Tubular Localization and Tissue Distribution of Peptide Transporters in Rat Kidney

David E. Smith,[1,4] Anna Pavlova,[2] Urs V. Berger,[2]
Matthias A. Hediger,[2] Tianxin Yang,[3]
Yuning G. Huang,[3] and Jürgen B. Schnermann[3]

Purpose. To define the tubular localization and tissue distribution of PEPT1 (low-affinity, high-capacity transporter) and PEPT2 (high-affinity, low-capacity transporter) in rat kidney.
Methods. mRNA expression of PEPT1 and PEPT2 was assessed with reverse transcription-polymerase chain reaction (RT-PCR) methods using cDNA prepared from microdissected nephron segments in rat. Tissue localization of rat renal PEPT1 and PEPT2 mRNA was further assessed by in situ hybridization with radiolabeled probes.
Results. RT-PCR analysis of microdissected segments from rat nephron showed that both PEPT1 and PEPT2 are confined to the proximal tubule. While PEPT1 is specific for early regions of the proximal tubule (pars convoluta), PEPT2 is overwhelmingly but not exclusively expressed in latter regions of the proximal tubule (pars recta). All other segments along the nephron were negative for PEPT1 or PEPT2 mRNA transcripts. These findings were supported by in situ hybridization results in which PEPT1 was selectively expressed in kidney cortex and PEPT2 in the outer stripe of outer medulla.
Conclusions. Contrary to current opinion, the data suggest that peptides are handled in a sequential manner in proximal regions of the nephron, first by the low-affinity, high-capacity transport system and second by the high-affinity, low-capacity transport system.

INTRODUCTION

Epithelial cells of mammalian intestine and kidney possess oligopeptide transporters in their apical membranes that are responsible for the absorption of peptide-bound amino acids and peptide-like drugs (1). In contrast to the small intestine in which there is no evidence for kinetic or molecular heterogeneity, the transport of oligopeptides in kidney occurs by more than a single system. Recently, the cDNAs encoding two families of H^+/peptide cotransporters have been reported in rabbit (2,3), human (4,5) and rat (6–8) along with molecular evidence for tissue specific expression. In particular, it is clear from these studies and others (9,10) that while PEPT1 is expressed in the intestine and to a smaller extent in kidney, PEPT2 is expressed in kidney but not intestine. The specific sites in the kidney

expressing these transporters have not been identified. Nevertheless, some investigators (11,12) have speculated that the peptide transporters are differentially expressed along the length of the nephron, with PEPT2 being primarily in the proximal parts of the nephron and PEPT1 being primarily in the distal parts of the nephron. Given the nutritional, therapeutic and pharmacologic importance of the renal peptide transport system, the purpose of the present study was to define the tubular localization and tissue distribution of PEPT1 and PEPT2 in rat kidney. In contrast to earlier predictions, we found that PEPT1 and PEPT2 were expressed solely in the proximal tubule, with PEPT1 being predominant in the early regions and PEPT2 being predominant in the latter regions.

MATERIALS AND METHODS

Microdissection

Male Sprague-Dawley rats (250–300 g) were anesthetized by an intraperitoneal injection of 120 mg/kg thiobutabarbital (Inactin; Byk-Gulden, Constance, Germany). After aortic blood flow to the left kidney was interrupted, the kidney was perfused through a cannula inserted into the abdominal aorta below the origin of the renal artery with 30 ml cold saline followed by perfusion with 30 ml culture medium [Dulbecco's modified Eagle's medium (DMEM); Sigma Chemical, St. Louis, MO] containing 1 mg/ml collagenase. The left kidney was then removed, cut into slices, and incubated in the DMEM/collagenase solution for 22 minutes at 37°C. Microdissection was performed with sharpened forceps at 4°C under a stereomicroscope. Lengths of the dissected segments were measured with an eyepiece micrometer. In general, 6 to 10 mm of tubule segments were dissected and pooled to constitute one sample. The following specimens were dissected from all slices: glomeruli (Glm), proximal tubules, medullary thick ascending limb (mTAL), cortical thick ascending limb (cTAL), macula densa-containing segment (MDCS), cortical collecting duct (CCD), outer medullary collecting duct (OMCD) and inner medullary collecting duct (IMCD). Within the proximal tubule, S1 segments are defined as the initial 2 mm of proximal convoluted tubule in dissections that included the glomerulus (PCT S1). S3 segments are defined as the outer medullary portion of the proximal straight tubule, identified by the transition to the thin descending limb (PST S3). Unidentified segments of proximal convoluted tubule (PCT) may contain S1 and S2 segments, but not S3 segments. Samples were placed in 100 μl guanidine isothiocyanate buffer (GITC buffer: 4 M guanidine isothiocyanate, 25 mM sodium acetate, pH 6.0, 0.8% β-mercaptoethanol), snap frozen in liquid nitrogen, and stored at −80°C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA Isolation

