In Situ Hybridization Evidence for Angiotensinogen Messenger RNA in the Rat Proximal Tubule

An Hypothesis for the Intrarenal Renin Angiotensin System

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Abstract

We examined angiotensinogen gene expression in rat kidney by in situ hybridization histochemistry. Using a rat cRNA probe to angiotensinogen, we demonstrated angiotensinogen mRNA to be localized predominantly in the proximal renal tubule, with considerably lesser amounts in distal tubular segments and glomerular tufts. Previous studies have localized renin immunoreactivity to the juxtaglomerular cells, glomerular tufts, and proximal tubules. Such findings provide further evidence for a local tissue renin angiotensin system within the kidney which may influence regional function. Based on our data, we hypothesize that a major site of angiotensin production is the proximal tubule. We postulate that angiotensin synthesized in and/or around the proximal tubule may directly modulate tubular transport of sodium, bicarbonate, and water. In addition to the proximal tubule, the specific localization of the renin angiotensin components elsewhere in the kidney would also support the other proposed regional functions of the intrarenal system, including modulation of tubuloglomerular balance. (*J. Clin. Invest.* 1990. 85:417–423.) renin • angiotensinogen • kidney • in situ hybridization • RNA

Introduction

Increasing evidence is mounting to support the existence of a complete renin-angiotensin system (RAS)[1] within multiple tissues, including the kidney (1–7). Within renal tissue, evidence of renin, angiotensin-converting enzyme, and angiotensins have been demonstrated using immunohistochemical (4, 8–12) and physiological techniques (13–18). Furthermore, renin and angiotensinogen mRNAs have been demonstrated concomitantly in renal tissue using the technique of Northern blot analysis (1, 6).

Studies using Northern analysis have shown that angiotensinogen mRNA is expressed in the renal cortex and medulla, while renin mRNA is expressed primarily in the cortex (19). Renal angiotensinogen and renin mRNAs appear to be regulated by sodium chloride diet (20). Additionally, there is evidence that angiotensinogen mRNA is regulated by hormones including steroids and androgen (21, 22). A local RAS within the kidney could be involved in the regulation of single nephron glomerular filtration rate, tubular function, glomerulotubular feedback, and/or intrarenal regional blood flow (2, 3). Until recently, physiological studies used whole animal or isolated kidney preparations, and could not distinguish between a local vs. circulating RAS. Even Northern analysis techniques could not localize the site(s) of an intrarenal RAS or the mechanism of local angiotensin II (AII) production. Although existing data do suggest the presence of such a complete RAS, the techniques used to date, while providing quantitation of whole tissue or regional mRNA content, fall short of demonstrating which cell types in a complex tissue express the renin and angiotensinogen genes.

The technique of in situ hybridization histochemistry demonstrates mRNA within tissue sections (23–26). Using this technique, Deschepper et al. found renin expression in the juxtaglomerular apparatus and confirmed this with immunohistochemistry (23). That study, however, did not examine angiotensinogen mRNA localization in the kidney.

Information on the localization of angiotensinogen mRNA is particularly important in the understanding of the intrarenal renin angiotensin system. If the renin and angiotensinogen genes are expressed within the same cells, intracellular AII may be formed, secreted, and act at surface AII receptors, suggesting an autocrine function. On the other hand, if renin and angiotensinogen mRNAs are produced in different cell types, the result might suggest extracellular site(s) of regional AII formation. The present investigation was designed to determine the cellular localization of angiotensinogen mRNA within the rat kidney and to study the regulation of angiotensinogen mRNA expression during sodium replete and deplete states using in situ hybridization. The findings may provide a framework for understanding the function of the intrarenal RAS, not only in normal physiologic states but in disease states, including kidney disease, congestive heart failure, and systemic hypertension.

Methods

Animals and tissue. Male 12–16-wk-old Wistar Kyoto WKY rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used for this study. Initial localization studies were performed in animals receiving a normal diet. Subsequently, we examined the regulation of sodium state on the localization and regulation of angiotensinogen mRNA expression by studying salt-depleted (n = 5) and salt-repleted rats (n = 5) that received either an NaCl diet (0.02%) or a diet consisting of 1.6% NaCl (Teklad, Madison, WI), respectively, for 14 d.

