Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat

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Abstract A retinol-binding protein (RBP) cDNA clone was used to examine the effect of retinol status on the level of RBP mRNA in the liver, and to explore whether extrahepatic tissues contain RBP mRNA. In the first series of experiments, poly (A^+) RNA was isolated from the livers of normal, retinol-depleted, and retinol-repleted rats and the levels of RBP mRNA in these samples were determined by both Northern blot and RNA Dot blot analyses. The levels of RBP mRNA in liver were similar in all three groups of rats. These findings confirm and extend previous studies which showed that retinol did not alter the in vivo rate of RBP synthesis or the translatable levels of RBP mRNA. In a second series of experiments, the RBP cDNA clone was used to survey poly (A^+) RNA isolated from 12 different rat tissues for RBP mRNA by Northern blot analysis. We found that, along with the liver, many extrahepatic tissues contained RBP mRNA. Kidney contained RBP mRNA at a level of 5–10% of that of the liver, and the lungs, spleen, brain, stomach, heart, and skeletal muscle contained 1–3% of that of the liver. Translation of kidney poly (A^+) RNA in rabbit reticulocyte lysates and immunoprecipitation of the translation products with anti-RBP antiserum resulted in a protein band of the same size as liver preRBP. These data suggest that RBP is synthesized in many extrahepatic tissues. It is possible that this extrahepatically synthesized RBP may function in the recycling of retinol from these tissues back to the liver or to other target organs. —**Soprano, D. R., K. J. Soprano, and D. S. Goodman.** Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. *J. Lipid Res.* 1986. **27:** 166–171.

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Vitamin A is transported in plasma from its storage site in the liver to peripheral target tissues as retinol bound to retinol-binding protein (RBP) (see Ref. 1 for a recent review). RBP is synthesized in the liver as a precursor, preRBP (approximately 24 kilodaltons), which is cotranslationally processed by microsomal membranes of the rough endoplasmic reticulum to mature RBP (20–21 kilodaltons) (2). RBP has one binding site for retinol and is secreted from the liver into plasma as the retinol-RBP complex (holo-RBP) (3, 4). Holo-RBP strongly interacts in the plasma with transthyretin and normally circulates as a 1:1 molar RBP-transthyretin complex.

The nutritional retinol status of rats appears to strongly influence the secretion of RBP without affecting RBP biosynthesis. In previous studies from this laboratory, it was shown that the secretion of RBP from the liver is blocked in rats that had been depleted of their retinol stores resulting in the accumulation of apoRBP in the liver and a concomitant decline in serum RBP levels (5, 6). Upon repletion of retinol-depleted rats with retinol, RBP is rapidly secreted from the liver (6). On the other hand, both the in vivo rate of liver RBP synthesis and the translatable levels of liver RBP mRNA remain unaltered, regardless of the retinol status of rats (7).

We now report the extension of these findings by the direct examination of RBP mRNA levels in rats of various retinol states, using a specific RBP cDNA probe. Our studies show that RBP mRNA levels in the liver are the same in normal, retinol-depleted, and retinol-repleted rats. In addition, we demonstrate that RBP mRNA is present in a large number of extrahepatic tissues and that kidney mRNA is capable of directing the synthesis of preRBP in vitro. These data suggest that RBP, previously thought to be made only in the liver, may be synthesized in a large number of extrahepatic tissues.

MATERIALS AND METHODS

Animals

For the studies involving vitamin A status, twelve male rats were obtained as weanlings from the Holtzman Co., Madison, WI and were randomly divided into three groups (called normal vitamin A, retinol-depleted, and retinol-repleted). Nine rats (retinol-depleted and retinol-repleted groups) were depleted of their liver vitamin A

Abbreviations: RBP, retinol-binding protein; SSC, 0.15 M NaCl, 0.015 M Na citrate, pH 7.5.

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stores by being fed a semipurified vitamin A-deficient diet similar to that previously described (7). The other three rats (normal vitamin A group) were fed the same semipurified vitamin A-deficient diet but supplemented with 4 mg of vitamin A (supplied as retinyl esters) per kg of diet. Serum vitamin A levels were monitored weekly until the rats fed the vitamin A-deficient diet exhibited serum retinol (vitamin A) levels below 3 μg/dl (approximately 8 weeks). At this time the animals were considered retinol-depleted (5). Three of the retinol-depleted rats and the normal vitamin A rats were anesthetized with ether, decapitated, and the liver and serum were collected. The remaining six of the retinol-depleted rats were repleted with retinol by oral administration of 150 μg retinol dissolved in 0.2 ml peanut oil (retinol-repleted group). Two rats were killed as above at 1 hr, at 2 hr, and at 4 hr after the administration of retinol. Total retinol levels in liver and serum were determined by high performance liquid chromatography (8) and RBP levels in the liver and serum were quantitated by a specific and sensitive radioimmunoassay (5).