RNA from glomerular and tubular samples were thawed in an ice slurry and sonicated for 15 seconds. Twenty micrograms of ribosomal RNA from Escherichia coli (Boehringer Mannheim, Indianapolis, IN) was added as carrier, and the sample in 100 μl of GITC buffer was layered onto a gradient

[1] College of Pharmacy and Upjohn Center for Clinical Pharmacology, The University of Michigan, Ann Arbor, Michigan 48109.
[2] Renal Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115.
[3] Department of Physiology, Medical School, The University of Michigan, Ann Arbor, Michigan 48109.
[4] To whom correspondence should be addressed. (e-mail: smithb@umich.edu)

of cesium chloride (100 µl of 97% and 20 µl of 40% cesium chloride in 25 mM sodium acetate buffer) in a 250 µl polycarbonate ultracentrifuge tube. Samples were centrifuged for 2 hours at 300,000 g in a Beckman TLA 100 ultracentrifuge (Beckman Instruments, Fullerton, CA) with a fixed-angle rotor. The RNA pellet was redissolved in 0.3 M sodium acetate and precipitated in ethanol.

Total RNA from whole kidney cortex was isolated with TRI reagent (Molecular Research Center, Cincinnati, OH). Briefly, tissue samples were homogenized in TRI reagent solution. After addition of chloroform and centrifugation, the homogenates separated into three phases: aqueous, interphase, and organic. RNA was precipitated from the aqueous phase by addition of isopropanol. Contaminating genomic DNA was removed with RNase-free DNase I (Gene-Hunter, Brookline, MA). The purified RNA was redissolved in diethyl pyrocarbonate-treated water containing 20 U of RNAsin.

Reverse Transcription

Reverse transcription was performed in the presence of 100 IU Moloney murine leukemia virus reverse transcriptase (Superscript; BRL, Gaithersburg, MD), 0.5 µg oligo(dT)$_{12-18}$ (Pharmacia, Piscataway, NJ), 20 IU RNAsin (Promega Biotech, Madison, WI), 10 mM dithiothreitol, 0.5 mM dNTP (Pharmacia), and 1% bovine serum albumin (Boehringer Mannheim) in the buffer provided by the manufacturer in a total volume of 20 µl. Prior to the addition of reverse transcriptase, dNTPs, and bovine serum albumin, the reaction mixture was incubated at 65°C for 5 minutes to allow the primers to anneal to the poly(A) tail of mRNA. cDNA was synthesized at 42°C for 1 hour and precipitated with 1 µl of linear acrylamide, 4 M ammonium acetate, and 100% ethanol. The pellets were redissolved in Tris-EDTA buffer at a dilution adjusted so that each 2 µl of cDNA corresponds to 1 mm of tubule or 1 glomerulus.

Polymerase Chain Reaction

The following primers were used for rat PEPT1 amplification: sense 5'-ATC CTG CAG GTG GAA ATC GAT-3' (bp 1159 to 1179) and antisense 5'-CTC GAT CTC GGC CGG GTT GA-3' (bp 2045 to 2064). These primers are located near the 3'-end of the coding region and bracket a 906 bp PCR product for rat PEPT1 (6). Since these primers were originally chosen based on regions of high homology between the rabbit and human species (2,4), specificity was confirmed by sequencing 245 bp of the PCR product from rat whole kidney cortex. For rat PEPT2 amplification, species-specific primers were constructed from its known sequence (8). The sequence of sense primer was 5'-CTC CCC CTA GTC CCC CTT TTC-3' (bp 66 to 86) and that of the antisense primer was 5'-GTA GCA TTT TTC CTC CCA GTA TTG-3' (bp 561 to 584). These primers are located near the 5'-end of the coding region and bracket a 519 bp PCR product containing diagnostic Dde I and Acc I restriction sites.

To control for variations in RNA isolation and reverse transcription efficiency, PCR amplification for β-actin was also performed. The primers for β-actin were chosen from the human published sequences (13). The sequences of the β-actin primers were sense 5'-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3' (bp 384 to 413) and antisense 5'-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3' (bp 705 to 734).

