Localization of retinol-binding protein messenger RNA in the rat kidney and in perinephric fat tissue

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Abstract The cellular localization of retinol-binding protein (RBP) messenger RNA (mRNA) in the kidney, and the developmental pattern of the renal expression of the RBP gene, were studied in the Sprague-Dawley rat. In situ hybridization studies were conducted with single-stranded cRNA probes, using sections of adult and young rat kidneys. These studies revealed specific localization of RBP mRNA in the outer stripe of the medulla, specifically localized in the S_3 segment of the proximal tubules. Northern blot analysis demonstrated that RBP mRNA was not detectable in the kidney before birth or during the first week postpartum, but was clearly detected by the end of the second week of age. No RBP mRNA was observed in the kidney by in situ hydridization at 12 days of age. At 26 days of age, however, RBP mRNA was clearly detected by the in situ hybridization technique, localized in the same anatomic region as that observed in the adult kidney. Transthyretin mRNA was not detected in the adult kidney. Previous studies have shown that immunoreactive RBP is localized in the convoluted segment of the proximal tubules of the rat kidney. The present results demonstrate that RBP mRNA in the kidney is localized in an anatomic region (the S_3 segment of the proximal tubules) different from that of immunoreactive RBP. In addition, an intense RBP mRNA hybridization signal was detected in the perinephric fat tissue of 26- and 40-day-old and adult rats. Further analysis of RNA from epididymal fat showed a level of RBP mRNA approximately 20% of that of liver. The function of RBP synthesized in the kidney and adipose tissue remains to be determined. We have previously hypothesized that RBP synthesized in extrahepatic tissue may function in the recycling of retinol back to the liver or to other target tissues. —**Makover, A., D. R. Soprano, M. L. Wyatt, and D. S. Goodman.** Localization of retinol-binding protein messenger RNA in the kidney and in perinephric fat tissue. *J. Lipid Res.* 1989. **30:** 171–180.

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Retinol-binding protein (RBP) is the plasma protein that transports retinol from its storage site in the liver to peripheral target tissues. RBP is a 20 to 21 kDa protein that has one binding site for retinol (see (1) for a recent review). RBP is synthesized in the liver and is mainly secreted from the liver into plasma as the retinol-RBP complex (holo-RBP) (1, 2). Holo-RBP strongly interacts in the plasma with another protein, transthyretin (TTR), and normally circulates as a 1:1 molar RBP-TTR complex (3–6).

The retinol nutritional status of rats strongly and specifically influences the secretion of RBP. Thus, secretion of RBP from the liver is specifically inhibited in rats that are depleted of their retinol stores, resulting in the accumulation of apoRBP in the liver and in the concomitant decline in serum RBP levels (1, 7, 8). Upon repletion of retinol-deficient rats with retinol, the secretion of RBP from the liver is specifically and rapidly stimulated. In contrast to these effects of retinol status on RBP secretion, RBP synthesis rate in liver is not altered by retinol depletion or repletion (9). The evidence for this conclusion includes the finding that RBP mRNA levels are not altered in liver, regardless of the retinol status of rats (10).

In a previous study from our laboratory, it was shown that RBP mRNA is present in a large number of extrahepatic tissues, as well as in liver, in the Sprague-Dawley rat (10). Kidneys contained RBP mRNA at a level of 5 to 10% of that of liver. RBP mRNA levels in the lungs, spleen, brain, heart, and skeletal muscle ranged from 1 to 3% of that of the liver. In addition, it was demonstrated that the preRBP was synthesized in vitro using kidney poly (A^+) RNA as a template (10).

The kidney has previously been shown to contain high levels of immunoreactive RBP (11). More recently, an immunohistochemical study of the rat kidney showed localization of RBP protein in the proximal convoluted tubules of the renal cortex (12). This RBP in the proximal convoluted tubular cells presumably represent RBP that

Abbreviations: RBP, retinol-binding protein; TTR, transthyretin; PBS, phosphate-buffered saline; mRNA, messenger RNA; CRBP, cellular retinol-binding protein; bp, base pair.

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had undergone glomerular filtration and tubular reabsorption (1, 11, 12).

The goal of the present study was to explore the specific localization of RBP mRNA in the kidneys of the adult, young, and newborn rat, using the technique of in situ hybridization. We report now that in both young and adult rats RBP mRNA is localized in the kidney in an anatomic site different from the site of localization of RBP protein. RBP mRNA is localized in the outer stripe of the medulla, mainly in the straight part (the S_3 segment) of the proximal tubules. Significant levels of RBP mRNA were also observed in perinephric and epididymal adipose tissue.

EXPERIMENTAL PROCEDURES

Animals

Male adult rats of the Sprague-Dawley strain were obtained from Camm Breeding Laboratories (Wayne, NJ). Rats were housed in individual pan-type cages with wood chip bedding and were given a commercial pelleted diet (Camm Research Institute) and water ad libitum. Pregnant female rats of the same strain, obtained at the 17th day of gestation, were housed individually under the same conditions. On the 20th day of gestation, one dam was anesthetized with ether, decapitated, and the fetuses were collected; the fetal kidneys were removed and utilized for study by Northern blot analysis. The pups from the other dams, ranging in age from 1 to 40 days postpartum, were used for the experiments presented in this study.