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Received for publication 14 December 1988 and in revised form 27 November 1989.

1. *Abbreviations used in this paper:* AII, angiotensin II; PAS, periodic acid-Schiff; RAS, renin-angiotensin system.

J. Clin. Invest.

0021-9738/90/02/0417/07 $2.00
Volume 85, February 1990, 417–423

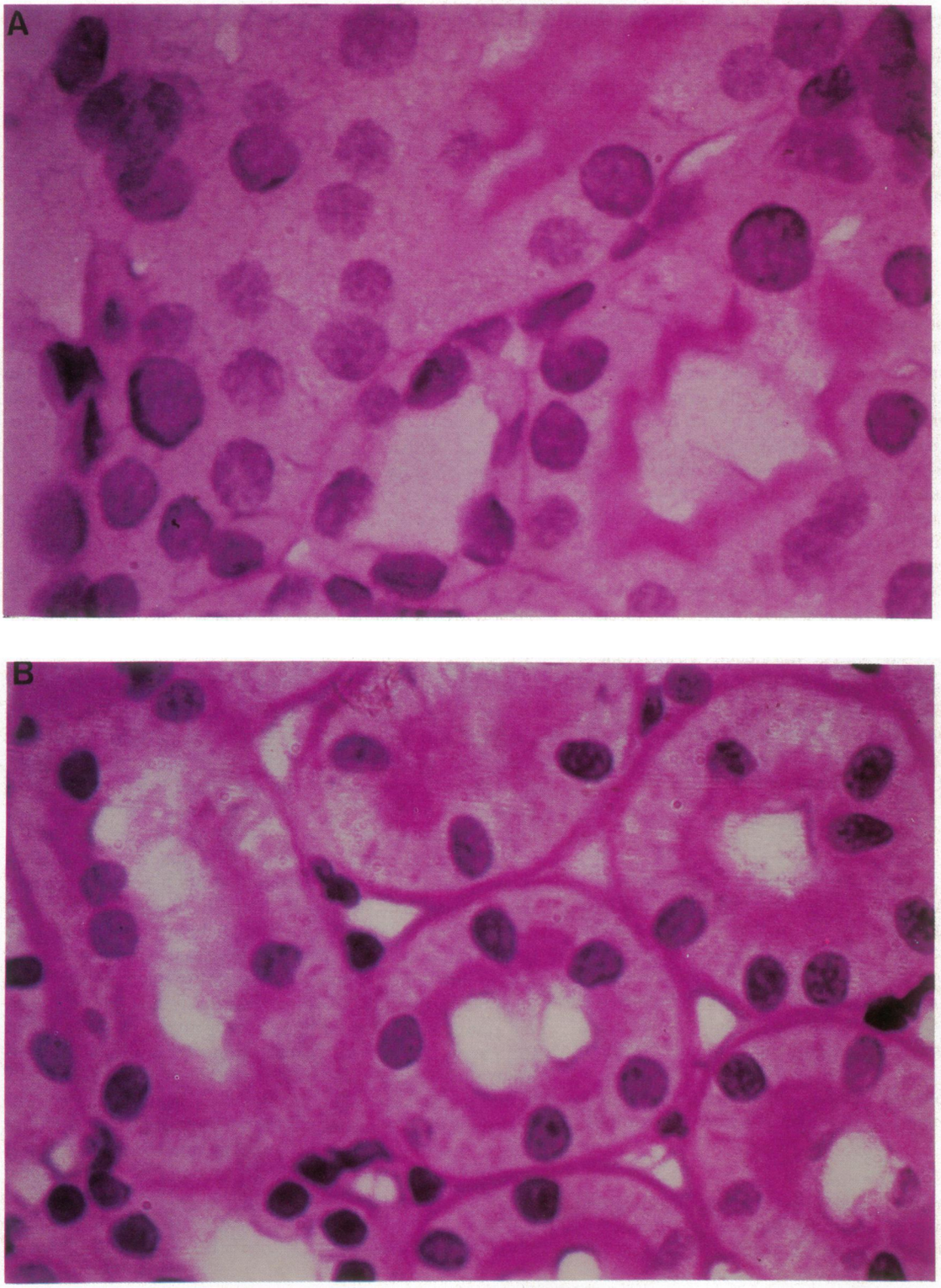


Figure 1. Angiotensinogen mRNA in the kidney (PAS stain). *A* shows kidney tissue incubated with control angiotensinogen probe and shows no hybridization (×100). *B* shows no hybridization in slides pretreated with RNAse (×100). In contrast, *C* shows positive hybridization of angiotensinogen cRNA in the proximal tubular cells (×100). Proximal tubule is identified by positive PAS staining in the luminal border. *D* shows heavy concentrations of grains in proximal tubules with minimal quantity of grains in the distal tubules (*arrows*). *P*, proximal tubules; *G*, glomerulus, *, distal tubule. As may be seen, *P* contains the vast majority of hybridization (×40).

Animals were anesthetized using ether and perfused in vivo via the left ventricle with 50 ml PBS followed by 50 ml of 4% paraformaldehyde. Tissue was placed in 4% paraformaldehyde, cleared in xylene, and embedded in paraffin. 5-μm sections were then cut on a rotary microtome and placed on poly-1-lysine–treated glass slides (0.01%) which were placed in 4% paraformaldehyde for 20 min, dipped in 3×