PCR reactions were performed in a total volume of 50 µl in the presence of 5 pmol of each oligonucleotide primer, 200 µM dNTP, 10 mM dithiothreitol, 50 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl, pH 8.3, 0.001% gelatin, 1.25 IU AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 1.5 µCi [^{32}P]dCTP (Amersham, Arlington Heights, IL). Mineral oil was layered on the top of each sample to prevent evaporation of the liquid. The samples were first denatured at 94°C for 3.5 minutes. The PCR cycle was programmed as follows: 94°C for 1 minute (melt), 55°C for 1 minute (anneal), and 72°C for 1 minute (extend). PCR was run for 25 cycles, and the last cycle was followed by additional incubation for 8 minutes at 72°C.

Analysis of PCR Products

After amplification, PCR products were subjected to size separation by polyacrylamide gel electrophoresis. The band intensity was determined by phosphorimaging with Phosphor Analyst software on a GS-250 Molecular Imager System (Bio-Rad, Hercules, CA). The PCR products for PEPT1 were identified by partial direct sequencing using the ABI 373 DNA sequencer and for PEPT2 by restriction digestion using Dde I and Acc I.

In Situ **Hybridization**

Rat kidney was collected, snap frozen at −30°C, and 12 µm sections were cut in a cryostat and captured onto positively-charged microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Sections were stored at −80°C before processing. The *in situ* hybridization procedure was performed as described (14) with minor modifications. Sections were brought to room temperature, and immersed for 20 minutes in 4% paraformaldehyde in 0.1 M PBS (phosphate-buffered saline). After rinses in PBS, sections were digested with 27 ng/ml of proteinase K for 5 minutes, rinsed again, acetylated in 250 ml 0.1 M triethanolamine containing 625 µl acetic anhydride, rinsed, dehydrated and air-dried. Probes were generated using ^{35}S-radiolabeled UTP (>1000 Ci/mmol; New England Nuclear, Boston, MA) and used in a hybridization buffer containing ~35,000 cpm/µl final probe concentrations. PEPT1 sense and antisense probes were synthesized from a PCR generated fragment of the PEPT1 sequence containing nucleotides 74–1670 and flanked by initiation sequences for RNA polymerase T7 and SP6, respectively. The PEPT2 sense and antisense cRNA probes were synthesized using T7 and T3 RNA polymerase from a full-length clone of PEPT2 in pBluescript (3.9 kb), after linearization with Sac II and Xbal I, respectively. Probes were hydrolyzed to an average length of 100 base pairs. Following hybridization, sections were washed with 5 × SSC, (sodium chloride/sodium citrate), 1 mM DTT (dithiothreitol) at 50°C for 30 minutes and with 2 × SSC, 50% formamide, 0.1 M DTT at 65°C for 20 minutes. After RNAase treatment (20 µg/ml) for 1 hour at 37°C, sections were washed with 2 × SSC and 0.1 × SSC at 37°C for 15 minutes each, and then dehydrated, air-dried and apposed to Kodak autoradiographic film for 2 days. Figures were printed directly from the autoradiographic image of the *in situ* labeling on the film.

RESULTS

Rat PEPT1/PEPT2 mRNA in Microdissected Tissue

Figure 1 is a representative gel displaying the localization of rat PEPT1 and PEPT2 mRNAs in microdissected nephron
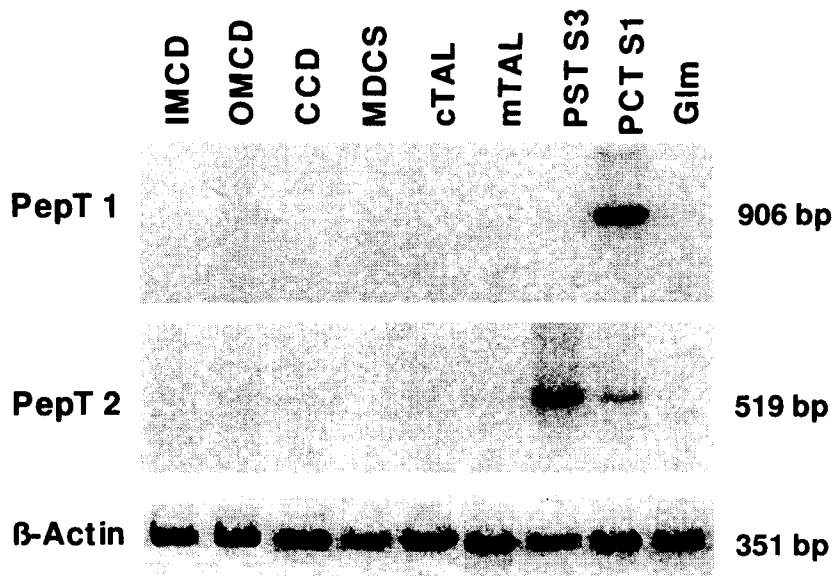
Fig. 1. Phosphoranalyzer image of PEPT1, PEPT2 and β-actin PCR products using cDNA prepared from microdissected nephron segments in rat. Glm, glomeruli; PCT S1, proximal convoluted tubule-S1 segment; PST S3, proximal straight tubule-S3 segment; mTAL, medullary thick ascending limb; cTAL, cortical thick ascending limb; MDCS, mascula densa-containing segment; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct.

