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Detection and Localization of Renin Messenger RNA in Human Pathologic Tissues Using In Situ *Hybridization*

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In order to investigate the synthesis of renin in human pathologic tissues, the authors used *in situ* hybridization to detect and localize renin messenger RNA (mRNA). The probe was a ^{35}S-radiolabeled 1.1-kb length complementary DNA of human renal renin. To compare the synthesis with the presence and the storage of renin, renin antigen was assessed by immunohistochemistry in the same tissues. The human pathologic tissues were as follows: two ischemic kidneys related to renovascular hypertension; two renal juxtaglomerular cell tumors; one extrarenal renin-secreting epithelioid sarcoma of soft tissues. In ischemic kidneys, the cells containing both renin mRNA and renin protein were found in numerous juxtaglomerular apparatus and in the wall of arterioles, shown by combined *in situ* hybridization and immunhistochemistry. Most of the tumor cells in the juxtaglomerular cell tumors and scarce tumor cells in the epithelioid sarcoma of soft tissues were positive by *in situ* hybridization and immunhistochemistry. These findings demonstrate that the presence of renin in these tissues is associated with local cellular production of renin. In particular, smooth muscle cells of the wall of arterioles are definitely capable of synthesizing renin. Moreover, in these tissues, gene expression (renin synthesis) and renin storage are concordant. (Am J Pathol 1988, 131:320–330)

RENIN PLAYS an important role in the control of blood pressure and salt and water balance. In normal conditions the main source of human renin is the juxtaglomerular apparatus in the kidney. Renovascular hypertension[1] and hypertension caused by renal juxtaglomerular cell tumors[2] are pathologic circumstances in which hypertension is clearly associated with an increased renal production of renin, mainly active renin.[3] Besides the renal renin system, reninlike enzymes have been described in other organs such as brain,[4] mouse submaxillary glands,[5] and fetal membranes.[6] Moreover, occasional extrarenal tumors have been rarely reported as sources of renin,[7–10] mainly secreted under the form of inactive prorenin.[11,12] *In situ* hybridization allows us to detect gene products such as messenger RNA (mRNA) encoding for hormones or enzymes and to localize them at the cellular level in tissue sections. Using *in situ* hybridization and immunohistochemistry, we investigated renin synthesis and storage in various pathologic human tissues implicated in an increased production of renin leading to hypertension.

Materials and Methods

Patients

Two patients presented with severe renovascular hypertension associated with unilateral renal artery stenosis. In one of the patients, plasma renin activity was 15 ng Angiotensin I/ml/hr. Renal tissue was obtained during surgery. Histopathologic study exhibited moderate diffuse nephroangiosclerosis.

Two juxtaglomerular cell tumors were obtained at

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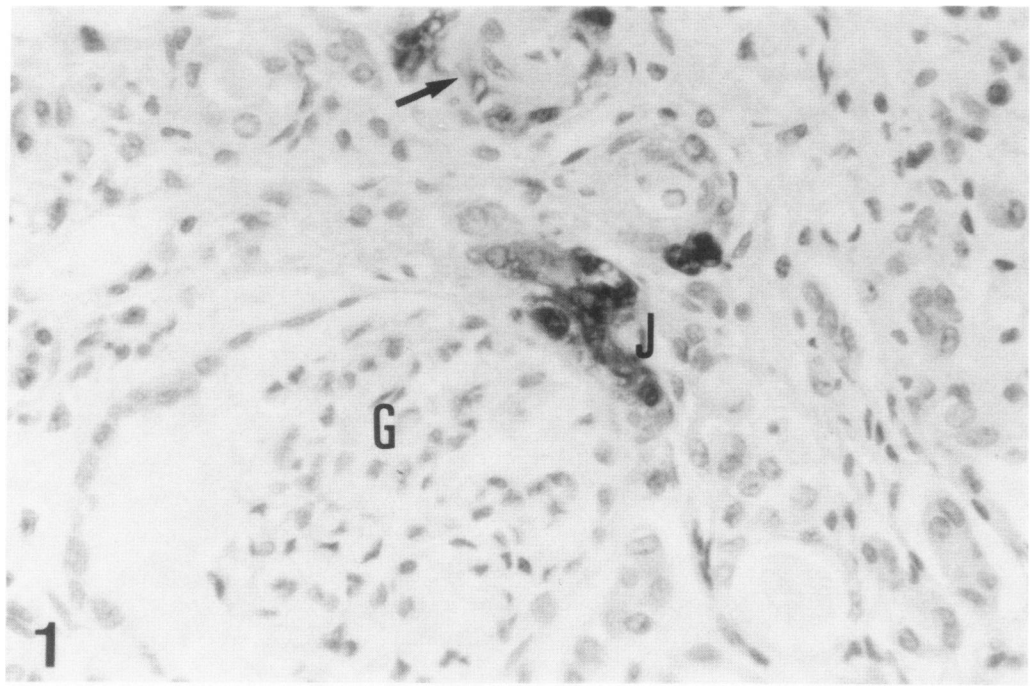