For the studies involving the tissue distribution of RBP mRNA, three male rats of the Sprague-Dawley strain (Camm Research Laboratories, Wayne, NJ) weighing 250–300 g were killed as above and the tissues were removed for the preparation of RNA.

Preparation of RBP-specific cDNA probe

Since human and rat RBP share 86% homology (9), we expected that a recently described (10) clone to human RBP would cross-hybridize under stringent conditions to rat RBP mRNA. This human RBP cDNA clone (cP2-6) was obtained as a generous gift from Dr. V. Colantuoni, EMBO Laboratory, Heidelberg, FRG. It contained a full-length human RBP cDNA cloned into the *Pst* 1 site of pBr 322 (10). Plasmid DNA was isolated by the alkaline lysis method (11). When this plasmid was digested with *Pst* 1 (Bethesda Research Labs., Gaithersburg, MD) two fragments, 721 and 227 bp, were generated in addition to the vector. The largest of these two fragments was isolated by electroelution from a 5% polyacrylamide gel, and nick-translated with [α-^{32}P]dATP and [α-^{32}P]dGTP (3000 Ci/mmol, Amersham, Arlington Heights, IL) to a specific activity of approximately 1 × 10^8 cpm/μg (12).

RNA isolation and analysis

Total RNA was prepared by the method of Tushinski et al. (13). RNA enriched in poly (A^+) RNA was obtained by oligo (dT)-cellulose affinity chromatography as described by Aviv and Leder (14) and quantitated by two independent methods: spectrophotometrically and by hybridization to [^{32}P]-labeled in vitro synthesized poly (dT) (15).

Dot blot analysis of poly (A^+) RNA was performed with a template manifold apparatus (Schleicher and Schuell, Inc., Keene, NH) to assure uniform dot size essentially as described by Thomas (16). Two μg of poly (A^+) RNA was denatured by incubation in 1 M glyoxal–10 mM Na phosphate buffer, pH 6.5, at 50°C for 1 hr. Twofold dilutions (1.0, 0.5, and 0.25 μg) of the denatured RNA samples were prepared with sterile water and spotted onto nitrocellulose paper equilibrated with 20× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.5). After all samples were spotted, the blot was air-dried, baked at 80°C for 2 hr, and treated with 20 mM Tris, pH 8.0, for 10 min at 100°C.

Northern blots were prepared by denaturing 10 μg of poly (A^+) RNA with 10 mM methylmercury hydroxide followed by electrophoresis in a 1% agarose gel as described by Bailey and Davidson (17). RNA was transferred to nitrocellulose paper in 20× SSC as described by Thomas (18) and the filters were baked at 80°C for 2 hr.

All nitrocellulose filters were prehybridized at 42°C for 4 to 8 hr in 50% formamide, 5× SSC, 1× Denhardt's (1× Denhardt's = 0.02% each of Ficoll, polyvinylpyrollidone, and bovine serum albumin), 50 mM Na phosphate, pH 6.5, and 250 μg/ml salmon sperm DNA. After prehybridization, the filters were hybridized in the same solution as the prehybridization with the addition of 5 × 10^5 cpm/ml (^{32}P)-labeled RBP cDNA and 5% dextran sulfate for 18 hr at 42°C. Following hybridization, filters were washed three times for 10 min each at room temperature with 2× SSC, and two times for 20 min each at 51°C with 0.1× SSC and 0.1% SDS. Filters were exposed to Kodak SB5 X-ray film at −70°C with intensifying screen and the appropriate exposures of radioautograms were quantitated by Computer Assisted Image Analysis Densitometry (Image Analysis #2000, Image Analysis Corporation, Deer Park, NY).

In vitro translation of liver and kidney mRNA

One μg of liver and 40 μg of kidney poly (A^+) RNA were translated in the rabbit reticulocyte lysate protein synthesizing system as previously described (2), except that the reticulocytes were obtained from Promega Biotec, Madison, WI. RBP was specifically immunoprecipitated with rabbit anti-rat RBP antiserum (4) in the same manner as previously described (2). The immunoprecipitated products were electrophoresed in SDS-15% polyacrylamide slab gels and fluorographs were prepared as previously described (2).

