The role of extrahepatic retinol binding protein in the mobilization of retinoid stores

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Abstract **Although the major tissue site of retinol binding protein (RBP) synthesis in the body is the liver, other sites of synthesis have been reported. The physiological role(s) of circulating RBP that is produced and secreted extrahepatically has not been systematically investigated. To address this question, we used as a model a mouse strain (hRBP$^{-/-}$) that expresses human RBP (hRBP) cDNA under the control of the mouse muscle creatine kinase promoter in an *rbp*-null background (RBP$^{-/-}$). By comparing hRBP$^{-/-}$, RBP$^{-/-}$, and wild-type mice, we asked whether extrahepatic RBP can perform all of the physiological functions of RBP synthesized in the liver. We demonstrate that extrahepatically synthesized hRBP, unlike RBP expressed in liver, cannot mobilize liver retinoid stores. Consistent with this conclusion, we find that circulating hRBP is not taken up by hepatocytes. RBP has been proposed to play an essential role in distributing hepatic retinoids between hepatocytes and hepatic stellate cells. We find, however, that the distribution of retinoid in the livers of the three mouse strains described above is identical. Thus, RBP is not required for intrahepatic transport and storage of retinoid. These and other observations are discussed.**—Quadro, L., W. S. Blaner, L. Hamberger, P. M. Novikoff, S. Vogel, R. Piantedosi, M. E. Gottesman, and V. Colantuoni. **The role of extrahepatic retinol binding protein in the mobilization of retinoid stores.** ***J. Lipid Res.*** **2004.** 45: **1975–1982.**

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Retinol is the major circulating retinoid (vitamin A and its analogs) form and is needed to maintain normal growth and development, immunity, reproduction, vision, and other important physiological processes (1). Retinol is transported from hepatic retinoid stores to target tissues exclusively by means of a specific 21 kDa transport protein, retinol binding protein (RBP) (2). Retinol is not biologically active per se, and within tissues it is oxidized to retinaldehyde and then to retinoic acid (3). Retinaldehyde is active in the visual cycle (4, 5). All-*trans*- and 9-*cis*-retinoic acid regulate the transcription of a variety of target genes (6, 7) through receptor-mediated events (8–13).

All retinoids present in the body originate from the diet (3). They are esterified in the intestine to retinyl ester and incorporated into chylomicrons along with other dietary lipids. Chylomicrons are secreted into lymph and then into the bloodstream, where they undergo structural rearrangements leading to the release of retinyl ester (14). The majority of chylomicrons are cleared by hepatocytes upon hydrolysis of retinyl ester into retinol (15). In the liver, newly formed retinol can be either stored in hepatic stellate cells (also called Ito cells) in the form of retinyl ester or secreted into the blood bound to RBP. RBP secretion from hepatocytes is a highly regulated process that is still not fully understood; it depends on retinol availability within the cell (16–19). Retinol-RBP circulates in the bloodstream in a 1:1 molar complex with transthyretin, a 55 kDa protein that is synthesized in and secreted from the liver (20). This ternary complex prevents retinol-RBP excretion by the kidney (2, 21). The mechanism through which tissues acquire retinol from circulating retinol-RBP is also subject to considerable debate.

Although the major site of RBP synthesis in the body is the hepatocyte, other adult organs and tissues are reported to express RBP (2). These include kidney, adipose, lacrimal gland, retinal pigment epithelium, testes, and brain (2). It is believed that the synthesis of RBP in extrahepatic tissues serves either to recycle retinol to liver (22–25) or to facilitate the uptake of retinol by tissues with blood tissue barriers (testes, eyes, and brain) (26–38). However, neither of these possibilities has been tested experimentally. Moreover, it is unclear whether circulating RBP derived from different tissues performs the same basic physiological functions as the hepatic protein (i.e., mobili-

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Abbreviations: hRBP, human retinol binding protein; MAP, multiple antigen peptide; RBP, retinol binding protein.

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zation of hepatic retinoid stores and delivery of retinol to tissues).

To address these questions, we used two mouse strains that we had previously generated. The RBP-knockout mouse strain ($RBP^{-/-}$) was obtained by targeted disruption of the genomic locus (39). The mice have dramatically reduced serum retinol levels (12.5% of wild-type animals) and impaired retinal function and visual acuity during the first months of life. Fed a retinoid-sufficient diet, they recover normal retinol levels and vision by 4 months of age. In contrast, their vision deteriorates and their circulating retinol concentration decreases if they are kept on a retinoid-deficient diet from weaning. Thus, the low levels of circulating retinol in these mice arise from recent dietary intake (39). Because they are dependent on a regular retinoid intake, the retinoid status of these animals is extremely tenuous (40, 41). Finally, because retinol mobilization from hepatic stores is compromised, $RBP^{-/-}$ animals accumulate retinol and retinyl ester in the liver at a higher rate than do wild-type mice (39, 42).

We also generated a transgenic mouse that expresses human RBP (hRBP) cDNA under the control of the mouse muscle creatine kinase promoter in the *rbp*-null background ($hRBP^{-/-}$) (43). The transgenic hRBP is produced at high levels, and, like endogenous murine RBP (mRBP), binds transthyretin and delivers retinol to tissues. Moreover, the hRBP suppresses the visual defect of the *rbp*-null mice and allows peripheral tissues to acquire normal levels of retinol (43).

The experiments reported here using mutant and wild-type mice address two important issues related to RBP and retinoid metabolism. First, we demonstrate that extrahepatically synthesized hRBP cannot mobilize hepatic stores. Only RBP synthesized in the liver can mobilize such stores. Second, we ask if RBP plays a role in mediating the distribution of retinoids between liver hepatocytes and stellate cells. RBP does not mediate cellular retinoid trafficking within the liver.

METHODS

Animals

$hRBP^{-/-}$ mice were generated and characterized as described by Quadro et al. (43).