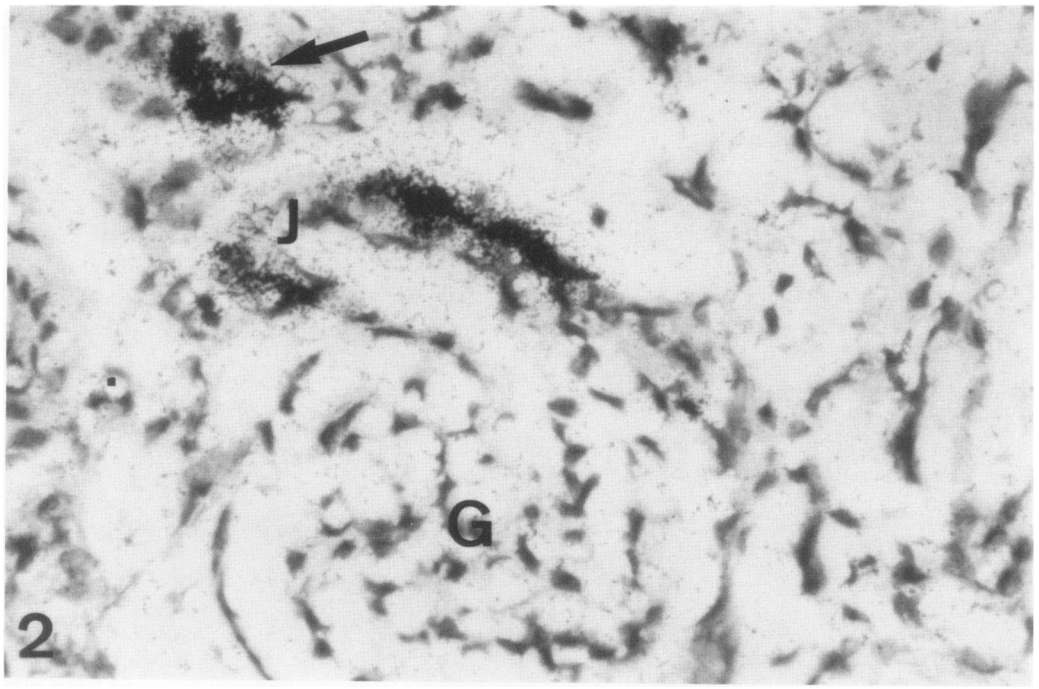


Figure 1—Ischemic kidney tissue section. The anti-human renin antibody strongly labels the juxtaglomerular apparatus (*J*) and the wall of an afferent arteriole (*arrow*). *G*, glomerulus. (Immunohistochemistry with PAP technique, ×500) **Figure 2**—Ischemic kidney tissue section. Demonstration of renin mRNA in the juxtaglomerular apparatus (*J*) and in an afferent arteriole (*arrow*). *G*, glomerulus. (*In situ* hybridization in formalin fixed frozen tissues, autoradiography, and Giemsa stain, ×500)

surgery from two patients with hypertension. The plasma total renin levels were 4000 pg/ml and 1400 pg/ml with, respectively, 91% and 61% of inactive renin. In the first tumor, tissue renin was 130 μg/g of tissue.

A 28-year-old female patient presented with hypertension and an extrarenal renin-secreting tumor. This tumor was characterized by unilateral pulmonary metastases from an epithelioid sarcoma of the soft tissues[13,14] of the thigh operated upon 6 years before. Plasma active renin was 200 pg/ml (normal values, 16.5 ± 6.4 pg/ml), and plasma total renin was 3000 pg/ml. Tumoral tissue was obtained from unilateral pneumectomy. In the tumor, tissue renin was 4 μg/g

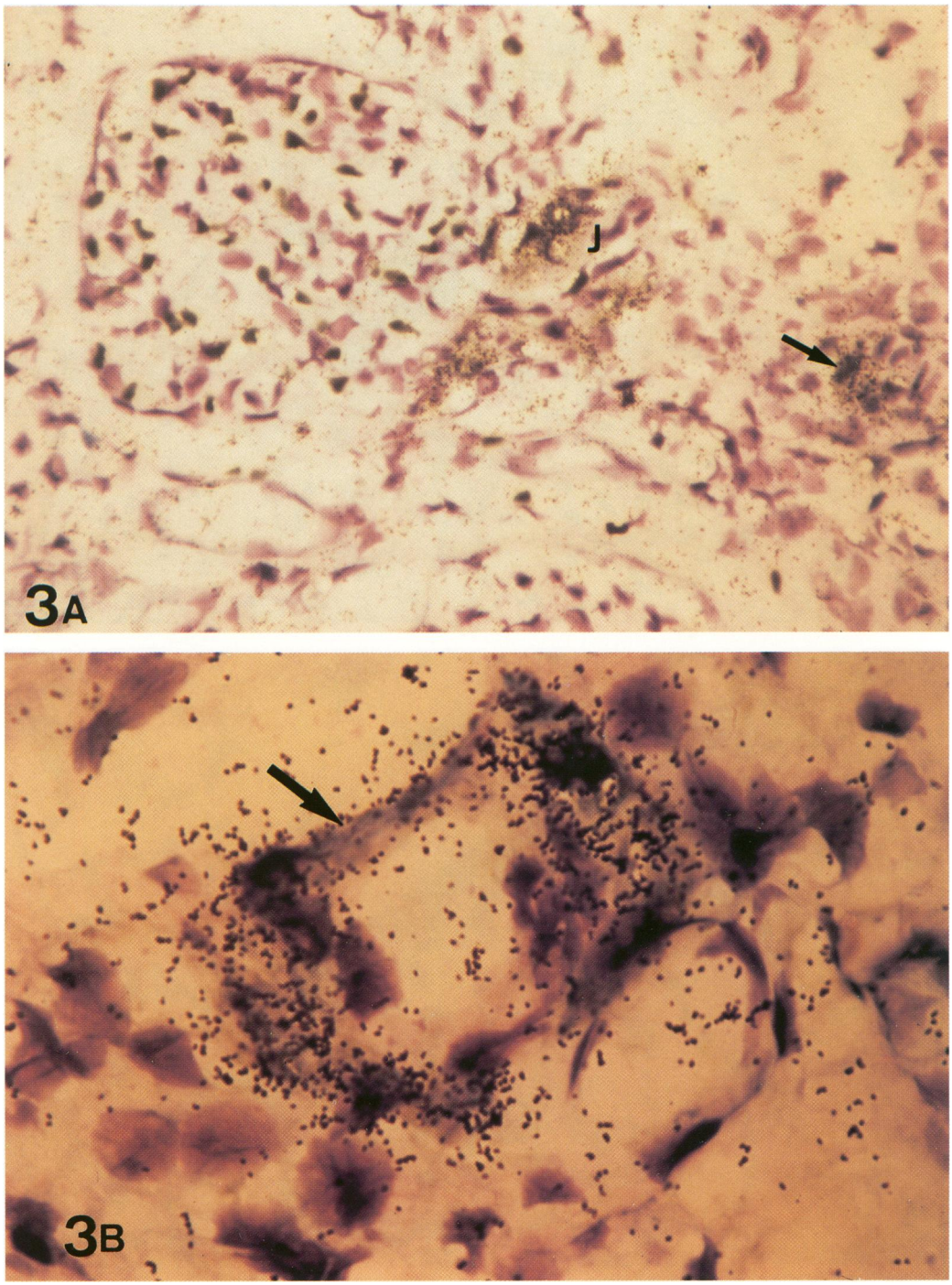


Figure 3A and B—Ischemic kidney tissue section. Combination of immunohistochemistry and *in situ* hybridization. Renin protein is characterized by a kaki reaction product, and renin mRNA is visualized by silver grains in the same cells of the juxtaglomerular apparatus (*J*) and arteriole (*arrow*). (Immunohistochemistry with PAP technique and *in situ* hybridization in formalin-fixed frozen tissues, **A,** ×320, and **B,** ×1200)

of tissue. Fixed paraffin-embedded tissue of the primary tumor was available from the surgical pathology files.