RESULTS

Liver RBP mRNA levels in normal, retinol-depleted, and retinol-repleted rats

In this study, three normal vitamin A and nine retinol-depleted rats were prepared. Six of the retinol-depleted

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rats were repleted orally with 150 μg of retinol. Pairs of retinol-repleted rats were killed 1 hr, 2 hr, and 4 hr after retinol repletion. Liver and serum levels of both total retinol and RBP were measured and found to be comparable to those previously reported for normal and retinol-depleted rats (5–7). In addition, after retinol repletion the liver total retinol, serum retinol, and serum RBP levels of the retinol-repleted rats all rose with time (liver RBP levels declined with the same time course) such that after 4 hr of repletion these levels were very similar to those reported after 45 min of IV injection of retinol (6, 7).

The size and amount of the rat RBP mRNA transcript was analyzed in normal and retinol-depleted rats by both Northern blot and Dot blot analysis of liver poly (A^+) RNA. A representative Northern blot of one pair of rats is shown in **Fig. 1**. The rat RBP mRNA from both the normal and the retinol-deficient rats migrated as a single band of approximately 1000 bases, very similar to that described for human RBP mRNA (10). In addition, no apparent differences in the amount of accumulated RBP mRNA were observed between normal and retinol-depleted rats.

The relative amounts of liver RBP mRNA in normal, retinol-depleted, and retinol-repleted rats were quantitated more carefully by Dot blot hybridization analysis. **Fig. 2** shows representative poly(A^+)RNA dots hybridized

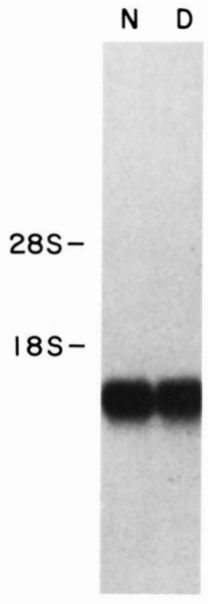

Fig. 1. Northern blot analysis of RBP mRNA isolated from the livers of normal and retinol-depleted rats. Ten μg of poly (A^+) RNA from normal (N) and retinol-depleted (D) rats was denatured with methylmercury hydroxide and electrophoresed in a 1% agarose gel. RNA was transferred to nitrocellulose paper and hybridized to nick-translated RBP cDNA.

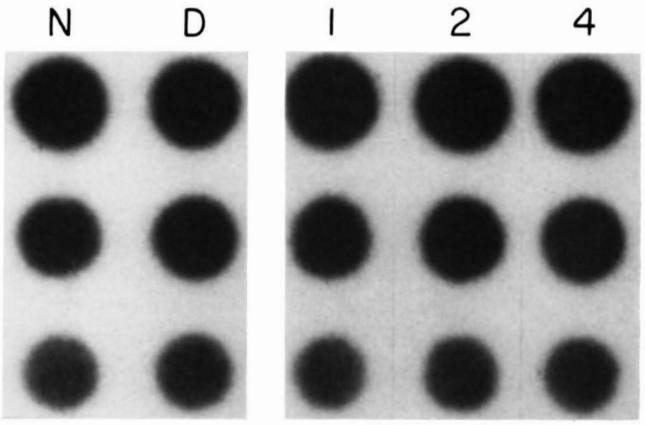

Fig. 2. RBP mRNA levels in livers of normal, retinol-depleted, and retinol-repleted rats. Poly (A^+) RNA was isolated from the livers of normal (N), retinol-depleted (D), and retinol-depleted rats repleted with retinol for 1 hr (1), 2 hr (2), and 4 hr (4). One μg and twofold dilutions of each RNA sample were dotted sequentially onto nitrocellulose paper and hybridized to nick-translated RBP cDNA.

with the RBP cDNA probe. In order to account for slight variations in the amount of poly(A^+)RNA bound to the nitrocellulose filter, total poly(A^+)RNA was measured by rehybridization of the identical blots with poly (dT). Analysis of the RBP mRNA levels (hybridization to RBP cDNA) compared to the total poly(A^+)RNA levels (hybridization to poly (dT)) demonstrated that the levels of liver RBP mRNA were very similar regardless of the retinol status of the rats. The relative integrated density units for RBP hybridization normalized for the poly (dT) hybridization for each group of rats (three normal, three retinol-depleted, and six retinol-repleted) were the following: normal 1.1 ± 0.2, retinol-depleted 1.1 ± 0.1, retinol-repleted 1 hr 1.1 ± 0.3, retinol-repleted 2 hr 1.0 ± 0.2, and retinol-repleted 4 hr 1.3 ± 0.1. Thus retinol does not appear to influence the level of RBP mRNA in the liver.