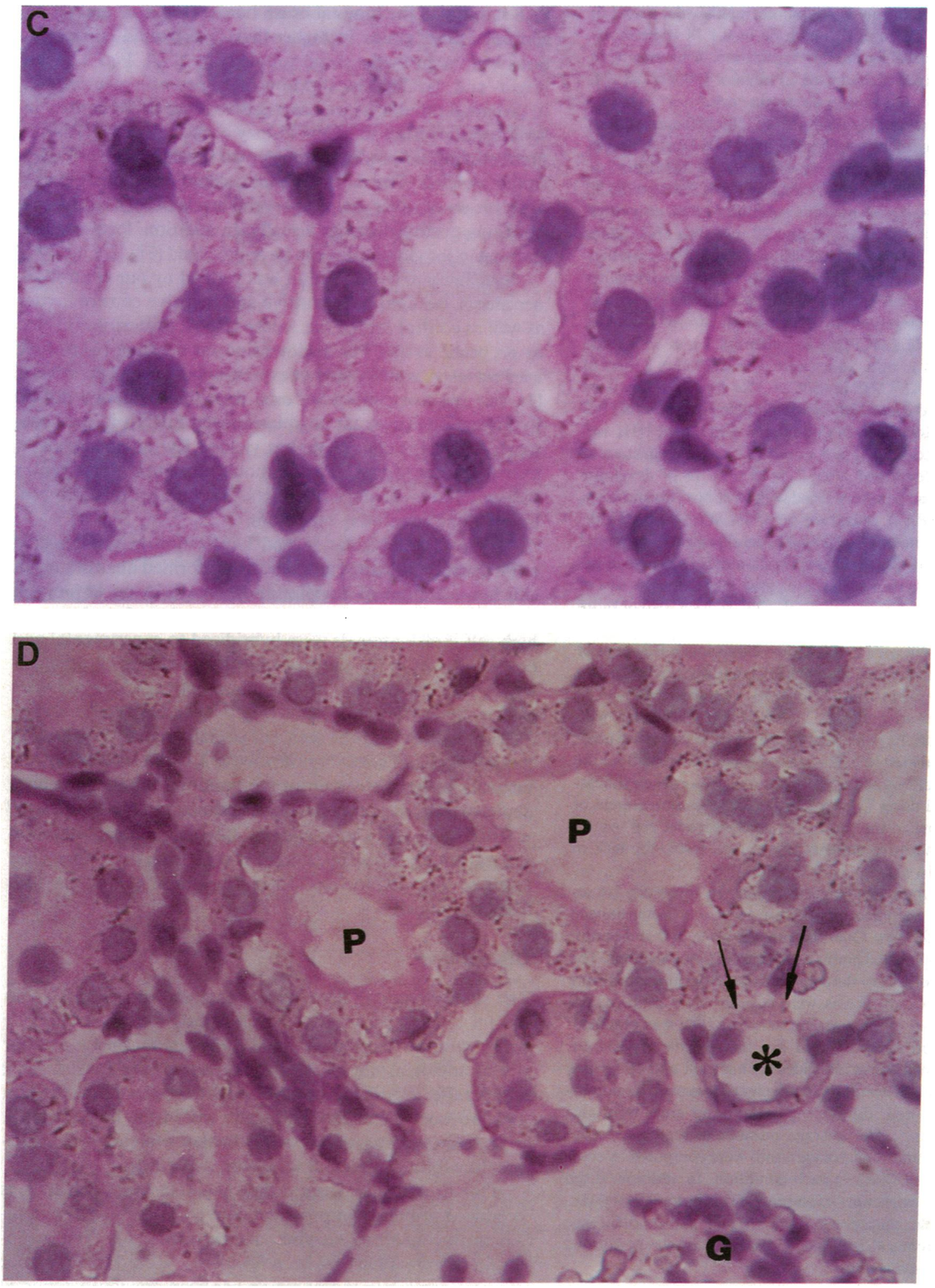


Figure 1. (Continued)

PBS, 1× PBS, 1× PBS for 5 min each, successively dehydrated with 30–100% ethanol, and stored at −20°C until hybridized.

Prehybridization. To prevent RNA degradation, all reagents were diethyl pyrocarbonate (DEPC; 0.1%) treated. All procedures, unless stated, were performed at room temperature. Slides were serially placed in 0.2 M HCl × 20 min, dH_2O for 5 min, and 2× standard saline citrate (SSC) for 15 min, followed by a 30-min wash in 0.1× SSC at 65°C (1× SSC = 0.15 M NaCl, 0.01 M Na citrate). After a 5-min dH_2O wash, slides were treated with proteinase K (50 μl/slide of 0.25 mg/ml proteinase K in 50 mM Tris HCl, pH 7.5, and 5 mM EDTA). Slides were rinsed in PBS, then fixed for 5 min in 4% paraformaldehyde, followed by PBS and glycine (2 μg/ml) PBS washes. Subsequently,

slides were again fixed for 20 min in 4% paraformaldehyde, rinsed in PBS, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min to reduce background hybridization. After rinsing in PBS, slides were successively dehydrated in ethanol (50, 60, 80, 94, 100%) with 0.3 M ammonium acetate for 5 min each.

Hybridization and posthybridization washes. 