Electron Microscopy

Small blocks of tissue from the two juxtaglomerular cell tumors and the pulmonary metastasis of the epithelioid sarcoma were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) 0.1 M (pH 7.4). After postfixation in 2% osmium tetroxide, the tissue samples were routinely processed for electron microscopy.

Immunohistochemistry

In all cases, tissues were fixed in aqueous Bouin's solution, embedded in paraffin, and tested with spe-

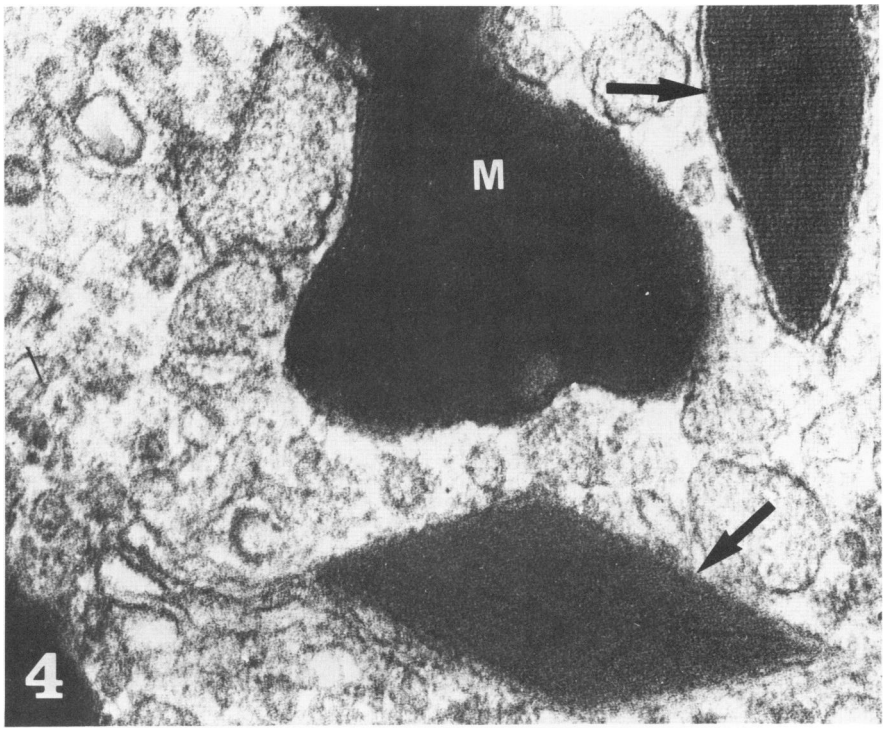


Figure 4—Juxtaglomerular cell tumor. Membrane-bound protogranules (*arrows*) and mature granules (*M*) in the cytoplasm of a tumor cell. (Electron microscopy, ×95,000)

cific rabbit anti-human renin antibody.[15] The paraffin was removed from the slides by dipping in xylene and ethanol three times for 5 minutes each bath. The unlabeled antibody peroxidase–antiperoxidase technique (PAP) was employed[2] with use of the anti-renin antibody at a dilution of 1:1000 and a commercial PAP kit (Biolyon, Lyon, France) following the steps recommended by the merchant. In the case of the pulmonary metastasis of the epithelioid sarcoma, the reactivity of the tumoral tissue to cytokeratin and Factor 8-related antigen (F8-RAg) was assessed. Monoclonal antibody to cytokeratin KL1 (Immunotech, laboratoires Luminy, Marseille, France) was used at a dilution of 1:100 and revealed with the alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique.[16] Briefly, the tissue sections were incubated for 30 minutes with KL1 antibody, rinsed in Tris buffer 0.05 M (pH 7.4), incubated with rabbit anti-mouse immunoglobulin antibody (Dako) at a dilution of 1:20 for 30 minutes and rinsed in Tris buffer. The sections were then incubated with APAAP complex (Dako) at a dilution of 1:50 for 30 minutes, washed in Tris buffer, and finally incubated with the alkaline phosphatase substrate containing 1 ng naphthol-AS-MX phosphate (Sigma), 100 μl dimethyl formamide, 4.9 ml Tris buffer 0.1 M (pH 8.2), levamisole 1 M, and 5 mg Fast Red TR (Sigma). The F8-RAg polyclonal antibody (Dako) was used at a dilution of 1:1000 and revealed with the PAP technique as described above.

