# Peritubular Cells Are the Site of Erythropoietin Synthesis in the Murine Hypoxic Kidney

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## Abstract

**Erythropoietin (Epo)-producing cells were identified in the murine hypoxic kidney by in situ hybridization. Profound anemia was induced in order to greatly increase Epo production. This resulted in high levels of Epo mRNA in the kidney. $^{35}$S-labeled DNA fragments of the murine Epo gene were used as probes for in situ hybridization. Control experiments conducted in parallel included kidneys of nonanemic mice, RNase-treated hypoxic kidney sections, and $^{35}$S-labeled non-Epo-related DNA. The Epo probe gave a specific hybridization signal in the hypoxic kidney in the cortex and to a lesser extent in the outer medulla. Glomerular and tubular cells were not labeled. All positive cells were identified as peritubular cells. Using immunofluorescence, we showed that cells with the same topography contained Factor VIII-related antigen. These data demonstrated that peritubular cells, most likely endothelial cells, constitute the major site of Epo production in the murine hypoxic kidney.**

## Introduction

Erythropoietin (Epo)[1] is the hormone that controls red blood cell production in mammals. Epo synthesis is regulated via feedback mechanisms involving tissue oxygen tension. Since the work of Jacobson et al. (1), evidence has accumulated suggesting that the kidney is the major source of Epo production (2). However, the identity of the kidney cells that are responsible for Epo biosynthesis is still a matter of controversy. A glomerular origin for renal Epo has been suggested based on immunohistochemical studies (3, 4) and on studies about Epo production by in vitro cultured glomerular (5) and mesangial (6) cells. Proximal tubular cells have also been considered by others as primary candidates for Epo production (7). However, all of the data reported so far were based upon indirect evidence of Epo production. The recent molecular cloning of the Epo gene has provided a powerful new tool to further analyze Epo biosynthesis (8–11). Using the murine Epo gene as a probe, a rapid Epo mRNA accumulation has been demonstrated in the kidney of adult rodents under hypoxic conditions (12, 13). Very recently, Schuster et al. have shown that Epo mRNA was not synthesized by the glomerular but by the tubular fraction of the hypoxic kidney (14). We used the in situ hybridization technique to identify the Epo-producing cells in the kidneys of profoundly anemic mice.

## Methods

*Induction of Epo production.* To increase the amount of kidney Epo mRNA and thus the in situ hybridization signal, ICFW mice were made profoundly anemic by 6-Gray irradiation followed 24 h later by an intraperitoneal injection of phenylhydrazine (60 mg/kg body wt). 9–10 d later (hematocrit < 10%), mice were bled for serum Epo titration and their kidneys were removed. One kidney of each mouse was frozen in liquid nitrogen for subsequent in situ hybridization, and the second one was processed for poly(A)$^+$ RNA isolation. The Epo level in the plasma of these anemic mice was shown to be between 6 and 10 IU/ml of serum by an in vitro bioassay using murine CFU-E-derived colonies in plasma clot and by an in vivo bioassay using $^{59}$Fe-incorporation in polycythemic mice.

*Cell culture.* The Epo-producing IW32 murine erythroleukemia cell line (15) was used as a positive control of Epo mRNA production. The cells were grown in alpha medium with 5% FCS for 3 d at 37°C, in presence of 5% $CO_2$ in air.

*Genomic DNA probes.* Two probes were used (Fig. 1). Probe a was a 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 pUC18 vector. A 265-bp Epo-insert/pUC18 Pst I-Eco RI purified fragment was derived from this construct. Probe b was a 1-kb Pst I-Pst I Epo genomic fragment containing part of exon IV and part of exon V.

*Northern blot analysis.* RNAs were extracted from crushed hypoxic and control kidneys or from pelleted IW32 cells using the hot phenol procedure (16). Total RNAs were purified by oligo(dT) affinity chromatography to obtain poly(A)$^+$ RNA-enriched preparations. 5 μg of poly(A)$^+$ RNAs were electrophoresed. After glyoxal denaturation and transfer to Gene Screen Plus membranes, the RNAs were hybridized with $^{32}$P-labeled probe b (specific activity, $2.10^8$ cpm/μg DNA). The filter was washed at 60°C for one-half hour in 2× standard saline citrate (SSC)-1% SDS, then at 60°C for one-half hour in 0.5× SSC-0.5% SDS, followed by two washes in 0.1× SSC, for one-half hour

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1. *Abbreviations used in this paper:* Epo, erythropoietin; VIII-RAg, VIII-related antigen.