Preparation of kidney sections

Adult rats were anesthetized by injection of ketamine-hydrochloride (80–100 mg/kg body weight) containing 4% acepromazine. The chest was opened and the systemic circulation was rinsed by cardiac perfusion with 20 ml cold isotonic saline, followed by perfusion with 4% cold paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (approximately 300 ml) for 20 min, through a catheter passed into the ascending aorta. After fixation, the kidneys were removed and further immersed in 4% paraformaldehyde for 6 to 12 hr at 4°C. The tissues were then immersed in sterile phosphate-buffered saline (PBS) containing 15% sucrose for 1 to 3 hr, at 4°C. The tissues were next embedded in OCT compound (Miles Laboratories, Naperville, IL) and frozen in isopentane cooled in liquid nitrogen. Serial sections of 5–10 micron thickness were cut on a cryostat and mounted on polylysine-coated slides. The slides were immediately frozen at −70°C in slide boxes containing desiccant capsules.

Rat pups of 1, 4, 6, 12, 26, and 40 days of age were anesthetized with Forane (isoflurane, USP, Anaquest, Madison, WI). The chest was opened and the systemic circulation was perfused with 3–100 ml of ice-cold 4% paraformaldehyde (depending on the size of the pup). The kidneys were dissected and immersed in 15% sucrose in PBS for 1–2 hr and then treated in the same way as described above.

Preparation of cRNA probes

Linearized rat RBP cDNA subcloned into pGem4 was used as the template to synthesize both sense and antisense strand rat RBP cRNA probes, as previously described (13). One μg of linearized rat recombinant RBP plasmid was used in the transcription reactions. ^{35}S-Labeled cRNA probes in both the sense and antisense orientation were synthesized in a 10-μl reaction mixture using 100 μCi ^{35}S-labeled UTP (sp act 1000–1400 Ci/mmol, New England Nuclear, Boston, MA), 10 μM UTP, 500 μM each of ATP, CTP, and GTP, 15 units RNAsin (Promega Biotec), 10 μM dithiothreitol, 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, and 15 units of either SP6 polymerase or T7 polymerase (Boehringer Mannheim, Indianapolis, IN). The reaction mixture was incubated for 1 hr at 40°C. The mixture was then diluted 10-fold with DNAse buffer (400 mM Tris, pH 8.0, 100 mM NaCl, 60 mM $MgCl_2$) and digested with 3 units of RNAse-free DNAse (RQ1 DNAse, Promega Biotec) at 37°C for 20 min to remove the plasmid template. After phenol extraction and ethanol precipitation, the RNA probes were stored in RNAse-free water at −70°C until used. Specific activity of the probe ranged from $1–2 \times 10^8$ cpm/μg. TTR cRNA probes were prepared in an analogous manner.

Hybridization of tissue sections

The hybridization procedure used here is a modification of the procedures described by Wilcox, Gee, and Roberts (14) and Fremeau et al. (15). Briefly, slides were removed from the −70°C freezer and covered immediately with proteinase K. The tissue sections were then washed in sterile water, followed by immersion first in 0.1 M triethanolamine and then immersion in 0.25% acetic anhydride in 0.1 M triethanolamine at room temperature. After the latter treatment, the slides were washed in 1 × SSC (150 mM NaCl, 15 mM Na citrate) two times, and the tissue sections were covered with prehybridization solution (50% formamide, 600 mM NaCl, 10 mM Tris, pH 7.5, 0.02% Ficoll, 0.02% polyvinylpyrollidone, 0.1% bovine serum albumin, 1 mM EDTA, 0.5 mg/ml denatured salmon sperm DNA, 0.5 mg/ml total yeast RNA, and 50 μg/ml yeast transfer RNA), and incubated for 120 min at 53°C in an airtight box. After the prehybridization step, the sections were covered with hybridization solution (the same as the prehybridization solution but including 10 mM dithiothreitol and 10% dextran sulfate) containing 125,000 cpm of heat-denatured ^{35}S-labeled RBP (or

TTR) cRNA probe (sense or antisense strand). The sections were hybridized for 16 hr at 53°C. After hybridization, the slides were washed in 2 × SSC at room temperature and treated with RNAse A and RNAse T_1 (Sigma Chemical Co., St. Louis, MO), (20 μg/ml and 2.2 μg/ml, respectively). The sections were then washed in 2 × SSC at 53°C, followed by a 3-hr wash in 0.1 × SSC containing 0.05% Na pyrophosphate and 14 mM β-mercaptoethanol at 53°C. The slides were further washed in the same solution at room temperature for 16–24 hr. The tissue sections were then dehydrated in graded alcohols and air dried. The slides were first exposed to XAR-5 film, and then dipped in a solution of NTB-2 nuclear emulsion and allowed to expose in a light-proof slide box. After exposure, the slides were developed, fixed, and finally stained with either Giemsa solution or hematoxylin–eosin. The sections were examined and photographed by dark field illumination, using an NTB 10 blue filter or bright field illumination.

RNA isolation and analysis

Total RNA was prepared by the method of Tushinski et al. (16). For some samples RNA enriched in poly (A^+) RNA was obtained by oligo (dT)-cellulose affinity chromatography, as described by Aviv and Leder (17). The relative amount of RBP-specific mRNA was analyzed either by Northern blot analysis or by RNase protection analysis.