500,000 cpm/slide of ^{35}S-labeled cRNAs for angiotensinogen and control ^{35}S-labeled probes (see below) were used. Approximate volume/slide of hybridization mixture was 50 μl. Hybridization buffer contained 50% formamide, 30 mM NaCl, 5 mM EDTA, 1× Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% BSA), and 20 mM Tris HCl. Slides were prewarmed to 42°C and probe-containing solution was applied. Slides were incubated for 4 h at 42°C in a sealed, humidified container into which hybridization buffer was placed to maintain moisture. Slides were washed successively in 4× PBS, treated with RNAse A in 500 mM NaCl, 10 mM Tris HCl (pH 8) buffer for 1 h at 37°C, and rinsed in buffer alone for 30 min. Slides were then washed in 2 liters of 2× SSC for 30 min, and 0.1× SSC at 45°C × 2 for 45 min. Slides were dehydrated, air fixed, and dipped into photographic emulsion (NTB-2 [Eastman Kodak Co., Rochester, NY] diluted 1:1 in 0.6 M NH_4 acetate). They were air dried for 2 h and stored in complete darkness at 4°C. The slides were developed after 7–14 d with D-19 developer (Eastman Kodak Co.), and counterstained with hematoxylin and eosin or periodic acid-Schiff (PAS), examined on a Nikon brightfield/darkfield microscope, and photographed. For the identification of proximal tubular cells, PAS staining was performed to stain the glycocalyx of the brush border of these cells. For each probe, four to six separate sets of slides were studied, run on separate days. Within each set, two or more slides/probe were used.