segments. As shown in this figure, PEPT1 is expressed solely in the S1 segment of proximal convoluted tubules. In contrast, PEPT2 is expressed overwhelmingly in the S3 segment of proximal straight tubules. Neither transporter is expressed anywhere else along the nephron. Thus, it appears that these two peptide transporters are confined to proximal cells and, as a result, proximal tubular segments were further analyzed. Figure 2 is a representative gel displaying the localization of PEPT1

and PEPT2 in PCT S1 segments, PST S3 segments, and undefined regions of the proximal convoluted tubules (S1 and/or S2 segments). As shown in this figure, PEPT1 is expressed in S1 segments and to a lesser degree in undefined segments of the PCT, but not in S3 segments. In contrast, PEPT2 is expressed in S3 segments and to a minor degree in undefined segments of the PCT, but not in S1 segments. Taken as a whole, heterogeneity in expression is evident with the low-affinity transporter
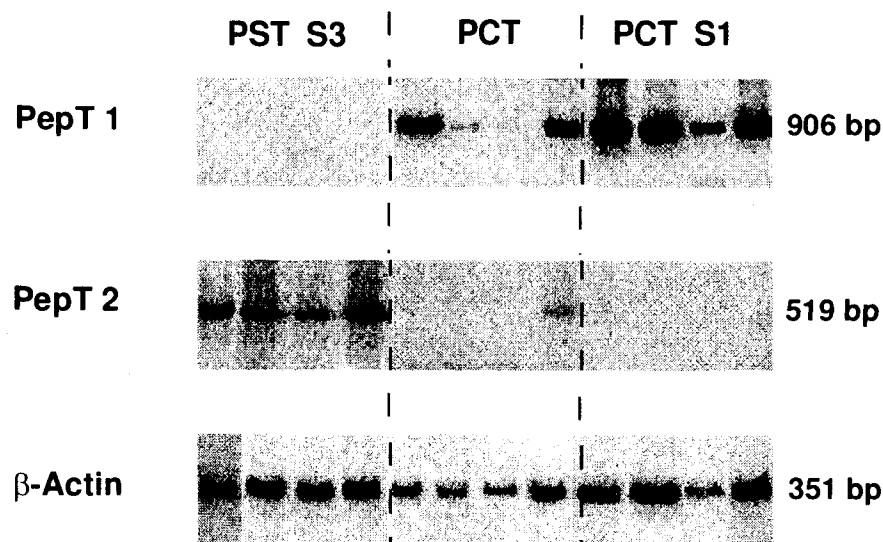

Fig. 2. Phosphoranalyzer image of PEPT1, PEPT2 and β-actin PCR products using cDNA prepared from microdissected proximal tubule segments in rat. PCT S1, proximal convoluted tubule-S1 segment; PCT, undefined regions of proximal tubule (S1 and/or S2 segments); PST S3, proximal straight tubule-S3 segments.

(PEPT1) being predominant in early regions of the proximal tubule (absent from S3 segments), and the high-affinity transporter (PEPT2) being predominant in latter regions of the proximal tubule.

PCR products for rat PEPT1 had the predicted size of 906 bp. The PCR product generated by amplification of the cDNA obtained from rat whole kidney cortex was confirmed by direct sequencing of 245 bp at the 3'end, which revealed identity with the published sequence from rat intestine (6). PCR products for rat PEPT2 had the predicted size of 519 bp (8). As shown in Figure 3, Dde I (151 and 368 bp) and Acc I (170 and 349 bp) yielded the expected digestion products, thus, verifying their identity.