For Northern blot analysis, 10 μg of poly (A^+) RNA was denatured and electrophoresed in a 1% agarose/formaldehyde gel, as described by Lehrach et al. (18). RNA was transferred to nitrocellulose paper as described by Thomas (19). All filters were hybridized to the oligo-labeled 548 bp rat RBP cDNA insert prepared by EcoR1 digestion of the rat RBP pGem4 clone (13), followed by electroelution from a 5% polyacrylamide gel. Prehybridization, overnight hybridization, and subsequent washing were carried out exactly as described previously (10). Filters were exposed to Kodak SB5 X-ray film at − 70°C with intensifying screens, and the appropriate exposures of autoradiograms were quantitated with a Hoeffer Model 1650 scanning densitometer. The peaks obtained were integrated using a planimeter resulting in a numerical value that was normalized to the level of RBP mRNA in the adult rat liver (which was assigned a relative value of 100%). To demonstrate that equal amounts of RNA were present in each sample subjected to electrophoresis, the blot was rehybridized with oligo-labeled 1150 bp mouse α-actin cDNA insert prepared by Pst I digestion of clone 91 (20), (obtained from Shirley Tilghman, Princeton University, Princeton, NJ), followed by electroelution from a 5% polyacrylamide gel. Prehybridization, hybridization, and quantitation were conducted in the same manner as described above.

For RNase protection analysis of the level of RBP mRNA, antisense strand (hybridizing) ^{32}P-labeled RBP cRNA probes were synthesized and solution hybridizations were performed essentially as described previously (13). Hybridized (protected) fragments were denatured and separated on 10% polyacrylamide/7 M urea gels. Gels were dried and exposed to SB5 film with intensifying screens at − 70°C. Bands were quantitated in the same manner as described above.

RESULTS

Cellular localization of RBP mRNA in the adult rat kidney

The major goal of this study was to determine the anatomic localization of RBP mRNA in rat kidney. Our previous study had demonstrated that RBP mRNA is present in rat kidney at levels of 5–10% of those in liver (10). In present study, the localization of RBP mRNA was determined by in situ hybridization using ^{35}S-labeled cRNA RBP probes. The hybrids were visualized as silver grains on a dark field image.

About 50 kidney sections of four different rats were examined for hybridization to ^{35}S-labeled RBP-cRNA or to ^{35}S-labeled TTR-cRNA (sense and antisense) probes. In all kidney sections that were hybridized with the antisense strand ^{35}S-labeled RBP cRNA probe, an intense hybridization signal was found specifically localized in one particular anatomic region of the kidney (shown in **Fig. 1b, d**), which was the outer stripe of the medulla. Only background levels of grains were observed in the inner medulla with the antisense strand ^{35}S-labeled RBP cRNA probe (Fig. 1b). As a control, the kidney sections were also hybridized with the sense strand ^{35}S-labeled RBP cRNA probe and very few nonspecifically distributed silver grains were (Fig. 1e). TTR antisense cRNA probe did not hybridized to any region of the kidney sections (Fig. 1f).

The outer stripe of the medulla, where the ^{35}S-labeled RBP cRNA probe specifically hybridized, contains the terminal portions of the pars recta (the S_3 segment) of the proximal tubule, the thick ascending limbs of the distal tubules, and collecting ducts (21). (For a detailed review of the structure of the nephron, see ref. 21). The grains (representing hybridization of ^{35}S-labeled RBP cRNA probe to RBP mRNA) were localized mainly on the cells of the S_3 segment of the proximal tubules. Very few (or no) grains were present in other parts of the proximal tubules (the S_1 and S_2 convoluted segments) which are localized in the outer cortex (Fig. 1b, d). In the rat, the histological transition from the S_2 to the S_3 segment of the proximal tubule is rather abrupt and occurs in the mid-