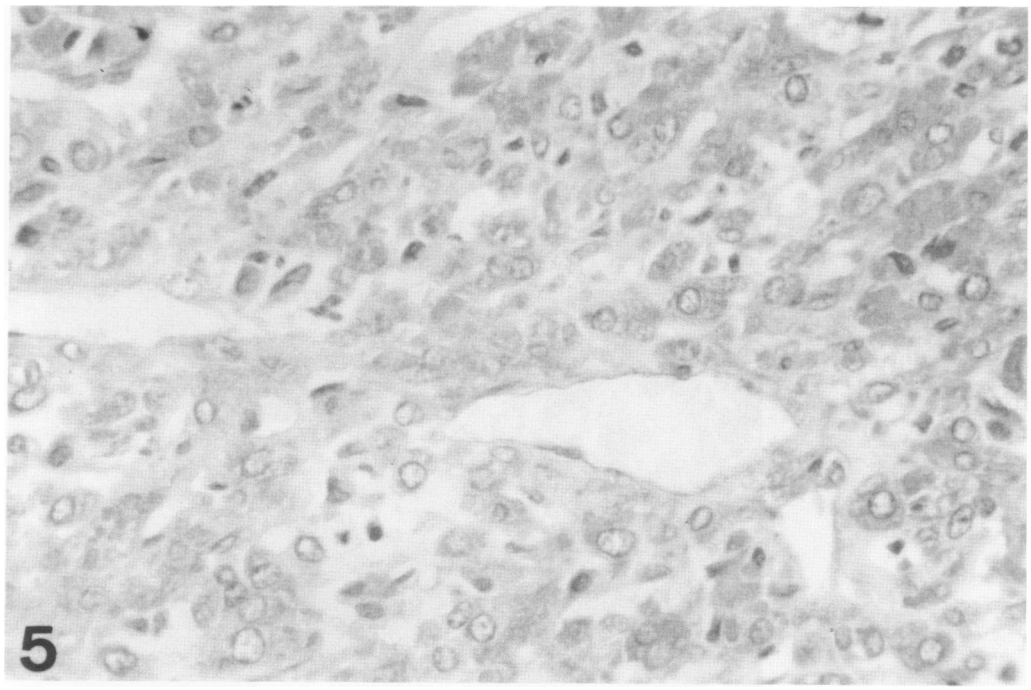
Controls consisted of the omission of the specific antibody.

Preparation of the Probe

A human cDNA fragment of 1.1-kb length, clone pHRn22,[17] was used as probe. The purified insert was labeled by the random primer elongation method,[18] with a commercially available kit from Amersham International UK. With ^{35}S-dCTP (400 Ci/mmol), the specific activity of the probe was about 2.10^8 cpm/μg of DNA. For control experiments, the plasmid pBR322, cut by the restriction endonuclease Alu I, was labeled under the same conditions to a similar specific activity and used as probe.

Preparation of the Tissues for *In Situ* Hybridization

For all the cases, tissues were rapidly obtained from the operative room, frozen, and stored in liquid nitrogen. Five-micron-thick frozen sections were fixed in 4% formalin in PBS 0.1 M (pH 7.4) for 15 minutes, dehydrated in graded alcohols, and stored at −20 C up to a few months. For the two ischemic kidneys and the renin-secreting metastatic epithelioid sarcoma, tissue samples were immediately fixed in 4% formalin in PBS for 3–6 hours at 4 C. One part of the specimens was washed in PBS, frozen in liquid



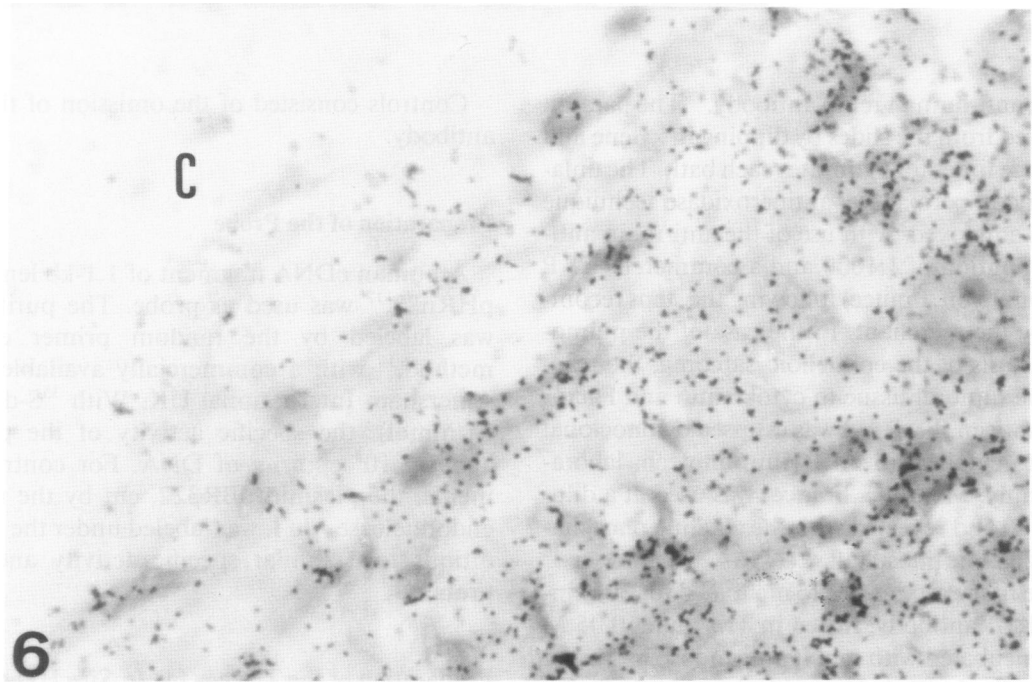


Figure 5—Juxtaglomerular cell tumor. The anti-human renin antibody labels most of the cytoplasms of the tumor cells. (Immunohistochemistry with PAP technique, ×500) **Figure 6**—Juxtaglomerular cell tumor. Detection of renin mRNA in the tumor cells. No signal is observed in the capsule (*C*). (*In situ* hybridization in Bouin's solution-fixed paraffin-embedded tissues, autoradiography, and Giemsa stain, ×1200)

nitrogen, and cut with a cryostat. The other part was dehydrated in alcohol and embedded in paraffin. As for immunohistochemistry, tissue samples fixed in Bouin's solution and embedded in paraffin were available for *in situ* hybridization. Paraffin was carefully removed.