Plasma clearance and liver uptake of [^{3}H]retinol

Three month old female $hRBP^{-/-}$, $RBP^{-/-}$, and wild-type mice received an oral bolus of [^{3}H]retinol (10^6 cpm/100 μl) in peanut oil via gavage. Plasma samples were obtained after centrifugation of the blood that had been collected into a tube containing EDTA at 14,000 *g*. Dissected tissues were immediately placed in liquid nitrogen and stored at −70°C until analysis. To assess [^{3}H]retinoid concentrations in total plasma, 20 μl of each plasma sample was transferred to a scintillation vial and dissolved in 20 ml of Hydroflor liquid scintillation counting solution. To analyze liver levels of [^{3}H]retinoids, tissues were weighed, homogenized in 3 volumes of PBS using a Polytron homogenizer (Brinkman Instruments, Westbury, NY), and extracted with chloroform-methanol (2:1, v/v). After centrifugation at 500 *g* for 10 min, the lower chloroform phase was transferred to scintillation vials and evaporated in a fume hood. The retinoid-containing lipid film remaining after evaporation of the chloroform was dissolved in 20 ml of Hydroflor liquid scintillation counting solution. ^{3}H-cpm present in plasma and tissues samples was measured in a Beckman LS 1800 liquid scintillation counter.

Preparation of protein extracts from mouse tissues and Western blot analysis

To survey RBP protein expression in mouse tissues, organs were removed after PBS perfusion and homogenized with a Waring blender in a buffer containing 20 mM potassium phosphate buffer, pH 7.0, 0.25 M sucrose, 50 mM NaCl, 5 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 mM benzamidine-HCl (5 ml/g wet weight). Nuclei were pelleted by centrifugation at 700 *g* for 5 min. All operations were performed at 4°C. Protein concentrations were determined by the method of Lowry et al. (44) using BSA as a standard. Western blot analysis was performed as described (39) by using a rabbit polyclonal anti-rat serum RBP (45).

Antibody production

A peptide corresponding to amino acids 185–201 of the primary sequence of mouse RBP (46) was synthesized by the Columbia University Howard Hughes Protein Core Facility. The peptide was linked to an eight-armed matrix (47), and this multiple antigen peptide (MAP) served as the immunogen. The MAP was sent to Pocono Rabbit Farm and Laboratory, Inc. (Canadensis, PA) for antibody production. For this purpose, a New Zealand White rabbit was injected intradermally with 1 mg of MAP in Complete Freund's Adjuvant. One booster intradermal injection of 100 μg of MAP in Complete Freund's Adjuvant was given on day 14. On day 28, the rabbit was given a subcutaneous injection of 100 μg of the MAP in Incomplete Freund's Adjuvant. Test bleeds began 42 days after the initial injection, followed by injection of 50 μg of MAP in Incomplete Freund's Adjuvant on day 56 and every 4 weeks thereafter. Test bleeds were taken 2 weeks after each injection (days 42, 70, and 98). On day 126 after the initial immunization, the rabbit was exsanguinated.

HPLC analysis

Retinol and retinyl ester concentrations in plasma and tissues were measured by reverse-phase HPLC as described (48, 49).

Diet and animal husbandry

A purified nutritionally complete control retinoid-sufficient diet (Purified Test Diet 5755; W. F. Fisher and Son, Inc.) containing 22 IU retinol/g diet and a retinoid-deficient but otherwise nutritionally complete diet (Purified Test Diet 5822; W. F. Fisher and Son, Inc.) containing by actual lot analysis <0.22 IU retinol/g diet were obtained from standard sources. All nutrients other than retinol were present in these two purified diets at the same concentrations. Mice used for these studies were bred and maintained continuously in a specific virus- and pathogen-free (barrier) facility operating on a 12 h dark/light cycle.

Light microscopy analysis

For light microscopic studies, the liver was removed from wild-type, $RBP^{-/-}$, and $hRBP^{-/-}$ mice under ether anesthesia. Approximately 1 mm slices of the liver were cut by hand and placed in a fixative containing 4% paraformaldehyde/2.5% glutaraldehyde/0.1 M cacodylate buffer for 5 h at 0°C with shaking. Frozen sections (~20 μm) were prepared from the liver slices using a freezing microtome and treated as follows: *1*) immersion in 0.5% oil red O/60% triethyl phosphate solution for 15 min at room temperature to detect neutral lipids in stellate cells and hepatocytes; *2*) staining in methyl-green pyronin for histology; and *3*)

preparation of toluidine blue-stained 1 μm sections after embedding into Epon, according to protocols established for electron microscopy, to confirm lipid distribution.

RESULTS

Circulating hRBP of extrahepatic origin cannot mobilize liver retinoid

To determine if RBP of extrahepatic origin can mobilize hepatic retinoid stores, we measured plasma and hepatic concentrations of [^{3}H]retinol at 2, 4, and 24 h after oral administration of a bolus dose of [^{3}H]retinol in peanut oil. **Figure 1A** shows the total radioactivity present in plasma at these time points. These values represent both the rate of clearance and secretion into plasma of the ingested [^{3}H]retinoid. Note that 2 h after administration, plasma [^{3}H]retinoid levels in RBP$^{-/-}$ and hRBP$^{-/-}$ mice were significantly lower than in wild-type animals. Triglyceride and cholesterol concentrations in plasma pools of all three strains were equivalent (data not shown), suggesting that their chylomicron retinoid clearance rates were similar. We also analyzed by HPLC [^{3}H]retinol levels in the circulation of all animals at the same time point (2 h). **Table 1** shows that ~70% of the ^{3}H label in the circulation of wild-type female animals was [^{3}H]retinol, compared