Analysis of Rat PEPT1/PEPT2 mRNA by *In Situ* Hybridization

In situ hybridization analysis of the distribution of PEPT1 and PEPT2 mRNA in kidney confirmed the selective localization of these two transporters to different parts of the proximal tubules. PEPT1 was only expressed in kidney cortex, which
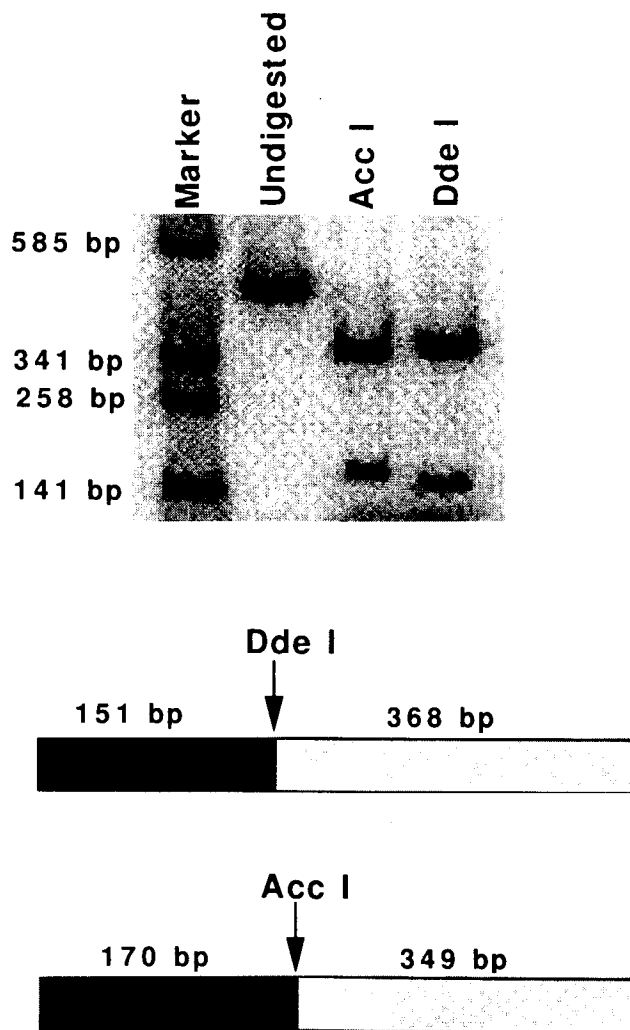

Fig. 3. Expected digestion products after restriction digestion of rat PEPT2 PCR products with Dde I and Acc I. Top, polyacrylamide gel for nondigested and digested products. Bottom, restriction map.

corresponds to the S1 and proximal S2 segments of the proximal tubule (Fig. 4A). In contrast, PEPT2 in situ signals were present in the outer stripe of outer medulla, which includes the medullary rays and which corresponds to the distal straight part of S2 and S3 segments (Fig. 4B). A qualitative assessment of the labeling intensities for the two transporters suggests that the mRNA for PEPT1 is less abundant than that for PEPT2. No labeling was observed when sense probes were used (not shown).

DISCUSSION

The transport properties of oligopeptides (i.e., activity and distribution) have been reported as analogous but opposite to that of glucose and the amino acids (11,15). In this regard, the concentration of di- and tripeptides is probably very low following filtration at the glomerulus. The filtrate instead would contain larger peptides and low molecular weight proteins in significant quantities. As such, these polypeptides are not substrates for the renal peptide transport system. However, as they pass down the length of the renal tubule, various peptidases in the brush border membrane can hydrolyze these polypeptides to generate di- and tripeptide substrates. As a result, the presence of a high-affinity system (PEPT2) in the proximal region and of a low-affinity system (PEPT1) in the distal region would be advantageous for peptide reabsorption under these physiological conditions. Although this scenario is conceptually appealing, there is little experimental data to support this hypothesis.

Using molecular probes, PEPT1 and PEPT2 have been reported in mammalian kidney. In rat (7), RT-PCR amplification of PEPT1 mRNA gave products for kidney cortex but not for kidney medulla. Western blot analysis subsequently confirmed the regioselectivity of rat PEPT1 protein in these tissues (10). For rat PEPT2 (8), Northern blot analysis revealed mRNA transcripts in kidney, especially in the medulla. Thus, it appears that in rat, PEPT1 is expressed solely in kidney cortex, while PEPT2 is expressed in cortex and, more abundantly, in medulla. With respect to rabbit (2,3) and human (4,5), both PEPT1 and PEPT2 have been detected in kidney, however, differences in tissue distribution have not been elucidated.