All the paraffin or frozen sections were harvested on clean sterilized glass slides. In order to increase adhesion of sections of fixed tissues on glass slides, the sections were covered with a solution of collodion (Collodion 4%, Merck) containing 1 volume of collodion, 25 volumes of ether, and 25 volumes of alcohol.[19] Un-

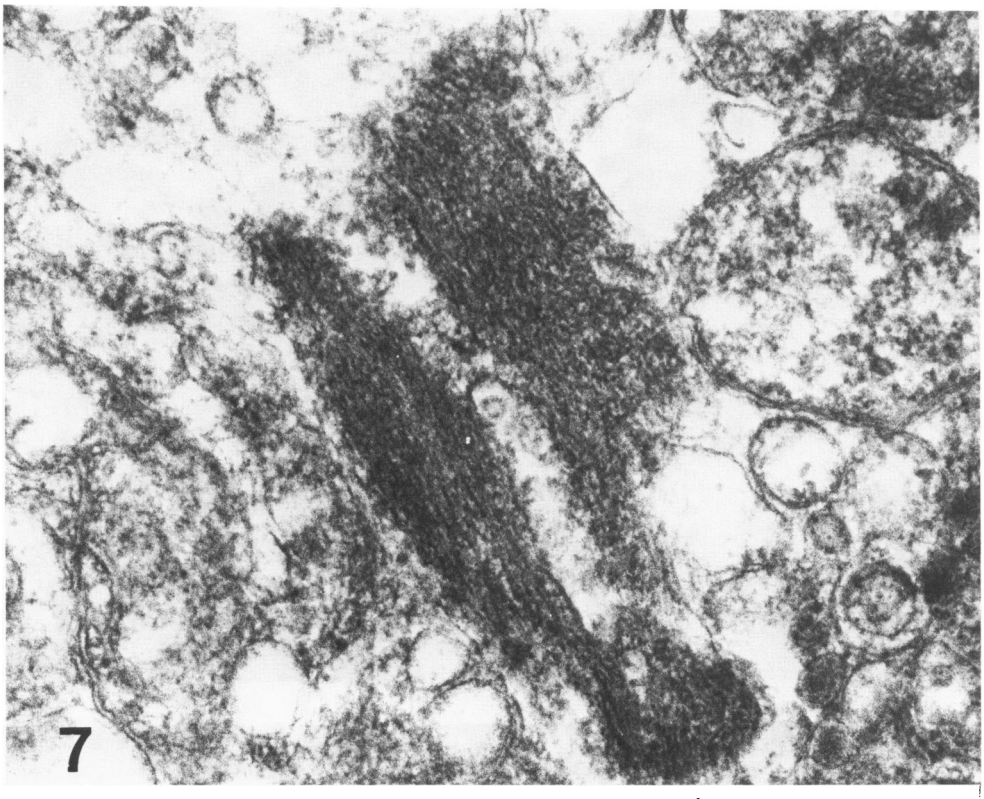


Figure 7—Renin secreting pulmonary metastasis of an epithelioid sarcoma of the soft tissues. Membrane-bound paracrystalline granules in the cytoplasm of a tumor cell. (Electron microscopy, ×95,000)

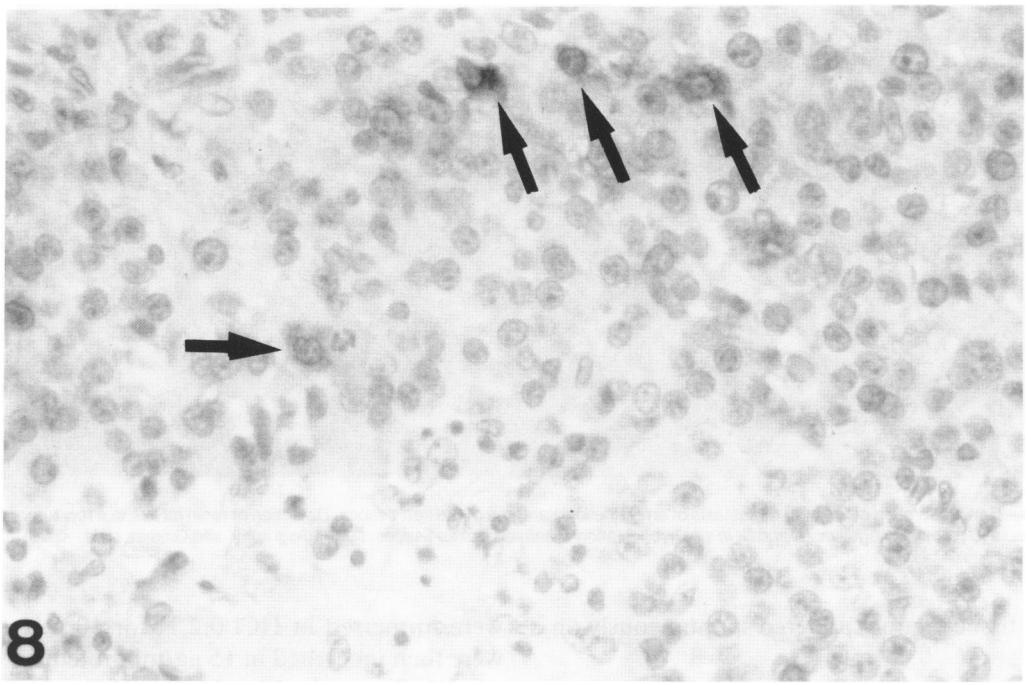


Figure 8—Renin-secreting pulmonary metastasis of an epithelioid sarcoma of the soft tissues. The anti-human renin antibody labels a few tumor cells. (Immunohistochemistry with PAP technique, ×500)