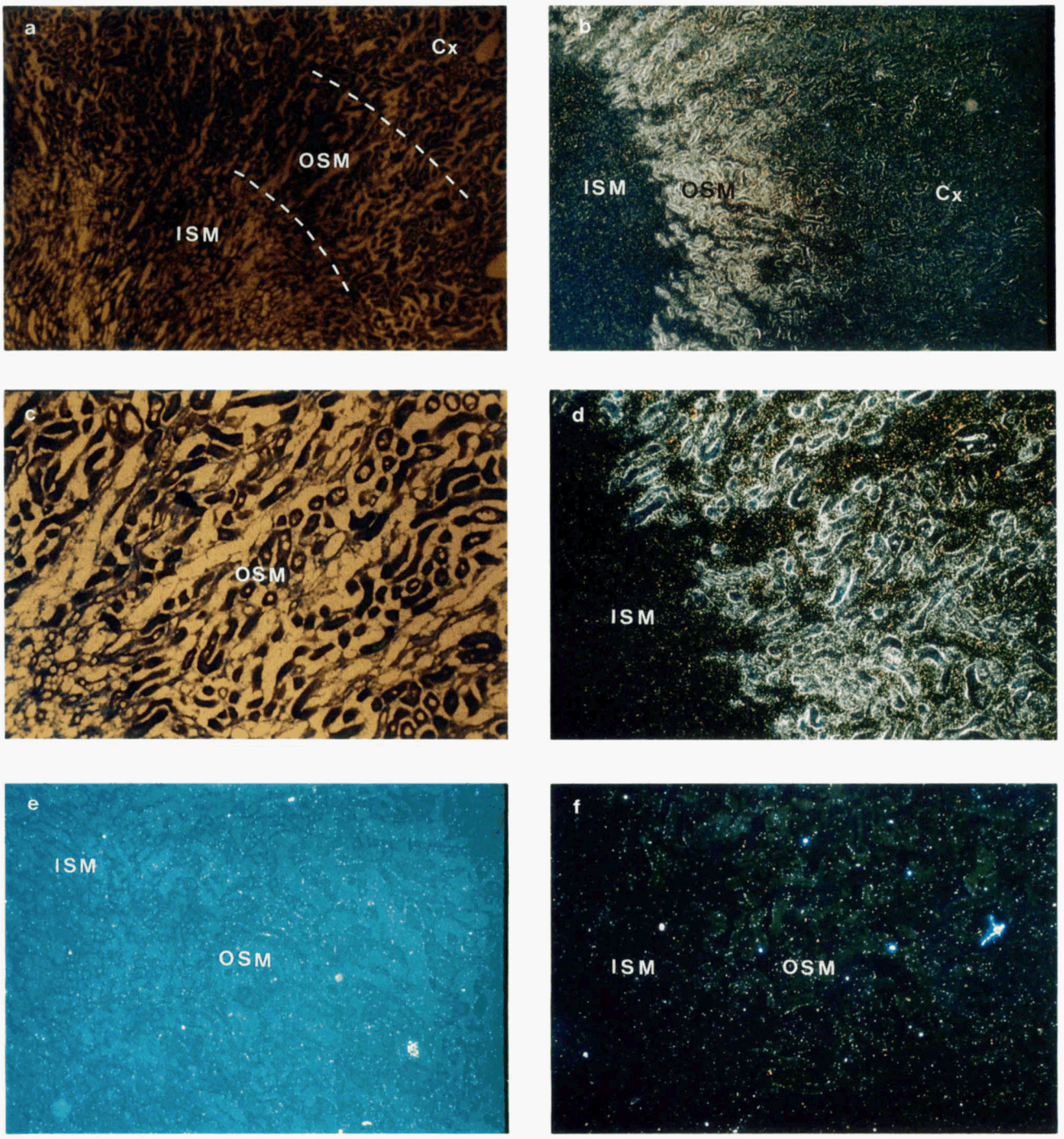

Fig. 1. Localization of RBP mRNA in the adult Sprague-Dawley rat kidney. Sections (a) and (c) are bright field micrographs of kidney transverse sections demonstrating the anatomical regions under study (outer stripe of the medulla (OSM) delineated between the dashed lines) (magnification: a, × 120; c × 300). Sections (b) and (d) are dark field micrographs of adjacent kidney sections hybridized to a ^{35}S-labeled RBP cRNA antisense probe (magnification: b, × 120; d, × 300). Note the intense hybridization signal (silver grains) over regions of the proximal tubules located in the outer stripe of the medulla (in the S_3 segment of the proximal tubules) whereas very few grains to none are present in the cortex or in the inner stripe of the medulla (ISM) (b and d). Section (e) is a dark-field micrograph of an adjacent kidney section hybridized to ^{35}S-labeled RBP sense strand (magnification × 300) and (f) is a dark field micrograph of an adjacent section hybridized to ^{35}S-labeled TTR antisense probe (as an additional control) (magnification × 300). Note that only background levels of silver grains are present, distributed nonspecifically over the tubules in both micrographs (e) and (f). All sections are Giemsa-stained; OSM, outer stripe of medulla; ISM, inner stripe of the medulla; Cx, cortex. (Note that the blue color in micrograph (e) is brighter than in (b), (d), and (f) due to differences in thickness of the coating emulsion. Magnification slightly reduced in reproduction.)

cortical region of the kidney. This anatomic transition coincides with the pattern of expression of RBP mRNA; thus, RBP mRNA was not seen in the outer regions of the cortex, but abruptly appeared in the midcortical region, distributed over that portion of the proximal tubules which was localized in the outer stripe of the medulla (Fig. 1b, d).

Developmental expression of RBP mRNA

Another question that we addressed in this study was: When does the expression of the RBP gene occur during development of the kidney? Kidney differentiation and development continues in the rat after birth, and new nephrons are developed during the first 4 weeks of life (22). During the first 8 days of age, nephron formation occurs through four distinguishable stages (23). Stage I corresponds to the renal vesicle in which the whole organ is ellipsoid. At this stage, the differentiated nephron has no glomerular anlage, and there is no connection between the renal vesicle and the collecting tubule. This stage lasts from birth to 48 hr of age. Stage II corresponds to the so-called S-shaped body, which consists of a glomerular anlage that is connected to the collecting tubule. This stage lasts until 4–5 days of age. Stage III is characterized by a spherical glomerulus. There is no difference between the height of the epithelial cells of the proximal tubule and the loop of Henle. The cells have very short microvilli. This stage lasts up to 7 days of age. Stage IV is characterized by a larger glomerulus than in stage III. Development of brush borders occurs in the proximal tubule, along with differentiation of the loop of Henle. This stage lasts until day 8 of age. During the second week of life, neogenesis of nephrons is a marked feature of the kidney, along with maturation and growth of the nephron. By 40 days of age, the glomerular filtration rate has increased 20-fold compared to the rate that is present at birth (24).