Probe preparation. We used a 500-kb angiotensnogen fragment of pRang 1650, a rat angiotensinogen cDNA (27) that we subcloned into Bluescribe vector. After linearizing with Eco RI, we transcribed with T_3 polymerase to generate an antisense cRNA which we labeled with [^{35}S]UTP (400 Ci/mmol). As controls for in situ hybridization we used: (*a*) slides that were incubated in hybridization buffer alone without the addition of probe; (*b*) cRNA sense probe for angiotensinogen (linearizing with Hind III and using T_7 polymerase); (*c*) RNAse treatment of slides before hybridizing to the probe; (*d*) anglerfish islet cell insulin cRNA probe; or (*e*) negative control tissues, i.e., testis, for lack of angiotensinogen mRNA (1).

Quantitation: histologic review/grain counts. In this study we attempted to quantitate the effect of sodium diet on angiotensinogen mRNA expression as follows: Quantitation was done by hand-counting the number of radiolabeled grains over areas of tissue sections. The investigators were blinded as to the sodium diets of the animals during the quantitation. Individual grains were counted over 20 proximal tubules per slide and an average grain count per slide was obtained. Two slides per rat were counted, and five rats per dietary sodium condition were examined. Thus, 10 slides for high salt and 10 for low salt animals were counted. Similarly, equivalent areas over 10 glomeruli per slide were counted. Equivalent background areas were also counted on each section. The average number of grains/structure ± SEM (i.e., tubule, glomerulus, background) were computed by summing total counts for all slides in each condition. In addition, distal tubular segments and vessels were examined in this fashion, but quantitation was not feasible, since few grains were seen and no difference was apparent on different sodium diets. Unpaired *t* test was performed between high and low salt groups.

Results

Fig. 1 shows light microscopic studies of renal angiotensinogen localization on kidney sections studied simultaneously. Fig. 1 *A* demonstrates control "sense probe" and Fig. 1 *B* shows RNAse pretreatment. Similar sections hybridized with angiotensinogen cRNA revealed angiotensinogen mRNA localized primarily to the proximal convoluted tubules as identified by PAS staining of the luminal brush border (Fig. 1, *C* and *D*). In addition, silver grains in higher-than-background concentrations were seen in the distal tubule (Fig. 1 *D*). Fig. 2 shows bright- and darkfield localization of angiotensinogen mRNA in proximal tubules, as well as negative sense probe control in serial sections from the same kidney cortical sample. (RNAse pretreatment, no probe, and anglerfish insulin probe provided similar results). The darkfield microscopic technique permits observation of silver grains at several levels of focus, thus emphasizing positive findings visually. In addition, focal positive staining in the glomerulus can be appreciated (Fig. 2, *E* and *F*).

Specificity of hybridization was verified by our control experiments: we used both positive and negative control tissues as well as control probes (i.e., sense cRNA, anglerfish mRNA, or no probe at all). For angiotensinogen negative control we used rat testis, which does not express angiotensinogen mRNA (1). For positive control we used liver that exhibited diffuse hybridization signals for angiotensinogen mRNA in the hepatocytes, whereas control probes yielded few or no signals in liver (data not shown). Furthermore, no signals were seen in kidney tissues with anglerfish cRNA or with hybridization solution alone containing no probes.

To study the regulation and localization in salt-depleted animals and salt-loaded animals, we performed quantitative in situ hybridization of angiotensinogen mRNA on kidneys of rats fed low or high salt diets. Kidney sections from low- and high salt intake animals were hybridized to experimental and control probes in the same in situ runs to ensure comparability. Typical low-power darkfield microscopy of kidney sections (4×) is shown for salt-depleted (Fig. 3 *A*) and salt-repleted (Fig. 3 *B*) animals. As may be seen, the overall "signal" is greater in the salt-depleted state. Specifically, a 2.1-fold increase in the intensity of signals is seen in the proximal tubule of sodium depleted rats as compared with sodium replete animals ($P < 0.005$) (Fig. 3 *C*). No apparent differences in angiotensinogen mRNA signals were detected in other sites that express low levels of this mRNA, i.e., distal tubule and glomerular tufts (data not shown).