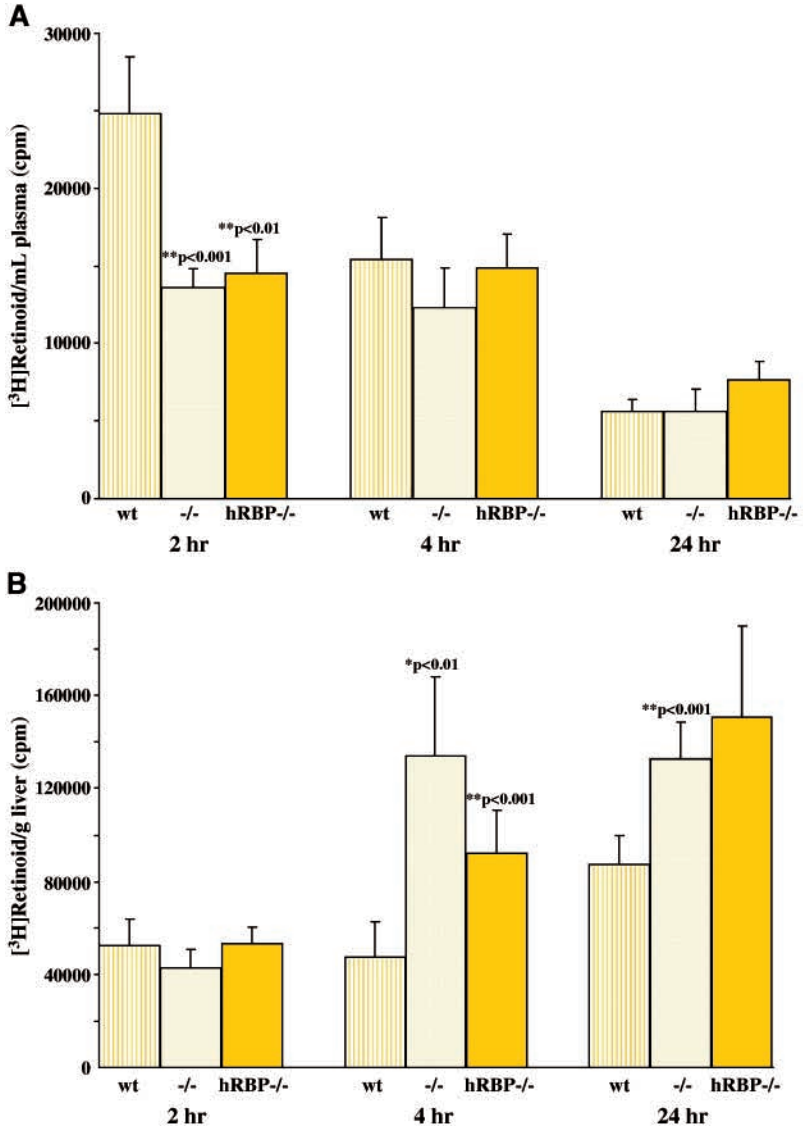

Fig. 1. Plasma response and tissue [^{3}H]retinoid uptake in mice that express human retinol binding protein in muscle but lack any endogenous mouse retinol binding protein (hRBP$^{-/-}$ mice). Comparison of plasma (A) and liver (B) [^{3}H]retinoid levels 2, 4, and 24 h after administration of a bolus dose of [^{3}H]retinol given by gavage in 0.1 ml of peanut oil to hRBP$^{-/-}$, RBP$^{-/-}$ (−/−), and wild-type (wt) mice. At each time, the values for each strain represent the mean ± SD obtained for five mice. Statistical comparisons were made by an unpaired Student's *t*-test. * $P < 0.01$ and ** $P < 0.001$ vs. the corresponding wild-type group. The data reported here reflect one representative experiment that was repeated three independent times yielding essentially similar results.

TABLE 1. Total plasma retinol 2 h after oral administration of [^{3}H]retinol to wild-type, RBP$^{-/-}$, and hRBP$^{-/-}$ mice

Genotype	[^{3}H]Retinol	Mice
	%	*n*
Wild type	72	5
RBP$^{-/-}$	28	5
hRBP$^{-/-}$	23	4

hRBP, human retinol binding protein; RBP, retinol binding protein. Values represent the percentage of total [^{3}H]retinol (retinol and retinyl ester) present in the circulation as [^{3}H]retinol. Plasma samples from female mice of each genotype were pooled. An identical duplicate experiment was carried out using age-matched male mice from each of the three strains. The data obtained from the male mice were the same as for the females.

with only ~25% of the ^{3}H label in female RBP$^{-/-}$ and hRBP$^{-/-}$mice. An identical study was carried out using male mice from the three strains and gave the same results. These data support the hypothesis that wild-type mice secrete newly absorbed [^{3}H]retinol back into the circulation. In contrast, RBP$^{-/-}$ and hRBP$^{-/-}$mice retain this retinol because they cannot mobilize hepatic retinoid stores. The levels of plasma radioactivity at 4 and 24 h were similar in the three strains. The distribution of this radioactivity between [^{3}H]retinol and derivatives was not determined.

Liver uptake curves of the three strains (Fig. 1B) support the above conclusion. At 2 h, liver [^{3}H]retinoid content was similar in all strains. Note that this observation does not conflict with our finding that wild-type animals secrete [^{3}H]retinol at this time point, because the amount of circulating [^{3}H]retinol is very small compared with the large amount of retinoid stored in liver (3). Failure to find a statistically significant decrease in hepatic retinoid levels does not, therefore, indicate the presence or absence of secretion. At 4 h, hepatic [^{3}H]retinol levels in RBP$^{-/-}$ and hRBP$^{-/-}$ mice were significantly higher than in wild-type animals. At 24 h, wild-type hepatic [^{3}H]retinol concentrations started to increase, likely reflecting reuptake of circulating retinol. Nevertheless, wild-type [^{3}H]retinol levels did not reach the levels of RBP-null mutants. Taken together, these results are consistent with the notion that RBP synthesized in the liver can mobilize liver stores but that RBP of extrahepatic origin cannot. In muscle and in other tissues analyzed, [^{3}H]retinol uptake curves were similar in the three mouse strains (data not shown).