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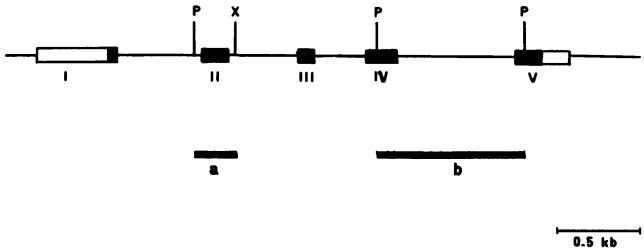


*Figure 1.* Schematic map of the mouse Epo gene and of the two DNA probes used. Exons are indicated by roman numbers. P, Pst I; X, Xho II.

at room temperature. The filter was exposed to x-ray film for 12 h at −70°C with an intensifying screen.

*In situ hybridization.* Sections (5 μm thick) of unfixed frozen kidneys from five anemic and two control mice were prepared. 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 (17). 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., St. Louis, MO) 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 Tris-HCl, pH 7.4, 1 mM EDTA, 600 mM NaCl, 0.02% Ficoll, 0.02% 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.), 400 μg/ml herring sperm DNA (Sigma Chemical Co.), 10 mM dithiothreitol, and 0.2 μg/ml of the radiolabeled probe denaturated at 100°C for 2 min. Probes a and b were $^{35}$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 d of exposure, the slides were developed in Kodak D 19, fixed with Kodak A 44, and stained with hematoxylin and phloxin. Three control experiments carried out in parallel 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.

*Immunohistochemical techniques.* Frozen sections of two kidneys, one from a normal and one from an anemic mouse, were fixed in cold aceton. Anti–human Factor VIII–related antigen (VIII-RAg) polyclonal rabbit antibody (Dakopatts, Copenhagen, Denmark) was used at a dilution of 1:100 in 0.1 M phosphate buffer saline (pH, 7.4). The indirect immunofluorescence technique was performed using a goat fluorescent anti–rabbit antibody (Institut Pasteur Production, Paris, France) at a dilution of 1:50. The monoclonal anti–F4/80 antibody, which recognizes a membrane antigen specific for murine monocytes-macrophages (18) (kindly provided by G. Milon, Pasteur Institut, Paris) was used at a dilution of 1:32 and was revealed using the peroxidase–anti-peroxidase technique.

## Results

On a Northern blot hybridized with Epo probe b, a 1.8-kb single band was detected in the poly(A)$^+$ RNAs extracted from the kidneys of anemic mice and from a murine Epo-producing erythroleukemia cell line. Under the same conditions, no Epo message was detectable in kidneys of normal mice (Fig. 2).

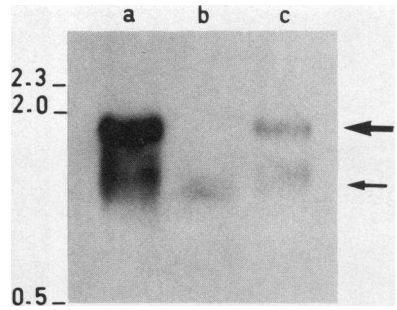


*Figure 2.* Northern blot of poly(A)$^+$ RNAs, hybridized with the Epo probe b mixed to a rat c-*Ha-ras* 1 probe as a gene transcription control. Lane *a*, pooled kidneys from anemic mice; lane *b*, pooled kidneys from control mice; lane *c*, IW32 Epo-producing cells. On the left of the figure are indicated the migration of Hind III-digested phage lambda size markers in kilobases. Large arrow, Epo mRNA; small arrow, *ras* mRNA.