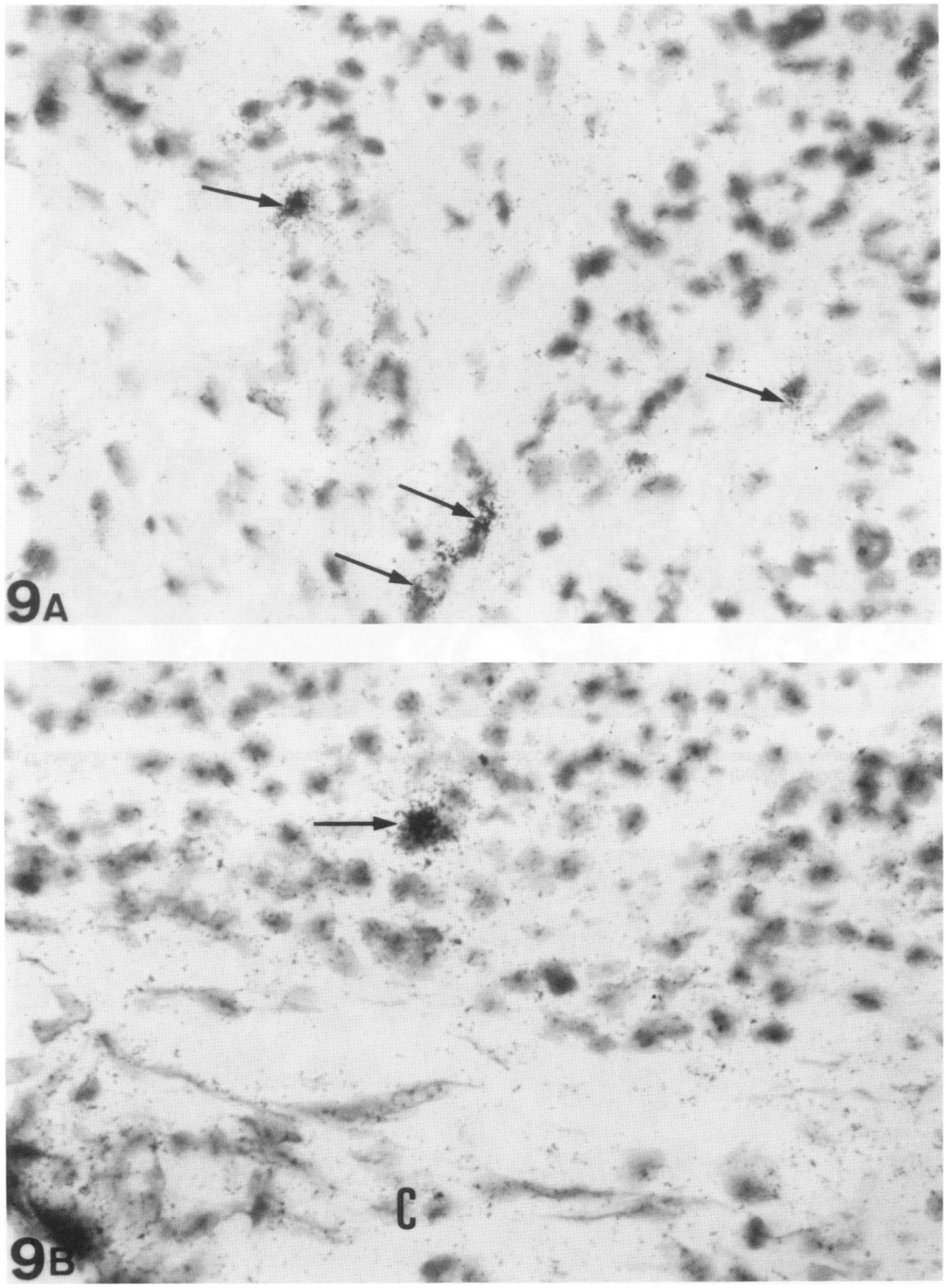
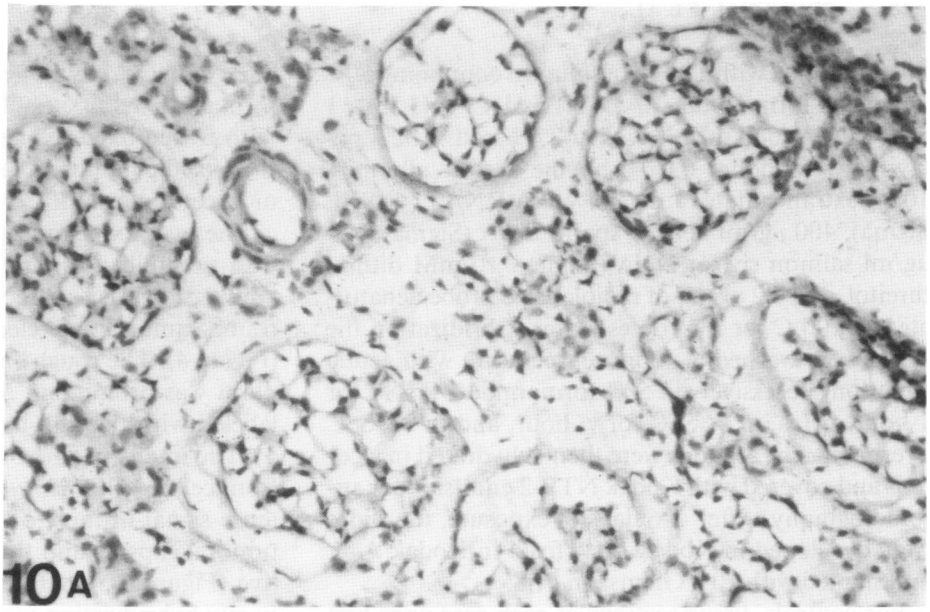


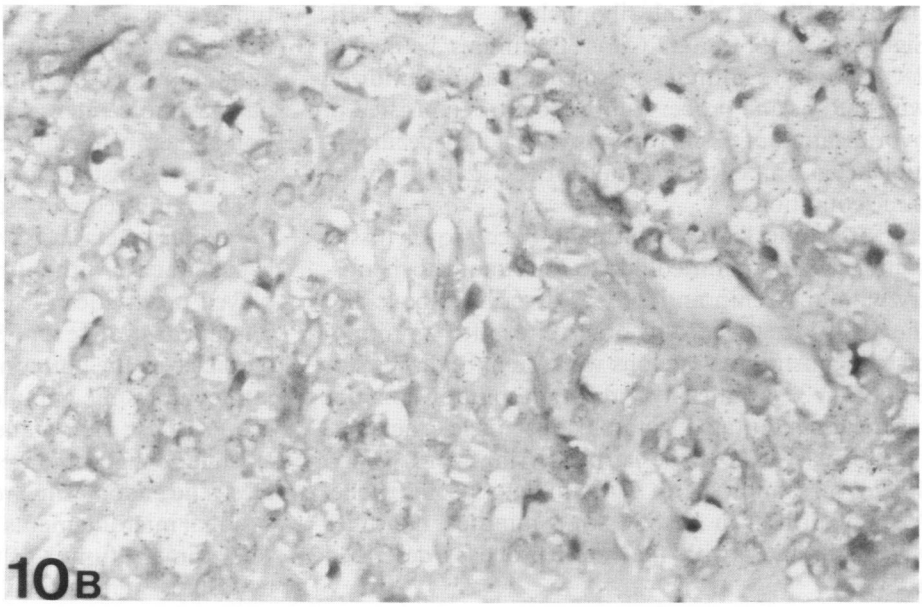
Figure 9A and B—Renin-secreting pulmonary metastasis of an epithelioid sarcoma of the soft tissues. Detection of renin mRNA in a few tumor cells (*arrows*), whereas the capsule (*C*) remains negative. (**A** and **B**, *in situ* hybridization in unfixed frozen tissues, autoradiography, and Giemsa stain, ×500)