Circulating hRBP is not taken up by liver

The data provided in Fig. 1 indicate that liver retinoid stores cannot be mobilized by hRBP secreted from muscle and suggest that RBP must be synthesized in liver to mobilize hepatic retinoid. Our data imply that circulating hRBP is not internalized by hepatocytes. To test this hypothesis, protein extracts were prepared from liver and muscle of hRBP$^{-/-}$ mice and from livers of wild-type and RBP$^{-/-}$ animals that were extensively perfused before dissection to remove blood from tissues. Western blot analysis was performed using a rabbit polyclonal anti-rat serum RBP (45) that cross-reacts with both endogenous mRBP and exogenous hRBP (**Fig. 2**). RBP was detected only in muscle extracts from hRBP$^{-/-}$ mice. As expected, no RBP was detected in liver extracts from RBP$^{-/-}$ mice, whereas a strong immunoreactive band was observed in extracts from wild-type mice. Critically, although the serum concentration of hRBP in serum from hRBP$^{-/-}$ mice (2.27 ± 0.43 mg/dl) is similar to the concentration of mRBP present in the circulations of wild-type mice (21, 43), no immunoreactive band, representing either hRBP or mRBP, was observed in extracts prepared from perfused livers of hRBP$^{-/-}$ mice. In separate experiments, we were unable to detect hRBP in liver extracts from hRBP$^{-/-}$ mice even when as much as 300 μg of total liver proteins was loaded onto the SDS-PAGE gel used for immunoblot analysis (data not shown). Our low limit of detection for hRBP in this assay was 1.5 ng. Using a conventional assumption that the wet weight of liver consists of 15% protein (50), we estimate that our low limit of detection for hRBP in hRBP$^{-/-}$ liver would be ~0.6 μg hRBP/g liver. Because wild-type mouse liver contains ~32 μg RBP/g tissue (49), if as little as 2% of the RBP present in liver is normally derived from uptake of extrahepatically synthesized RBP, we would have been able to detect this by Western blot. Thus, the results obtained suggest that circulating RBP of extrahepatic origin is not internalized to any significant extent by liver cells. Immunohistochemical analysis of liver sections from hRBP$^{-/-}$ mice using a polyclonal anti-human serum RBP (51) also failed to detect hRBP, confirming these results (data not shown).

Circulating hRBP and mobilization of retinoid from peripheral stores

We next explored the role of extrahepatic RBP in maintaining normal serum and tissue retinol concentrations

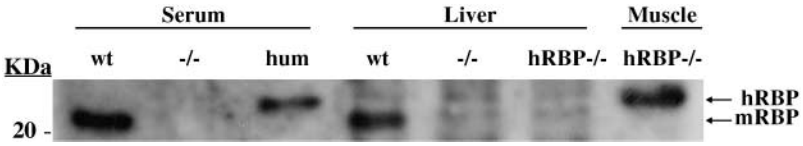

Fig. 2. Western blot analysis of liver and muscle protein extracts from hRBP$^{-/-}$ mice. Analysis was performed using 50 μg of liver and 10 μg of muscle protein that was extracted from tissues of mice that had undergone a whole body perfusion with ice-cold saline immediately before death. Three microliters of a 1:10 dilution of serum from wild-type (wt) and RBP$^{-/-}$ (−/−) mice and 3 μl of a 1:10 dilution of human serum (hum) were loaded as controls. Western blot analysis was performed using rabbit polyclonal anti-rat serum RBP (45). The position of a protein marker is indicated at left. The concentration of hRBP in serum of hRBP$^{-/-}$ mice is approximately the same (~2.0 mg RBP/dl) as the concentration of mouse RBP (mRBP) present in wild-type mice (21, 43).

during extended periods of dietary retinoid deprivation. Six week old hRBP$^{-/-}$, RBP$^{-/-}$, and RBP$^{+/-}$ (heterozygous) females were placed on a retinoid-deficient diet or a retinoid-sufficient diet for 1 month. Retinol and retinyl ester levels in serum, liver, lung, and muscle were then determined by HPLC. The results of this experiment are shown in **Tables 2** and **3**. As expected, serum retinol levels in animals on a retinoid-deficient diet remained constant in RBP$^{+/-}$ mice and decreased close to zero in RBP$^{-/-}$ mice. Dietary retinoid deprivation reduced serum retinol levels by 50% in hRBP$^{-/-}$ mice (Table 2).

Retinoid deprivation for 1 month did not significantly reduce liver retinoid levels in any of the three strains analyzed (Table 3). The low liver retinoid levels of hRBP$^{-/-}$ compared with age- and sex-matched heterozygous and knockout animals reflects the amount of time they were kept on a retinoid-sufficient diet before vitamin deprivation (Table 3). The retinoid content of the stores is, in fact, related to the amount of retinoid ingested with the diet (reviewed in Ref. 14). In contrast, lung retinoid stores remained constant in RBP$^{+/-}$ mice maintained on a retinoid-deficient diet but decreased by 70% in RBP$^{-/-}$ mice and by 90% in hRBP$^{-/-}$ animals (Table 3). It is possible that failure to mobilize liver retinol stores may activate a pathway that draws on the lung deposits, which represent an important retinoid storage site in mouse (52–54). Depletion of nonhepatic peripheral stores may account for the decrease of serum retinol levels in hRBP$^{-/-}$ mice after 1 month on a retinoid-deficient diet (Table 2).

Retinoid distribution in the liver of RBP$^{-/-}$ and hRBP$^{-/-}$ mice is normal

We previously demonstrated that RBP$^{-/-}$ mice take up retinoid normally from the diet and accumulate hepatic retinoid stores at a rate only slightly greater than do wild-type mice (39, 42). To identify the liver cells that accumulate retinoid in the RBP$^{-/-}$ mice, liver sections from 5 month old wild-type and RBP$^{-/-}$ male mice were stained with oil red O. This dye, which specifically recognizes lipid-rich droplets, is commonly used to stain and identify retinoid-rich droplets present in hepatic stellate cells (55) (**Fig. 3A**, **B**). No structural abnormalities were observed in the livers of RBP$^{-/-}$ mice. Furthermore, high magnification of these sections indicated that both the number and the size of the lipid droplet-containing cells were equivalent in wild-type and RBP$^{-/-}$ mice. Toluidine blue staining of Epon-embedded sections from the same mice demonstrated that the stellate cells in the perisinusoidal space were fully laden with retinoid-rich droplets (Fig. 3D, E). Analysis of multiple sections indicates that both the number and the distribution of stellate cells of RBP$^{-/-}$ and wild-type mice were similar.