RBP mRNA levels in tissues

Next, we wished to determine whether any tissues in addition to the liver contained RBP mRNA. Poly (A^+) RNA was obtained from various rat tissues and the size and amount of the RBP mRNA was determined by Northern blot analysis. **Fig. 3** shows that many tissues contained RBP mRNA. As expected, RBP mRNA was most abundant in the liver. The levels of RBP mRNA in extrahepatic tissues could be grouped into three categories (see **Table 1**): kidney, which contained 5–10% of that of the liver level; lung, spleen, brain, stomach, heart, and skeletal muscle, which contained 1–3% of that of the liver level; and large intestine, small intestine, testes, and pancreas, which contained undetectable levels by the methods employed (less than 1% of that of the liver level).

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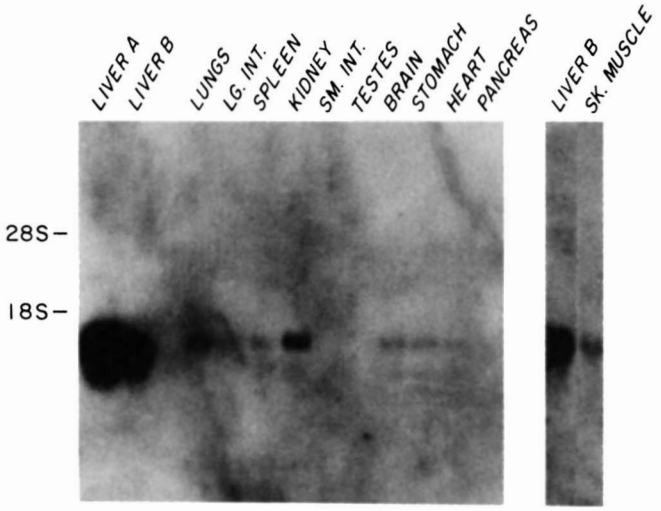

Fig. 3. Distribution of RBP mRNA in a variety of tissues of the rat. Ten μg of poly (A^+) RNA from each of the indicated tissues except liver (where both 10 μg (liver A) and 2 μg (liver B) were used) was denatured with methylmercury hydroxide and electrophoresed in a 1% agarose gel. RNA was transferred to nitrocellulose paper and hybridized to nick-translated RBP cDNA.

In vitro translation of liver and kidney mRNA

To investigate whether the RBP mRNA detected in the extrahepatic tissues was capable of translation into RBP, we chose to translate kidney mRNA, since it contained the highest levels of RBP mRNA detected by hybridization. Forty μg of poly (A^+) RNA from the kidney and 1 μg of poly (A^+) RNA from the liver was translated in rabbit reticulocyte lysates and the translation products were immunoprecipitated with rabbit anti-rat RBP antiserum. As shown in **Fig. 4**, a protein band of approximately 23 to 24 kilodaltons (preRBP) was immunoprecipitated with anti-RBP antiserum from both the kidney and liver translation products. In the liver sample a small amount of processed RBP was detected migrating at 20,000 daltons. Often a small amount of processed RBP is observed even in the absence of dog pancreas microsomal membranes in the translation mixture (2).

DISCUSSION

These studies were undertaken to study two aspects of RBP metabolism. First, we wished to directly measure liver RBP mRNA levels in normal, retinol-depleted and retinol-repleted rats. Second, we were interested in determining whether RBP mRNA was present in any extrahepatic tissues of the rat.

Previous work from our laboratory has shown that retinol plays an important role in the process of RBP secretion from the liver (5, 6). However, the rate of RBP synthesis and the translatable levels of RBP mRNA were not altered by retinol (7). In the present studies, we directly measured liver RBP mRNA levels in normal, retinol-depleted, and retinol-repleted rats using an RBP cDNA clone. We found that the levels of RBP mRNA were very similar in all three groups of rats. Thus, retinol does not appear to influence the accumulated level of liver RBP mRNA. It would appear that retinol does not control the expression of the RBP gene in the liver; however, additional studies in which the transcription rate of the RBP gene is directly measured are necessary to definitely demonstrate this.