In the present study, we demonstrate for the first time that the renal peptide transporters, namely PEPT1 and PEPT2, are differentially distributed along the length of the nephron. Using microdissected tubular segments from rat coupled to RT-PCR analysis, we show that PEPT1 and PEPT2 are not only confined to the proximal tubule, but that their tubular localization is heterogeneous. In particular, PEPT1 was found to be specific for early parts of the proximal tubule (pars convoluta) whereas PEPT2 was expressed preferentially (but not exclusively) in latter parts of the proximal tubule (pars recta). The data in microdissected tubules were corroborated by our studies using in situ hybridization, and further suggest that PEPT2 is more abundant in mammalian kidney as compared to PEPT1. Neither transporter is present anywhere else along the length of the nephron. As a result, it appears that peptides and peptidomimetics are handled in a sequential manner in proximal regions of the nephron, first by the low-affinity, high-capacity transport system and second by the high-affinity, low-capacity transport system. Although the physiologic significance of these findings is uncertain at present, one can speculate that peptides are preferentially reabsorbed in kidney by PEPT2 due to the trans-
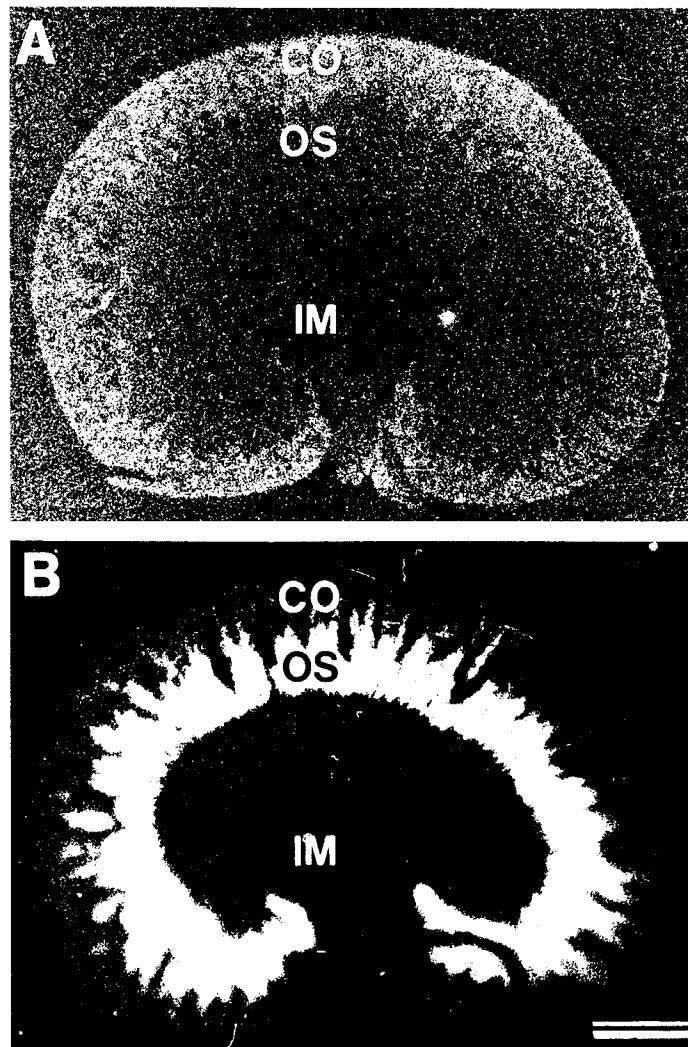
Fig. 4. In situ hybridization for PEPT1 and PEPT2 mRNA in rat kidney. A) PEPT1 mRNA is selectively expressed in kidney cortex (CO), corresponding to S1 and proximal S2 tubules. B) PEPT2 mRNA is primarily expressed in the outer stripe of outer medulla (OS), which corresponds to distal S2 and S3 segments. No labeling was observed in the inner medulla (IM), or when sense probes were used. Bar = 250 μm.

porter's high-affinity nature and greater abundance over PEPT1. This contention is supported by *in vivo* microinfusion experiments (16) in which glycylsarcosine was found to be reabsorbed mainly from late portions of the rat proximal tubule.

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