Extrahepatic expression of hRBP affected neither the structure of the liver nor its ability to accumulate and store retinoid (Fig. 3C, F). Finally, higher magnification of liver sections from hRBP$^{-/-}$ animals indicated that both the number and the size of the lipid droplet-containing cells as well as the number and the size of the droplets present in hepatic stellate cells were indistinguishable from wild-type and RBP$^{-/-}$ mice

TABLE 2. Serum retinol levels of RBP$^{+/-}$, RBP$^{-/-}$, and hRBP$^{-/-}$ mice maintained on a retinoid-deficient diet for 1 month

Genotype	Diet	Retinol
		μg/dl
RBP$^{+/-}$	Control	17.2 ± 1.5
	Retinoid-deficient	16.0 ± 2.6
RBP$^{-/-}$	Control	2.2 ± 0.3
	Retinoid-deficient	0.9 ± 0.6[a]
hRBP$^{-/-}$	Control	28.0 ± 2.6
	Retinoid-deficient	12.5 ± 2.1[b]

Retinol levels were determined by reverse-phase HPLC and are expressed as means ± SD. Statistical significance was determined by Student's unpaired *t*-test. Four 6 week old female mice were analyzed in each group.

[a] $P < 0.01$ vs. the corresponding control diet group.

[b] $P < 0.001$ vs. the corresponding control diet group.

TABLE 3. Total retinol levels in tissues of RBP$^{+/-}$, RBP$^{-/-}$, and hRBP$^{-/-}$ mice maintained on a retinoid-deficient diet for 1 month

Tissue	Genotype	Diet	Total Retinol
			μg/g tissue
Liver	RBP$^{+/-}$	Control	97.9 ± 25.9
		Retinoid-deficient	106.7 ± 21.5
	RBP$^{-/-}$	Control	174.6 ± 85.7
		Retinoid-deficient	77.5 ± 52.7
	hRBP$^{-/-}$	Control	38.9 ± 8.2
		Retinoid-deficient	43.6 ± 9.5
Lung	RBP$^{+/-}$	Control	59.6 ± 22.3
		Retinoid-deficient	73.7 ± 18.1
	RBP$^{-/-}$	Control	61.1 ± 15.8
		Retinoid-deficient	18.9 ± 7.3[a]
	hRBP$^{-/-}$	Control	100.9 ± 35.9
		Retinoid-deficient	7.0 ± 5.9[a]
Muscle	RBP$^{+/-}$	Control	n.d.
		Retinoid-deficient	n.d.
	RBP$^{-/-}$	Control	n.d.
		Retinoid-deficient	n.d.
	hRBP$^{-/-}$	Control	0.2 ± 0.1
		Retinoid-deficient	0.1 ± 0.1

Retinol levels were determined by reverse-phase HPLC and are expressed as means ± SD. Statistical significance was tested by Student's unpaired *t*-test. Four 6 week old female mice were analyzed for each group. hRBP$^{-/-}$ mice were maintained on a retinoid-sufficient diet for 13 days between weaning and the start of retinoid deprivation, whereas RBP$^{+/-}$ and RBP$^{-/-}$ mice were kept for 22 days after weaning on a retinoid-sufficient diet before the beginning of the experiment. n.d., not detectable (<0.1 μg/g tissue).

[a] $P < 0.01$ vs. the corresponding control diet group.

DISCUSSION

The major physiological role of RBP is to deliver retinol to tissues and to mobilize hepatic retinoid stores to ensure a constant supply of retinoid to tissues, especially in times of insufficient dietary intake (2, 40). The role that hepatic RBP plays in the maintenance of this homeostasis is well established, whereas that played by extrahepatically synthesized RBP has not been explored. The experiments reported here provide new insights into the mechanisms of mobilization of retinoid stores by RBP. We demonstrate

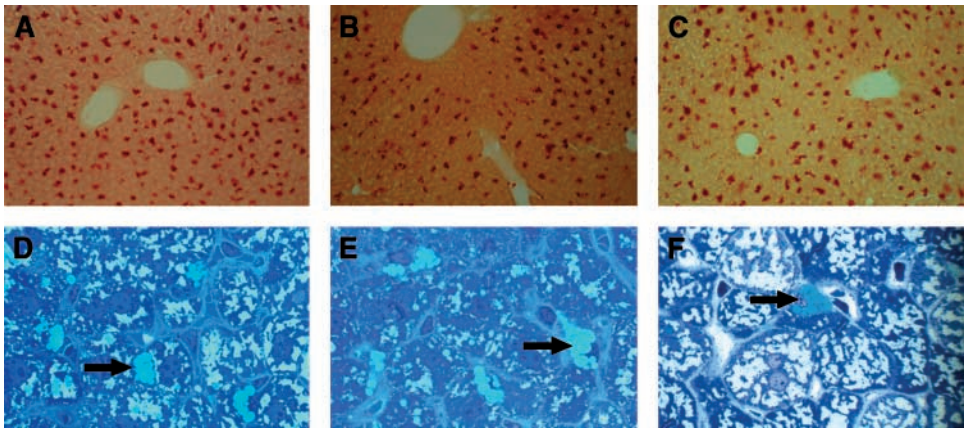

Fig. 3. Cellular pattern of retinoid storage is normal in livers of RBP$^{-/-}$ and hRBP$^{-/-}$ mice. Oil red O staining (top panels) and toluidine blue staining of Epon-embedded sections (bottom panels) from 5 month old wild-type (A and D), RBP$^{-/-}$ (B and E), and hRBP$^{-/-}$ (C and F) male mice. In the top panels, sinusoidal stellate cells are distended with lipid in all three strains of mice. All of the stellate cells throughout the hepatic cord (from the portal triad area to the central vein area) in the three strains of mice have accumulated essentially similar amounts of lipid. Little accumulation of lipid is evident in the hepatocytes throughout the hepatic cord. In the bottom panels, the accumulation of lipid droplets in the stellate cells is more distinctively delineated. D, E, F: View with an oil-immersion microscope (400×). The arrows indicate lipid droplets present in hepatic stellate cells. The large white areas are glycogen granules present in adjoining hepatocytes.