Discussion

The studies reported here localize angiotensinogen mRNA in the rat kidney, providing data previously not available. The demonstration that the proximal convoluted tubule is a major site of angiotensinogen mRNA expression is a novel finding which raises intriguing possibilities for the site of production and function of AII formed locally within the kidney. Thus, the present investigation provides further evidence and understanding for a complete RAS within the kidney.

Our in situ data suggest that angiotensinogen mRNA is produced primarily in proximal convoluted tubule where angiotensin-converting enzyme, angiotensin I, AII, and angiotensin receptors have all been found (4). Indeed, the presence of angiotensinogen in this region has been previously reported (9). While renin mRNA has been reported to be localized in the juxtaglomerular cells, renin has also been demonstrated by immunohistochemistry or by radioimmunoassay in cultured cells to be present in the afferent and efferent arterioles, interlobular arteries, mesangial cells, and proximal connecting and cortical collecting tubules (4, 28). It has also been demonstrated in interstitial fluid (13). The absence of renin mRNA in proximal and collecting tubules (23) suggests that the vast ma-

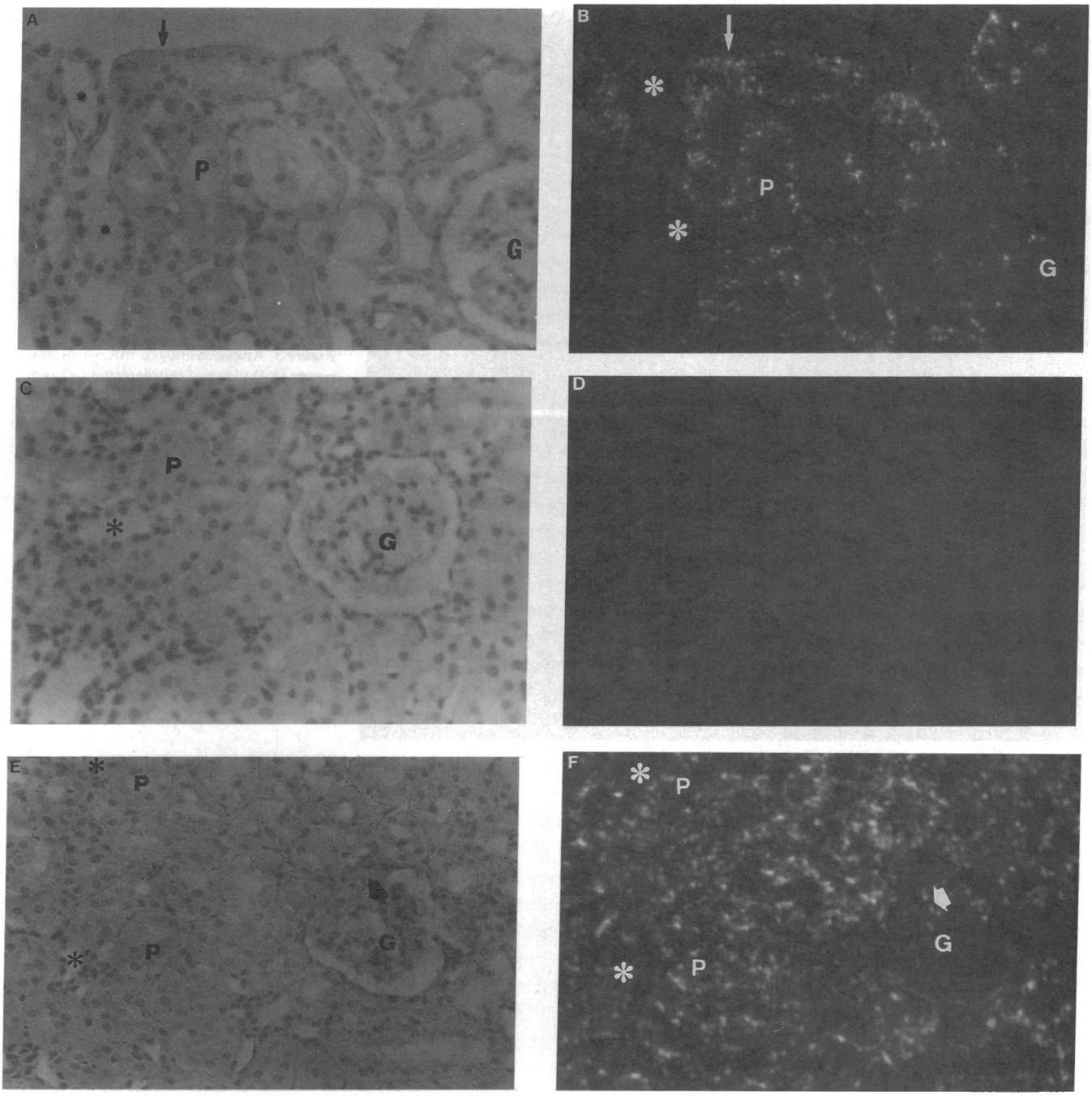
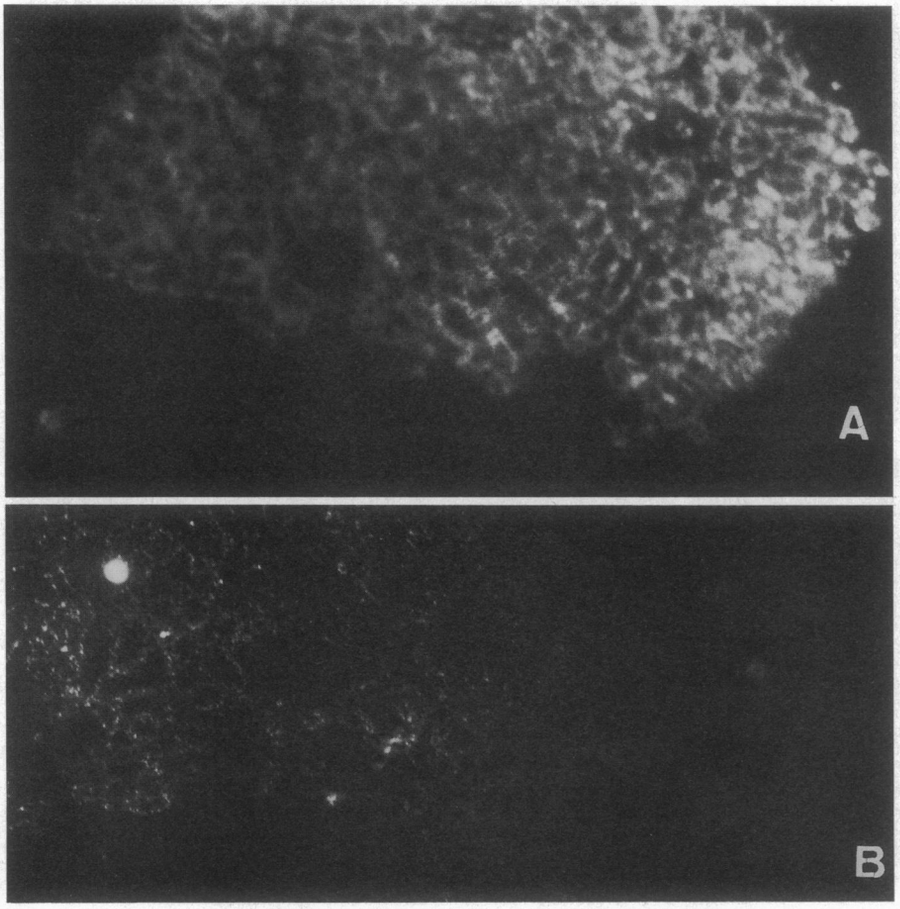


Figure 2. Angiotensinogen mRNA in proximal tubule is emphasized by darkfield microscopy. Two representative examples from separate experiments are shown. *A* shows brightfield and *B* darkfield of the same slide (ang-n probe) (×20). *C* and *D* show negative probe under brightfield and darkfield on the same slide. RNAse pretreatment, no probe at all, and anglerfish insulin probe gene gave similar negative results. *E* and *F* represent brightfield and darkfield of a separate experiment (×20). Note that in addition to positive signals in the proximal tubule, they are also seen focally in the glomerulus (*arrow*). *P*, proximal tubule; *G*, glomerulus; *, distal tubule.

jority of renin at these sites may be taken up or transported. With angiotensinogen present in proximal tubule, filtered renin, or renin delivered to this region by interstitial fluid, may produce local angiotensin I. Angiotensin I could subsequently form AII via local proximal tubular angiotensin converting enzymes present in brush border, basolateral membrane, or the surrounding interstitial fluid. AII could then activate local proximal tubular AII receptors.