Considering these postnatal developmental stages, we addressed the question of whether there is a correlation between the postnatal developmental stages of the rat kidney and the expression of RBP mRNA. Northern blot analysis was carried out with kidney RNA of young male rats at different ages (before birth at 20 days of gestation, and at 1, 2, 5, 7, 14, 19, 25, and 40 days postpartum) and of adult rats (60–80 days of age), The results of the study, shown in **Fig. 2**, indicate that RBP mRNA was not detected in the prenatal kidney or in the newborn rat kidney during the first 7 days postpartum. At the end of the second week after birth (14 days of age), however, RBP mRNA was clearly detectable. From this age and thereafter, the levels of RBP mRNA were quite variable (Fig. 2), but in general were in the range of levels seen in adult rat kidneys [3–10% of the adult liver levels (see Fig. 2), similar to levels previously reported (10)]. It should be

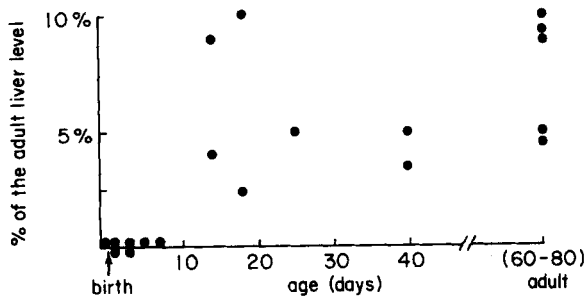

Fig. 2. Relative levels of kidney RBP mRNA during rat development. Ten μg samples of kidney poly (A^+) RNA isolated from rats at the indicated ages were separated on formaldehyde/agarose gels, transferred to nitrocellulose paper, and probed with oligo-labeled rat RBP cDNA. The intensity of hybridization was quantitated by densitometric scanning and normalized to the adult liver RBP mRNA levels, which were set at 100%.

noted that, in this study, all visible fat was dissected away from the kidneys before homogenization and RNA isolation. Thus, the hybridization signals reported in Fig. 2 represent RBP mRNA in the kidney itself and not in associated perinephric fat (see below). Actin mRNA levels were determined on the same blot and were found to be very similar for all samples, indicating that nearly equal amounts of the total mRNA were analyzed in each sample.

In addition to the Northern blot analysis, we carried out an in situ hybridization study on kidney sections of rats of different ages, including rats 1, 4, 6, 12, 26, and 40 days of age. Sections of two different rats were examined at each time point. No hybridization signal was detected for RBP mRNA in any of the sections of the rats of 1, 4, 6, and 12 days of age (sections were exposed up to 7 weeks). RBP mRNA was, however, clearly detected by in situ hybridization of kidney sections of 26-day-old rats (5 weeks exposure) (**Fig. 3a,c**). The hybridization signal was localized in the same anatomic region as that seen in the adult kidney, namely in the outer stripe of the medulla. The same pattern of expression was observed at 40 days of age (Fig. 3b,d).

RBP mRNA in the perinephric and epididymal fat

In the process of hybridization of the antisense RBP ^{35}S-labeled cRNA probe to adult kidney sections, we detected an intense signal over the adipocytes of the perinephric fat (**Fig. 4c**). The signal was detected in all the assays in which kidney sections that included perinephric fat were hybridized to the RBP cRNA probe (antisense strand). The sense strand did not give any hybridization signal over the adipose tissue (data not shown). In addition, the perinephric fat did not hybridize to the antisense strand ^{35}S-labeled TTR cRNA probes (data not shown). An intense hybridization signal was detected as well in the perinephric fat of 26- and 40-day-old

