; RMC, rat mesangial cells; RT-PCR, reverse transcription-polymerase chain reaction.

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