that RBP synthesized in muscle is unable to mobilize hepatic retinoid stores. Thus, after administration of a bolus dose of [^{3}H]retinol, wild-type mice secrete [^{3}H]retinol-RBP from liver back into the circulation. In contrast, RBP$^{-/-}$ and hRBP$^{-/-}$ mice do not resecrete [^{3}H]retinol; instead, they accumulate it in liver (Fig. 1). Consistent with the failure of circulating hRBP to mobilize hepatic retinol, we detect no hRBP in livers of hRBP$^{-/-}$ mice by Western blot (Fig. 2) or by immunohistochemical analysis (data not shown). These data suggest that circulating hRBP is not taken up by hepatocytes. Several authors have reported endocytosis of RBP by different cells in culture (F9 embryonal carcinoma, HepG2 hepatocarcinoma, Caki-1 kidney carcinoma, HeLa cells) (56, 57) or by parenchymal and stellate cells from rat liver after intravenous administration of large bolus doses of purified hRBP (500 μg/rat) (24, 25). These studies suggested that internalization of circulating RBP by liver allows the recycling of retinol from peripheral tissues to liver and apo-RBP to drain hepatic retinol stores. Other authors, in contrast, reported that retinol uptake from RBP by liver cells in vitro does not depend specifically on its binding to RBP (58, 59). Our present data unequivocally demonstrate that circulating hRBP of extrahepatic origin, and, presumably, circulating RBP of hepatic origin as well, is not taken up by hepatocytes and, as consequence, cannot mobilize hepatic retinol. Our experiments, however, do not solve the question of whether circulating RBP can deliver retinol to liver, as it does to the eye.

We also demonstrate that mobilization of retinoid from lung stores occurs in animals that cannot mobilize liver retinoid deposits. Thus, RBP$^{-/-}$ mice show a 70% decrease and hRBP$^{-/-}$ mice show a 90% decrease in lung deposits after 1 month of retinoid deprivation (Table 3). We recently reported that RBP$^{-/-}$ mice maintained on a retinoid-deficient diet from the time of weaning (21 days) for up to 6 months show a decline in retinoid levels in liver and lung at a rate comparable to that in wild-type mice maintained on a similar dietary regimen (41). We also show that in the absence of RBP, alternative pathways of hepatic retinoid turnover seem to be activated to render available to the peripheral tissues the retinoid otherwise trapped in the liver (41). The mechanism responsible for the decline in lung retinoid stores in the present study is not clear. We are now investigating whether similar mechanism(s) may play a role in the mobilization of lung retinoid stores.

A considerable body of evidence in the literature supports the notion that RBP plays an important role in facilitating the transfer of newly absorbed retinol between hepatocytes and stellate cells, the major hepatic site of retinoid storage (24, 57, 60–64). The data we present in this study clearly rule out this assumption. We find no quantitative or qualitative differences in the pattern of retinoid storage in liver sections from wild-type and RBP$^{-/-}$ mice (Fig. 3). In previous reports (39, 43, 65), we demonstrated that liver retinoid concentrations in wild-type and RBP$^{-/-}$ mice were similar. The present experiments extend this finding and provide the first strong evidence that RBP is not required for shuttling retinol from hepatocytes to stellate cells (14). Our data support the model proposed by Ghyselinck et al. (66). They showed that stellate cells of CRBP-I-null mice do not accumulate retinol (66), suggesting that CRBP-I and not RBP plays a pivotal role in facilitating the normal intercellular trafficking of retinol within the liver. Our work also argues against the idea that RBP synthesized outside the liver and secreted into the circulation plays an important physiological function in recycling retinol from the periphery back to the liver for storage or to other target tissues (22–25).

In conclusion, we demonstrated that although circulating RBP of extrahepatic origin supports retinol delivery to the eye and other tissues (43), it cannot draw on liver retinol stores, presumably because it is not taken up by hepatic cells. We propose that RBP must be synthesized within the liver to mobilize hepatic retinoid stores. Our data also show that RBP is not involved in the intercellular trafficking of retinol between hepatocytes and hepatic stellate cells. This leaves open the question of what role RBP synthesized extrahepatically plays in retinoid metabolism. We assume that this RBP has a function specific to its tissue of origin, but this remains to be determined.