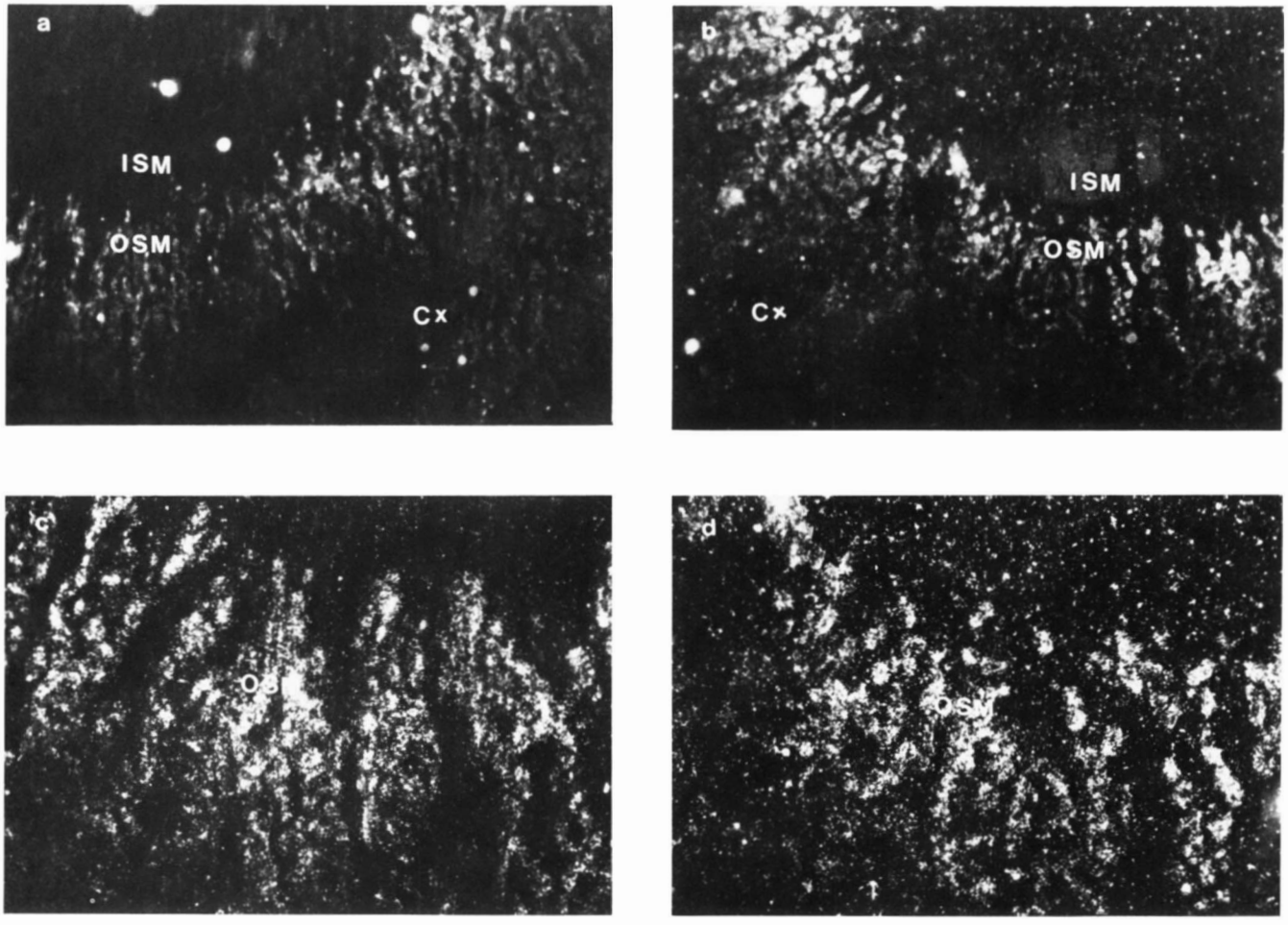

Fig. 3. Localization of RBP mRNA in the kidneys of 26- and 40-day-old rats. All transverse sections were hybridized to a ^{35}S-labeled RBP antisense probe. (All are dark field micrographs, hematoxylin–eosin stained). Sections (a) and (c) demonstrate hybridization signals (silver grains) localized in the outer stripe of the medulla (OSM) of 26-day-old rat kidneys (magnification: a, × 120; c, × 300). Sections (b) and (d) demonstrate an intense hybridization signal in the same anatomic location of 40-day-old rat kidney sections (magnification: b, × 120; d, × 300). Note the higher density of tubules that hybridized to ^{35}S-labeled RBP-cRNA in the 40-day-old rat kidney (b and d) compared to 26-day-old rat kidneys (a and c); OSM, outer stripe of the medulla; ISM, inner stripe of the medulla; Cx, cortex. Magnification slightly reduced in reproduction.

rats (Fig. 4a,b). Adipose tissue was not assayed previously (10) by Northern blot analysis for the presence of RBP mRNA.

To determine whether the positive in situ hybridization observed in perinephric fat tissue was specific for RBP mRNA and whether RBP mRNA is present in other fat tissue, we isolated total RNA from the epididymal fat pad and determined the level of RBP mRNA by RNase protection analysis. **Fig. 5** shows that RNA isolated from both the liver and the epididymal fat pad protected the same size rat RBP band. Thus, the hybridization signal in fat corresponds to authentic RBP mRNA as opposed to cross-hybridization to a related RNA. Furthermore, the level of RBP mRNA in the epididymal fat pad was approximately 20% of that found in the liver.

DISCUSSION

The kidneys are known to play in important role in the normal metabolism of RBP [see (1) for review]. In patients with chronic renal disease, the plasma levels of both RBP and retinol are greatly elevated, although the TTR levels remain normal (2). Free RBP, not complexed with TTR, is small enough to be filtered readily by the renal glomeruli, whereas TTR and the RBP-TTR complex are not. Thus, formation of the RBP-TTR complex serves to protect RBP by preventing its glomerular filtration and subsequent renal catabolism (2, 3). Although the proportion of RBP in plasma present as free RBP is normally very small, it is sufficient to permit a significant amount of RBP to be filtered by the glomeruli and metabolized by