Smith, Muto, and Goodman (19) previously demonstrated by radioimmunoassay the presence of immunoreactive RBP in a large number of tissues; however it was not possible in that study to distinguish RBP synthesized de novo in a given tissue from RBP associated with the tissues (but originally synthesized in the liver). In order to directly address this question, poly (A^+) RNA was isolated from 12 rat tissues and assayed for RBP mRNA by Northern blot analysis and hybridization with RBP cDNA. As expected, the liver contained the largest amount of RBP mRNA; however, many of the other tissues studied also demonstrated positive hybridization with the RBP cDNA. Quantitation of the blots revealed that the kidney contained 5–10% of that of the liver, and that the spleen, lungs, heart, brain, stomach, and skeletal muscle contained 1–3% of that of the liver. In all cases of positive hybridization with the RBP cDNA, the size of the RNA molecule was identical to that of the liver. Furthermore, poly (A^+) RNA isolated from the kidney was capable of directing the synthesis of preRBP in vitro in rabbit reticulocyte lysates. It is not known whether this extrahepatic RBP mRNA is present throughout the cells of these tissues or whether it is localized in specific cell types. Localization of RBP mRNA within specific cells of tissues awaits in situ hybridization studies.

TABLE 1. Relative amounts of RBP mRNA in various rat tissues

Tissue	RBP mRNA Abundance[a]
Liver	100%
Kidney	5–10%
Lungs	1–3%
Spleen	1–3%
Brain	1–3%
Stomach	1–3%
Heart	1–3%
Skeletal Muscle	1–3%
Large Intestine	<1%
Small Intestine	<1%
Testes	<1%
Pancreas	<1%

[a]Quantitated by Computer Assisted Image Analysis Densitometry of appropriate exposures of three Northern blots including the one shown in Fig. 3. For each blot the amount of RBP mRNA in liver was taken as 100%.

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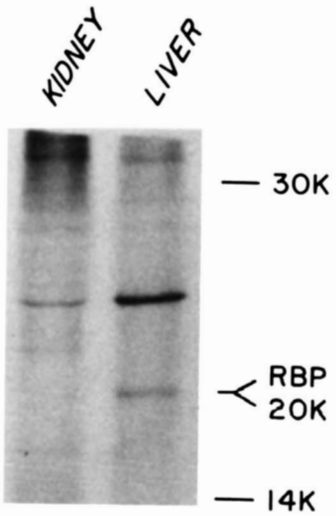

Fig. 4. Translation of liver and kidney mRNA and immunoprecipitation of RBP. One μg of poly (A^+) RNA from the liver and 40 μg of poly (A^+) RNA from the kidney were translated in the rabbit reticulocyte lysate protein synthesizing system. RBP was immunoprecipitated with rabbit anti-rat RBP serum. Immunoprecipitated products were analyzed on SDS-15% polyacrylamide slab gels and fluorographed. Molecular weight markers used were: lactalbumin (14,000), soybean trypsin inhibitor (20,000), carbonic anhydrase (30,000), and purified serum RBP.

It should be noted that, although the amount of RBP mRNA in each of the individual tissues is small, the composite amount of RBP mRNA and potentially the amount of RBP secreted from extrahepatic tissues is not trivial, especially when considering the mass of skeletal muscle in the body. Thus a significant portion of the RBP in plasma could potentially be of extrahepatic origin.

Recently the mRNA of a number of liver secretory proteins has been observed in extrahepatic tissues. Along with RBP, extrahepatic mRNA has been reported for apolipoprotein E (20, 21), transthyretin (22), albumin (23), and transferrin (24). In each case, the pattern and the level of the specific mRNA is distinct. This would suggest that the presence and pattern of RBP mRNA in extrahepatic tissues is not a general aspect of liver secretory proteins but rather a specific pattern of distribution pertinent to RBP metabolism and, presumably, function.

Kinetic studies of the turnover and metabolism of RBP in vivo in humans (25, 26), in cynomolgus monkeys (27), and in rats (28) consistently suggest that an RBP molecule that has delivered its retinol subsequently undergoes catabolism (in the kidneys) and is not reutilized. On the other hand, there is evidence suggested by De Luca et al. (29) and Lewis, Green, and Underwood (30) that there may be quantitatively significant recycling and reutilization of retinol in the animal body. If this is true, then the possibility that retinol is recycled from extrahepatic tissues as the retinol-RBP complex, and that this involves RBP newly synthesized in the extrahepatic tissues, should be considered. Such a scheme would be consistent with the present finding of RBP mRNA in a number of extrahepatic tissues. Accordingly, we hypothesize 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. Future studies will be needed to explore this and other possible hypotheses.

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