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REFERENCES

1. Napoli, J. L. 1996. Biochemical pathways of retinoid transport, metabolism, and signal transduction. *Clin. Immunol. Immunopathol.* **80 (Suppl.):** 52–62.
2. Soprano, D. R., and W. S. Blaner. 1994. Plasma retinol-binding protein. *In* The Retinoids: Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, New York. 257–282.
3. Blaner, W. S., and J. A. Olson. 1994. Retinol and retinoic acid metabolism. *In* The Retinoids: Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, New York. 229–256.
4. Wald, G. 1968. The molecular basis of visual excitation. *Nature.* **219:** 800–807.
5. Saari, J. C. 1999. Retinoids in mammalian vision. *In* The Handbook of Experimental Pharmacology. Retinoids. H. Nau and W. S. Blaner, editors. Springer-Verlag, Heidelberg, Germany. 563–588.
6. Clagett-Dame, M., and L. A. Plum. 1997. Retinoid-regulated gene expression in neural development. *Crit. Rev. Eukaryot. Gene Expr.* **7:** 299–342.
7. Gudas, L. J., M. B. Sporn, and A. B. Roberts. 1994. Cellular biology and biochemistry of the retinoids. *In* The Retinoids: Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, New York. 443–520.
8. Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans. 1995. The nuclear receptor superfamily: the second decade. *Cell.* **83:** 835–839.
9. Pfahl, M., and F. Chytil. 1996. Regulation of metabolism by retinoic acid and its nuclear receptors. *Annu. Rev. Nutr.* **16:** 257–283.
10. Leblanc, B. P., and H. G. Stunnenberg. 1995. 9-Cis retinoic acid signaling: changing partners causes some excitement. *Genes Dev.* **9:** 1811–1816.
11. Chen, J. D., and R. M. Evans. 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature.* **377:** 454–457.
12. Kurokawa, R., M. Soderstrom, A. Horlein, S. Halachmi, M. Brown, M. G. Rosenfeld, and C. K. Glass. 1995. Polarity-specific activities of retinoic acid receptors determined by a co-repressor. *Nature.* **377:** 451–454.
13. Glass, C. K., M. G. Rosenfeld, D. W. Rose, R. Kurokawa, Y. Kamei, L. Xu, J. Torchia, M. H. Ogliastro, and S. Westin. 1997. Mechanisms of transcriptional activation by retinoic acid receptors. *Biochem. Soc. Trans.* **25:** 602–605.
14. Vogel, S., M. V. Gamble, and W. S. Blaner. 1999. Biosynthesis, absorption, metabolism and transport of retinoids. *In* The Handbook of Experimental Pharmacology. Retinoids. H. Nau and W. S. Blaner, editors. Springer-Verlag, Heidelberg, Germany. 31–95.
15. Goodman, D. S. 1965. Cholesterol ester metabolism. *Physiol. Rev.* **45:** 747–839.
16. Dixon, J. L., and D. S. Goodman. 1987. Effects of nutritional and hormonal factors on the metabolism of retinol-binding protein by primary cultures of rat hepatocytes. *J. Cell. Physiol.* **130:** 6–13.
17. Dixon, J. L., and D. S. Goodman. 1987. Studies on the metabolism of retinol-binding protein by primary hepatocytes from retinol-deficient rats. *J. Cell. Physiol.* **130:** 14–20.
18. Perozzi, G., E. Mengheri, V. Colantuoni, and S. Gaetani. 1991. Retinoid intake and in vivo expression of the genes involved in retinol transport. *Eur. J. Biochem.* **196:** 211–217.
19. Marinari, L., C. M. Lenich, and A. C. Ross. 1987. Production and secretion of retinol-binding protein by a human hepatoma cell line, HepG2. *J. Lipid Res.* **28:** 941–948.
20. Monaco, H. L., M. Rizzi, and A. Coda. 1995. Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein. *Science.* **268:** 1039–1041.
21. van Bennekum, A. M., S. Wei, M. V. Gamble, S. Vogel, R. Piantedosi, M. E. Gottesman, V. Episkopou, and W. S. Blaner. 2001. Biochemical basis for depressed serum retinol levels in transthyretin-deficient mice. *J. Biol. Chem.* **276:** 1107–1113.
22. Soprano, D. R., K. J. Soprano, and D. S. Goodman. 1986. Retinol-binding protein messenger RNA levels in the liver and in extrahepatic tissues of the rat. *J. Lipid Res.* **27:** 166–171.
23. Green, M. H., J. B. Green, and K. C. Lewis. 1987. Variation of retinol utilization rate with retinoid status in the rat. *J. Nutr.* **117:** 694–703.
24. Gjoen, T., T. Bjerkelund, H. K. Blomhoff, K. R. Norum, T. Berg, and R. Blomhoff. 1987. Liver takes up retinol-binding protein from plasma. *J. Biol. Chem.* **262:** 10926–10930.
25. Senoo, H., E. Stang, A. Nilsson, G. M. Kindberg, T. Berg, K. R. Norum, and R. Blomhoff. 1990. Internalization of retinol-binding protein in parenchymal and stellate cells of rat liver. *J. Lipid Res.* **31:** 1229–1239.
26. McGuire, B. W., M. C. Orgebin-Crist, and F. Chytil. 1981. Autoradiographic localization of serum retinol-binding protein in rat testis. *Endocrinology.* **108:** 658–667.
27. Shingleton, J. L., M. K. Skinner, and D. E. Ong. 1989. Characteristics of retinol accumulation from serum retinol-binding protein by cultured Sertoli cells. *Biochemistry.* **28:** 9641–9647.
28. Herbert, J., T. Cavallaro, and R. Martone. 1991. The distribution of retinol-binding protein and its mRNA in the rat eye. *Invest. Ophthalmol. Vis. Sci.* **32:** 302–309.
29. Martone, R. L., E. A. Schon, D. S. Goodman, D. R. Soprano, and J. Herbert. 1988. Retinol-binding protein is synthesized in the mammalian eye. *Biochem. Biophys. Res. Commun.* **157:** 1078–1084.
30. Ong, D. E., J. T. Davis, W. T. O'Day, and D. Bok. 1994. Synthesis and secretion of retinol-binding protein and transthyretin by cultured retinal pigment epithelium. *Biochemistry.* **33:** 1835–1842.
31. Flood, M. T., P. Gouras, J. E. Haley, and W. S. Blaner. 1982. Human retinal pigment epithelium in vitro: organization, ultrastructure, and biochemistry. *Birth Defects Orig. Artic. Ser.* **18:** 53–66.
32. Flood, M. T., C. D. Bridges, R. A. Alvarez, W. S. Blaner, and P. Gouras. 1983. Retinoid utilization in human retinal pigment epithelial cells in vitro. *Invest. Ophthalmol. Vis. Sci.* **24:** 1227–1235.
33. Das, S. R., and P. Gouras. 1988. Retinoid metabolism in cultured human retinal pigment epithelium. *Biochem. J.* **250:** 459–465.
34. Zetterstrom, R. H., A. Simon, M. M. Giacobini, U. Eriksson, and L. Olson. 1994. Localization of cellular retinoid-binding proteins suggests specific roles for retinoids in the adult central nervous system. *Neuroscience.* **62:** 899–918.
35. MacDonald, P. N., D. Bok, and D. E. Ong. 1990. Localization of cellular retinol-binding protein and retinol-binding protein in cells comprising the blood-brain barrier of rat and human. *Proc. Natl. Acad. Sci. USA.* **87:** 4265–4269.
36. Aldred, A. R., C. M. Brack, and G. Schreiber. 1995. The cerebral expression of plasma protein genes in different species. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **111:** 1–15.
37. Ruberte, E., V. Friederich, P. Chambon, and G. Morriss-Kay. 1993. Retinoic acid receptors and cellular retinoid binding proteins. III. Their differential transcript distribution during mouse nervous system development. *Development.* **118:** 267–282.
38. Zheng, W., Y. M. Lu, G. Y. Lu, Q. Zhao, O. Cheung, and W. S. Blaner. 2001. Transthyretin, thyroxine, and retinol-binding protein in human cerebrospinal fluid: effect of lead exposure. *Toxicol. Sci.* **61:** 107–114.
39. Quadro, L., W. S. Blaner, D. J. Salchow, S. Vogel, R. Piantedosi, P.