The histological structure of the hypoxic kidneys did not differ from the normal structure of control kidneys. On the sections of hypoxic 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. 3 *a*). At higher magnifications (Fig. 3 *b* and *c*), all the positive cells appeared to be in a peritubular location, along the capillary lumen with their nuclei protruding into the lumen. In the cortex, most of the peritubular cells significantly hybridized with the Epo probe. Cells of the arteries, arterioles, and veins were negative. In the outer medulla, about half of the peritubular cells were labeled. No significant labeling was observed in the inner medulla (data not shown). Identical results were observed in all of the five anemic mouse kidneys tested in four separate experiments. Both probes a and b gave the same pattern of hybridization, although a lower background was obtained with probe a. 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 (data not shown). Furthermore, no specific labeling could be observed in kidney sections from two normal mice (data not shown).

To determine the lineage of peritubular cells we used antibodies directed against Factor VIII-RAg and against the murine monocyte-macrophage specific antigen F4/80. Kidney sections of hypoxic and control mice had similar immunolabeling pattern. Using anti-Factor VIII-RAg antibody, a strong positivity was observed in areas identified as sections of peritubular capillaries (Fig. 3 *d*) in the cortex and the medulla. Endothelial cells of the large vessels were also labeled. Anti-F4/80 antibody, which strongly reacted with Kupffer cells in the liver of the same mice, did not react with cortical peritubular capillaries (data not shown).

## Discussion

The present data demonstrate that Epo mRNA synthesis is induced by anemia in kidney cells which are neither glomerular nor tubular but peritubular cells. Indeed, we find that the Epo mRNA producing cells are located outside the tubular basement membrane, along the vascular lumen with their nucleus often protruding into the lumen, indicating that these peritubular cells are most probably capillary cells (19). Furthermore, in the human kidney, peritubular capillary cells are known to be genuine endothelial cells containing Factor VIII-RAg (20). Our immunofluorescence data show that this is also