fixed frozen tissue sections adhered spontaneously on glass slides.

In Situ Hybridization

In situ hybridization was performed by a method derived from Fournier et al.[20–22] The tissue sections were immersed in HCl 0.2 N for 10 minutes. They were then incubated in 15 μg/ml proteinase K (Protease XI, Sigma) in 20 mM Tris-HCl (pH 7.4) and 2 mM calcium chloride at 37 C for 15 minutes. After permeation, the sections were washed in distillated water and dehydrated in alcohols. They were then hybridized under a sealed coverslip for 24 hours at 37 C





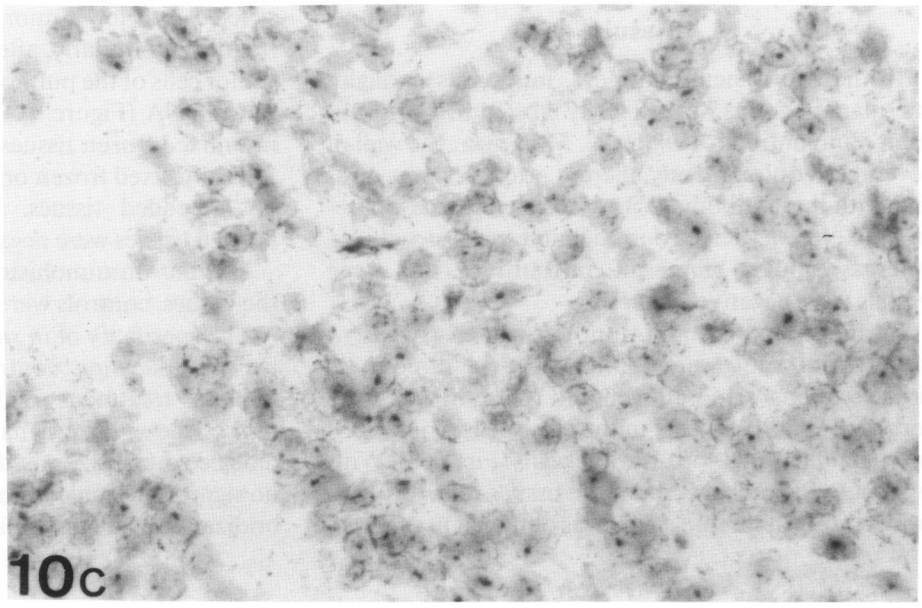


Figure 10A–C—Controls of the specificity of *in situ* hybridization. No signal is detected in cells. **A**—Ischemic kidney tissue section hybridized with pBR322. **B**—Juxtaglomerular cell tumor. **C**—Renin-secreting pulmonary metastasis of an epithelioid sarcoma of the soft tissues treated with ribonuclease. (*In situ* hybridization, preparation of tissues the same as in the positive tests, autoradiography, and Giemsa stain, **A,** ×230; **B,** ×500; **C,** ×500)

in a moist box with 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 tRNA (Type III, Sigma), 400 μg/ml herring sperm DNA (Sigma), 400 μg/ml salmon sperm DNA (Sigma), 10 mM dithiothreitol, and 0.2 ng/ml of radiolabeled probe denaturated at 100 C for 2 minutes. After hybridization the slides were washed at room temperature with gentle agitation successively in 50% formamide–4× SSC for 1 hour, twice in 2× SSC for ½ hour, and in 2× SSC overnight. The sections were then dehydrated in alcohols and covered with Kodak NTB-2 emulsion for autoradiography. After 4–6 days of exposure, the slides were developed in Kodak D19, fixed in Kodak A44, and stained with 10% Giemsa in PBS.

For controls, tissue sections of each case were first treated with 50 μg/ml ribonuclease A (Type III, Sigma) in 2× SSC at 37 C for 30 minutes, washed in 2× SSC for 15 minutes, permeated, and hybridized with the specific probe. Another control consisted of hybridization of tissue sections with a nonspecific radiolabeled probe, the plasmid pBR 322 used in the same conditions.

In ischemic kidneys, formalin-fixed frozen tissue sections were submitted first to immunohistochemistry with anti-renin antibody as described above and then hybridized after this procedure. After immunohistochemistry, the sections were prehybridized[23] for 2 hours at 37 C with a hybridization solution containing heat-denaturated heterologous nucleic acids (2 mg/ml yeast tRNA, 400 μg/ml herring sperm DNA, 400 μg/ml salmon sperm DNA) and no specific probe. After two rinses in 2× SSC for 5 minutes, the slides were hybridized with the renin probe as described above.

Results

In the two ischemic kidneys, numerous juxtaglomerular apparatus were strongly labeled with anti-human renin antibody (Figure 1). Moreover, the wall of several arterioles, mainly afferent arterioles, was positive with this antibody. With *in situ* hybridization, renin mRNA was detected in the same renal structures, whereas tubules, glomeruli, interstitium, and large vessels were negative (Figure 2).