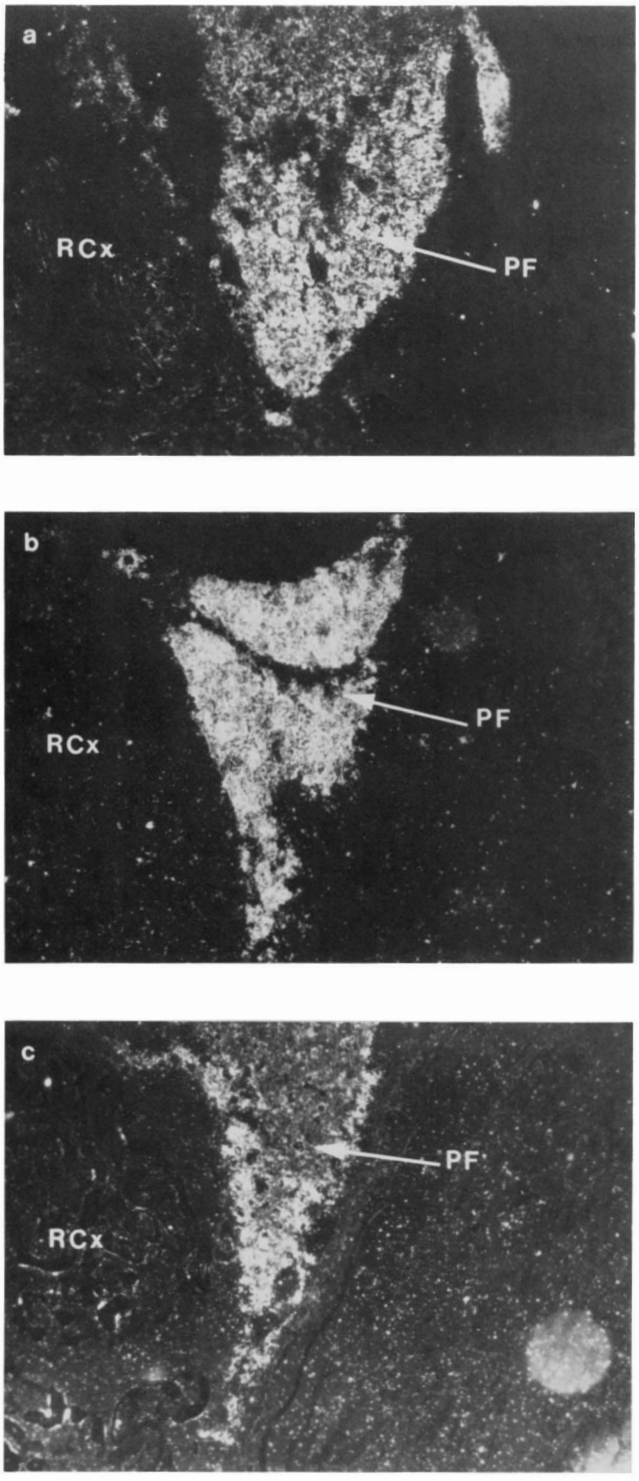

Fig. 4. Localizatin of RBP mRNA in the 26-day-old [micrograph (a)], 40-day-old [micrograph (b)], and adult [micrograph (c)] rat perinephric adipose tissue. (Transverse section, dark field micrograph, magnification × 300). Note the dense pattern of silver grains over the perinephric fat tissue of all ages. The signal appears stronger in (a) and (b) than in (c), partly because these sections were exposed for 5 weeks, whereas the adult tissue (c) was exposed for 4 weeks; PF, perinephric fat; RCx, renal cortex. Magnification slightly reduced in reproduction.

the kidneys each day. Patients with severe chronic renal disease have a reduced overall glomerular filtration rate, and hence a reduced metabolic clearance rate and an elevated plasma level of RBP. Normally, very little RBP appears in the urine, as nearly all of the filtered RBP is reabsorbed and degraded by the renal tubules (2, 25). In patients with impaired tubular function and tubular proteinuria, however, low molecular proteins, including RBP, are excreted in the urine in relatively large amounts (25, 26).

Previous radioimmunoassay studies in the rat showed that the kidneys contained the highest level of RBP observed in any of the tissues studied (11). Most (76%) of the RBP in kidney homogenates was recovered in the soluble supernatant fraction (11). Cell fractionation studies of the distribution of vitamin A in rat kidneys showed that most of the vitamin A resided in cortex tubular cells (27). More recently, immunohistochemical studies from our laboratory showed that immunoreactive RBP was localized in the proximal convoluted tubular cells of the renal cortex (12). Similar studies with similar findings have also been carried out with human kidney tissue (28). These data all support the conclusion that immunoreactive RBP in the kidney mainly represents RBP that had undergone glomerular filtration and tubular reabsorption.

The goal of the present study was to determine the anatomic localization of RBP mRNA in the rat kidney. It was previously reported from our laboratory that RBP mRNA is present in many extrahepatic tissues, and that the kidney had the highest relative level of RBP mRNA in any of the adult extrahepatic tissues studied (10). In the present study, using the technique of in situ hybridization, we localized RBP mRNA in the outer stripe of the renal medulla, specifically in the cells of the S_3 segment of the proximal tubules. Our results, therefore, demonstrate that RBP mRNA in the kidney is localized in an anatomic region (the S_3 segment of the proximal tubules) different from that of immunoreactive RBP (which is in the convoluted portion of the proximal tubules).

The proximal tubule of the rat is divided into three distinct morphologic segments (21, 29). The first segment, S_1, corresponds to the initial portion of the pars convoluta. The second segment, S_2, includes the terminal portion of the pars convoluta and the first part of the pars recta. The third segment, S_3, is the remainder of the pars recta before the transition into the thin loop of Henle. These three segments have also been identified in the mouse (30), rabbit (31, 32), and monkey (33). In the rat, the transition from the S_2 to S_3 segment is rather abrupt. The cells of the S_3 segment lack lateral interdigitations, mitochondria are smaller, the lysosomes are few in number, and the endocytotic apparatus is less developed than in the S_1 and S_2 segments. In the rat, the brush border of this segment is taller than that of S_1 and S_2 seg-

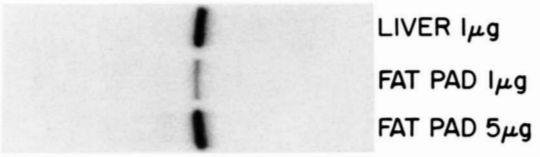

Fig. 5. Expression of RBP mRNA in epididymal fat pad. Total RNA was isolated from the epididymal fat pad and the liver of adult male rats. The levels of RBP mRNA in a 1-μg sample of liver RNA and in both a 1-μg and a 5-μg sample of the fat pad RNA were determined by RNase protection analysis using a ^{32}P-labeled antisense strand rat RBP cRNA probe.

ments. The cells contain a prominent Golgi apparatus, and have a high acid phosphatase activity (29). The exact function of the S_3 segment is not known. This segment is not believed to be involved in protein reabsorption to any significant degree and is less involved in active transport and water reabsorption than the pars convoluta (21). Recently, it was reported that the mRNA of α_1-antitrypsin (a plasma protease inhibitor, predominantly synthesized in liver) is also localized in the outer stripe of the medulla, in the kidney in the *Mus caroli* mouse (34).