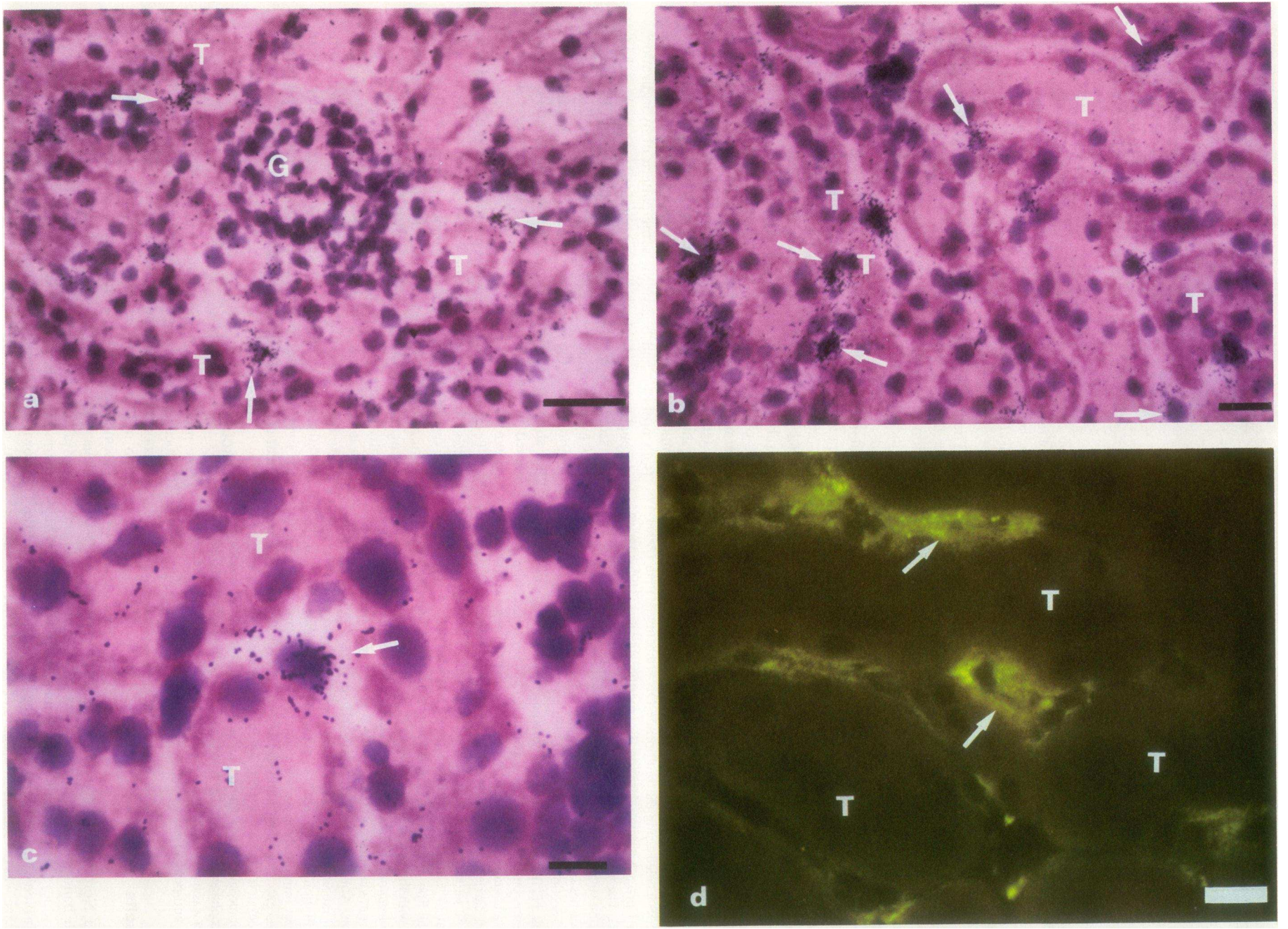


*Figure 3.* (*a–c*) In situ hybridization with the Epo probe a in anemic mouse kidney sections. (*a*) In the cortex, positive cells are indicated by arrows. Glomerulus (G) and tubules (T) are negative. (bar, 50 $\mu$m). (*b* and *c*) Higher magnifications show the peritubular location of positive cells (some of them are indicated by arrows). (*b*) Bar, 25 $\mu$m; (*c*) bar, 15 $\mu$m. (*d*) Indirect immunofluorescence with anti–Factor VIII-RAg antibody in anemic mouse kidney sections: labeling of peritubular capillary endothelial cells (bar, 15 $\mu$m).

the case in the murine kidney. Since most of the cortical peritubular cells of the murine hypoxic kidney were stained by both an Epo-specific probe and an anti-Factor VIII-RAg antibody, we suggest that the Epo-producing cells are endothelial cells. Bone marrow and spleen macrophages have been also described to produce Epo in culture (21). However, we find that macrophages are not the Epo-producing cells in the hypoxic kidney. Thus, Epo-producing cells are mainly located around the proximal tubules of the cortex and to a lesser extent in the outer medulla. In contrast, kidney macrophages identified by a monoclonal antibody (18) have been located in the medulla and in the vicinity of the glomeruli. Finally, no macrophages are found within the hybridization areas using the same antibody. Our data are in complete agreement with the recent work demonstrating the expression of Epo mRNA by the tubular extracts of the hypoxic kidneys (14). Since the protocol we used to induce anemia resulted in a 300-fold increase in the level of serum Epo (6–10 U/ml vs. 20–30 mU/ml in serum from normal mice), we cannot rule out the possibility that a basal Epo production occurs in other cells than the peritubular cells, these latter being the only cells highly stimulated by hypoxia. Moreover, the localization of Epo production to the peritubular cells does not exclude a role for the other kidney cells in the storage of Epo. Indeed, previous data suggesting that glomerular cells produce Epo were mostly obtained using immunofluorescence techniques (3, 4), which do not distinguish between production and storage of a protein.

The identification of peritubular, most probably capillary endothelial cells as the Epo-producing cells under hypoxia has interesting physiological implications. The demonstration that these cells are the site of Epo production under hypoxia does not necessarily imply that they are the oxygen-sensitive cells. Such oxygen-sensitive cells might still be the tubular epithelial cells, which are known to be exquisitely sensitive to hypoxia (22). In the latter hypothesis, the decreased oxygen tension would be detected by the tubular epithelial cells, which would in turn induce an Epo mRNA increase in their neighboring peritubular cells.

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