AII has multiple effects on the proximal tubule. It has been recently reported that AII is a powerful stimulus for sodium and in bicarbonate resorption in the early proximal convoluted tubule S_1 segment (14). Using an isolated perfused rabbit tubule preparation, Schuster et al. (18) have demonstrated a direct effect of AII on the proximal convoluted tubule in which net volume flux is influenced. AII's effect on sodium transport has been shown to follow a similar pattern. Since AII does not



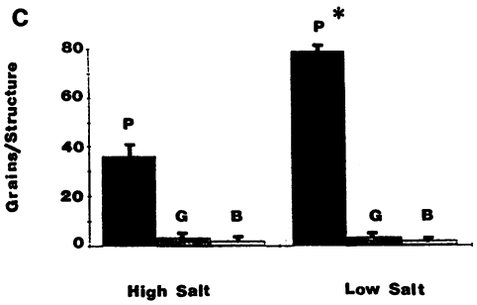


Figure 3. Angiotensinogen mRNA in low-power (×4) views of salt-depleted (*A*) and salt-repleted (*B*) animals. The overall intensity of grains is obviously higher in the salt-depleted kidneys. Quantitation of angiotensinogen mRNA concentration in various kidney structures under salt-depleted compared with salt-repleted conditions (see Methods) is shown in *C*. *y* axis shows grain counts/structure. Low salt–treated animals displayed a higher number of grains over the proximal tubule as compared with high salt. *P*, tubule; *G*, glomerulus; *B*, background; $*P < 0.005$, salt-replete vs. salt-deplete.

appear to be acting via an electrogenic mechanism, it might affect electroneutral Na^+Cl^- cotransport or Na^+/H^+ exchange. Recently Liu and Cogan (14) have reported that modulation of AII activity can greatly alter bicarbonate absorption in the early (S_1) proximal tubule, probably via a peritubular signal. In their studies, Cogan and Liu administered subpressor doses of AII or competitive inhibitor and noted that AII stimulated both sodium and bicarbonate reabsorption (14). Additionally, there is evidence for a luminal site of action for AII in the proximal tubule, as well as AII binding sites on the brush border and basolateral membrane (17, 18). Our data imply that the AII in the proximal tubule is probably generated from angiotensinogen locally available. Angiotensinogen expression within the proximal tubule would provide a ready local source of angiotensin. The high concentration of angiotensin I, AII, and renin in the interstitium suggests that this is possible. This physical location would provide a ready means for on site AII generation to modulate proximal renal tubular function.

Angiotensinogen mRNA is found in lesser amounts in glomeruli, distal tubule, and possibly intrarenal vessels. Chai et al. (29) have found a high density of AII receptors in glomeruli as well as vasa recta bundles. Angiotensin-converting enzyme is found in vascular endothelium throughout the kidney. Thus, AII produced via the local RAS might have a role in glomerular contractility, glomerular tubular feedback, and distal tubular function.

Additional studies of regulation of these mRNA species may be helpful in ultimately determining the functional aspects of their posttranslational products. The present study demonstrates that a low sodium diet results in an increase in angiotensinogen mRNA content, primarily in the proximal tubular cells. This finding would suggest an increased produc-

tion of local angiotensin, which may play a role in reestablishing sodium homeostasis. By combining quantitative in situ hybridization studies with physiological perturbations, the cell-specific function of the intrarenal RAS may be further elucidated.

Acknowledgments

We thank Ms. Donna MacDonald and Ms. Judy Wright for their secretarial assistance.

This work is supported by National Institutes of Health (NIH) grants HL-35610, HL-35792, HL-19259, HL-35252, and HL-40210, NIH Specialized Center of Research in Hypertension grant HL-33697, the Milton Fund, and a grant from RJR-Nabisco, Inc.

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