Considering the prominent Golgi apparatus in the cells of the S_3 segment, one might suggest that this segment of the proximal tubules is highly active in the synthesis of secretory proteins. The potential contribution of these cells to the total amount of RBP in the body is small, compared to liver, since the number of cells in the S_3 segment that express RBP mRNA, and hence that might synthesize and secrete RBP protein, is low compared to the number of liver hepatocytes. However, the results reported here suggest that the number of copies of RBP mRNA per S_3 segment cell may be comparable to, or higher than, the number of copies per hepatocyte.

The significance of RBP synthesis in this particular segment of the proximal tubule is unclear. It was previously suggested that RBP synthesized in extrahepatic tissues may function in the recycling of retinol from these tissues back to the liver or to other target tissues (10). Considerable evidence now exists that quantitatively significant recycling and reutilization of retinol occurs in the animal body (1, 35, 36). Accordingly, it was hypothesized (10) that "when retinol leaves an extrahepatic tissue, a new molecule of RBP is synthesized locally, retinol is added to this molecule in the microsomes, and the holo-RBP is secreted into the plasma for delivery of retinol back to the liver or to other extrahepatic tissues."

It seems reasonable that the kidney might play a significant role in the conservation, recycling, and reutilization of retinol in the body. Quantitatively significant amounts of retinol are presumably filtered by the glomeruli and reabsorbed by the proximal convoluted tubules each day as the retinol-RBP complex (holo-RBP). The exact amounts of both RBP and retinol that participate in this process each day are, however, not known. Cellular retinol-binding protein (CRBP) is also present in relatively high concentrations in the cells of the proximal convoluted tubules of the renal cortex (12). It has been suggested (12) that CRBP may serve to bind and conserve some of the retinol that is filtered and reabsorbed as the retinol-RBP complex, and that CRBP in the tubular cells may play a role in retinol reutilization.

The findings reported here present a puzzling problem. If the kidney is involved in retinol conservation and reutilization, and if extrahepatically synthesized RBP is involved in retinol recycling, then one would have anticipated that RBP would be synthesized in the same site where retinol is localized. Our results clearly show, however, the RBP mRNA (and hence presumably RBP synthesis) is localized in the S_3 segment of the proximal tubule, a site quite removed from the site (mainly the S_1 segment) of retinol reabsorption and localization. Therefore, if RBP synthesized in the kidney is involved in the recycling of retinol from the kidney, either a molecule of retinol from the S_1 segment must be transported to a molecule of newly synthesized RBP in the S_3 segment, or the reverse must occur. No information is available as to whether or not such molecular movements (of retinol and/or RBP) might occur in the kidney, or as to the mechanisms that might be involved. Alternatively, RBP synthesized in the S_3 segment of the proximal tubule might serve some other, as yet unknown, physiological function. Future research is clearly needed in order to address these questions.

RBP mRNA was first detected in the kidney by Northern blot analysis by the end of the second week of life. The levels in young rats were variable (2–10% of adult liver level), but in general, were similar to adult levels (3–10% of adult liver level). This may indicate that the gene is expressed to a maximal extent (for this tissue) as soon as it becomes transcriptionally active. At 26 days postpartum, RBP mRNA was already specifically localized to the outer stripe of the medulla. At this stage, neogenesis of nephrons is completed and the proximal tubules are functionally differentiated.

RBP expression in the perinephric fat was discovered in the process of hybridization of a whole cross-section of the kidney to a RBP ^{35}S-labeled cRNA probe. An intense hybridization signal was detected over the adipocytes. In addition, we detected a significant amount of RBP mRNA in epididymal fat by RNase protection analysis. The function of RBP in adipose tissue is not known. It is known that a small but significant amount of chylomicron vitamin A, injected intravenously into rats as labeled retinyl ester in chylomicrons, is taken up from the circulation by adipose tissue (37). A quantitatively similar proportion of injected chylomicron cholesteryl esters (of the

order of 5–10% of such injected esters) is recovered in adipose tissue after chylomicron clearance from the circulation (38, 39). Nestel, Havel, and Bezman (40) showed that, in hepatectomized dogs, the adipose tissue removed about 40% of the chylomicron cholesteryl esters. It is well known that adipose tissue is rich in lipoprotein lipase, a key enzyme involved in chylomicron triglyceride hydrolysis, and is also relatively rich in other lipid ester hydrolase activities (41, 42). From studies with doubly-labeled cholesteryl esters, Quarfordt and Goodman (39) suggested that cholesteryl esters are taken up intact by the fat tissue and subsequently undergo hydrolysis in situ. Finally, it is known that a small but definite amount of vitamin A activity is present in fat deposits (43). Taken together, these various data suggest that adipose tissue plays a quantitatively small, but definite, role in the uptake, and probably also in the tissue storage, of chylomicron retinol and cholesterol. If this is true, then RBP, newly synthesized in adipose tissue, might indeed play a role in the mobilization of retinol from adipose tissue, and in retinol recycling in the body. Further studies are needed to explore whether the expression of the RBP gene is a general phenomenon in adipocytes, or whether it only occurs in certain adipose depots. The question of the possible nutritional regulation (particularly by vitamin A status) of RBP gene expression in the kidney and adipose tissue also merits future investigation.

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