Furthermore, when immunohistochemistry and *in situ* hybridization were combined in the same renal tissue sections, renin antigen and renin mRNA were localized simultaneously in the same cells of juxtaglomerular apparatus and arteriole walls (Figure 3). With respect to the preparation of the tissues for *in situ* hybridization, an intense signal was obtained in the ischemic kidneys only in frozen unfixed tissues or frozen formalin-fixed tissues. Tissue preservation was better with frozen fixed tissues. Formalin- or Bouin's solution-fixed paraffin-embedded tissues yielded negative or uncertain results.

The two juxtaglomerular cell tumors exhibited ultrastructurally a variable amount of intracytoplasmic granules, either paracrystalline protogranules or round, amorphous, electron-dense mature granules (Figure 4). The rough and smooth endoplasmic reticulum was prominent. Most of the tumor cells were labeled with the anti-human renin antibody (Figure 5). The intensity of the immunolabeling was variable from cell to cell. *In situ* hybridization detected an intense signal in most of the tumor cells, whereas the tumoral capsule and the adjacent kidney were negative (Figure 6). Contrary to immunohistochemistry, *in situ* hybridization yielded an even signal in all the tumor cells. For the two juxtaglomerular cell tumors, unfixed frozen tissues and Bouin's solution-fixed paraffin-embedded tissues were tested. Although the signal was more intense in unfixed frozen tissues, fixation and embeddeding combined a satisfactory signal and a better histologic preservation.

The renin-secreting sarcoma of soft tissues and its pulmonary metastasis were made of masses of irregular ovoid cells without necrosis. The tumor cells displayed positivities for cytokeratin, whereas the cells remained negative for F8-RAg. At the electron-microscopic level, the pulmonary metastasis showed scanty intracytoplasmic granules in scarce tumor cells (Figure 7). These membrane-bound granules had a paracrystalline shape and were consistent with renin granules. The endoplasmic reticulum was prominent. Occasional microfilaments were observed in cytoplasms. The primary tumor of the thigh and its pulmonary metastasis were labeled with immunohistochemistry with anti-human renin antibody. The labeling was restricted to a few tumor cells scattered in the tumor (Figure 8). Similarly, after *in situ* hybridization, sparse tumor cells of the pulmonary metastasis contained renin mRNA (Figure 9). The signal was more intense in unfixed frozen tissues but remained satisfactory in formalin-fixed frozen or Bouin's solution-fixed paraffin-embedded tissues. Formalin-fixed paraffin-embedded tissues were negative.

For the immunohistochemical procedures, in all the tissues, controls were negative.

The specificity of *in situ* hybridization was assessed by the following controls (Figure 10). The ribonuclease treatment resulted in the absence of detection of the specific signal. Hybridization with the radiolabeled pBR322, used as a nonspecific probe, gave no significant signal in the tissues. Moreover, after hybridization with the renin probe, the signal was con-

stantly detected in the same particular tissue structures, whereas other structures, such as adjacent lung or kidney, tumoral capsule, renal tubules were negative.

Discussion

Our results demonstrate that human renin mRNA can be detected in various human pathologic tissues by *in situ* hybridization. In this study, the importance of the preparation of tissues for *in situ* hybridization is emphasized. We observed that a light formalin fixation, either after cryostat sectionning or in whole specimens, yielded the more intense signal. After fixation and paraffin embedding, a less intense signal could inconstantly be detected, as observed by others.[21,24] All the specimens lightly fixed in formalin and embedded in paraffin were negative, suggesting that, if paraffin embedding is required, the fixation of tissues must be strong enough to stabilize mRNA and resist through the steps of embedding. Thus, when *in situ* hybridization is negative, the role of the tissue preparation must be questioned before the absence of a specific mRNA is certain.

In ischemic kidneys, *in situ* hybridization localized renin mRNA in numerous juxtaglomerular apparatus and in the walls of arterioles, probably in smooth muscle cells of afferent arterioles. So far, in human tissues, renin system has been investigated morphologically by immunohistochemistry.[15,25,26] Immunohistochemical studies are in agreement with our immunolocalization of renin in an increased number of juxtaglomerular apparatus and in recruited arterioles of ischemic kidneys. However, immunohistochemical detection of an antigenic protein is unable to differentiate local synthesis from endocytosis. When renin is detected by immunohistochemistry in juxtaglomerular apparatus of ischemic kidneys, renin synthesis in this structure is not questionable because the juxtaglomerular apparatus is the well-established site of renin synthesis in the kidney.[27] When renin is detected by immunohistochemistry, outside the juxtaglomerular apparatus, in arterioles, the actual synthesis of renin needs other demonstration. *In situ* hybridization definitely demonstrates that the cells of these structures are capable of synthesizing renin. In particular, the smooth muscle cells of the wall of arterioles are involved in the synthesis of renin. This fact is in agreement with the smooth muscle cell origin of the juxtaglomerular apparatus cells.[27] In ischemic kidneys, *in situ* hybridization and immunohistochemistry were combined in the same tissue sections. This resulted in the simultaneous localization of renin mRNA and renin protein in the same cells of juxtaglomerular apparatus and arterioles. Thus, in these cells renin production and renin storage are concordant. Recently, in agreement with our results, *in situ* hybridization performed in rats with a mouse submaxillary gland renin cDNA, localized renin mRNA in the juxtaglomerular apparatus of the kidney of normal rats.[28]

In the renin-secreting tumors, the signal detected by *in situ* hybridization shows that tumor cells are sites of renin synthesis. The tumor cells are similarly labeled by immunohistochemistry. This suggests that the same tumor cells are involved in renin production and in renin storage. In the juxtaglomerular cell tumors, the intensity of the immunolabeling is variable as well as the amount of cytoplasmic renin granules reflecting the cellular variability of renin secretion. This contrasts with the hybridization signal evenly distributed in all the tumor cells, suggesting that all the tumor cells are equally implicated in renin synthesis.

The renin-secreting sarcoma of soft tissues has histologic and immunohistochemical features of an epithelioid sarcoma of soft tissues.[14,29] Renin secretion has not been previously reported in this tumor, the histogenesis of which is unknown. In this case, renin synthesis cannot argue for any cellular origin of the epithelioid sarcoma of soft tissue, because several tumoral cell types have been involved in renin synthesis,[7-10] and the regulation of gene expression may be modified in malignant cells.

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