Gouras, S. Freeman, M. P. Cosma, V. Colantuoni, and M. E. Gottesman. 1999. Impaired retinal function and retinoid availability in mice lacking retinol-binding protein. *EMBO J.* **17:** 4633–4644.
40. Quadro, L., L. Hamberger, V. Colantuoni, M. E. Gottesman, and W. S. Blaner. 2003. Understanding the physiological role of retinol-binding protein in retinoid metabolism using transgenic and knockout mouse models. *Mol. Aspects Med.* **24:** 421–430.
41. Quadro, L., L. Hamberger, M. E. Gottesman, V. Colantuoni, R. Rajashekhar, and W. S. Blaner. 2004. Transplacental delivery of retinoid: the role of retinol-binding protein and lipoprotein retinyl ester. *Am. J. Physiol. Endocrinol. Metab.* **286:** E844–E851.
42. Paik, J., S. Vogel, L. Quadro, R. Piantedosi, M. E. Gottesman, K. Lai, L. Hamberger, M. De Morais Vieira, and W. S. Blaner. 2004. Retinoid: overlapping delivery pathways to tissues from the circulation. *J. Nutr.* **134 (Suppl.):** 276–280.
43. Quadro, L., W. S. Blaner, L. Hamberger, R. N. Van Gelder, S. Vogel, R. Piantedosi, P. Gouras, V. Colantuoni, and M. E. Gottesman. 2002. Muscle expression of human retinol-binding protein (RBP). Suppression of the visual defect of RBP knockout mice. *J. Biol. Chem.* **277:** 30191–30197.
44. Lowery, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 263–275.
45. Muto, Y., J. E. Smith, P. O. Milch, and D. S. Goodman. 1972. Regulation of retinol-binding protein metabolism by retinoid status in the rat. *J. Biol. Chem.* **247:** 2542–2550.
46. Jessen, K. A., and M. A. Satre. 1998. Induction of mouse retinol binding protein gene expression by cyclic AMP in Hepa 1-6 cells. *Arch. Biochem. Biophys.* **357:** 126–130.
47. Mertz, J. R., P. W. Banda, and A. L. Kierszenbaum. 1995. Rat sperm galactosyl receptor: purification and identification by polyclonal antibodies raised against multiple antigen peptides. *Mol. Reprod. Dev.* **41:** 374–383.
48. Blaner, W. S., S. R. Das, P. Gouras, and M. T. Flood. 1987. Hydrolysis of 11-cis- and all-trans-retinyl palmitate by homogenates of human retinal epithelial cells. *J. Biol. Chem.* **262:** 53–58.
49. Wei, S., V. Episkopou, R. Piantedosi, S. Maeda, K. Shimada, M. E. Gottesman, and W. S. Blaner. 1995. Studies on the metabolism of retinol and retinol-binding protein in transthyretin-deficient mice produced by homologous recombination. *J. Biol. Chem.* **270:** 866–870.
50. Blaner, W. S., H. F. Hendriks, A. Brouwer, A. M. de Leeuw, D. L. Knook, and D. S. Goodman. 1985. Retinoids, retinoid-binding proteins, and retinyl palmitate hydrolase distributions in different types of rat liver cells. *J. Lipid Res.* **26:** 1241–1251.
51. Blaner, W. S. 1990. Radioimmunoassays for retinol-binding protein, cellular retinol-binding protein, and cellular retinoic acid-binding protein. *Methods Enzymol.* **189:** 270–281.
52. Redlich, C. A., J. N. Grauer, A. M. Van Bennekum, S. L. Clever, R. B. Ponn, and W. S. Blaner. 1996. Characterization of carotenoid, retinoid, and alpha-tocopherol levels in human lung tissue and pulmonary macrophages. *Am. J. Respir. Crit. Care Med.* **154:** 1436–1443.
53. Nagy, N. E., K. B. Holven, N. Roos, H. Senoo, N. Kojima, K. R. Norum, and R. Blomhoff. 1997. Storage of retinoid in extrahepatic stellate cells in normal rats. *J. Lipid Res.* **38:** 645–658.
54. Chytil, F. 1992. The lungs and vitamin A. *Am. J. Physiol.* **262:** L517–L527.
55. Wake, K. 1980. Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and retinoid-storing cells in extrahepatic organs. *Int. Rev. Cytol.* **66:** 303–353.
56. Matarese, V., and H. Lodish. 1993. Specific uptake of retinol-binding protein by variant F9 cell lines. *J. Biol. Chem.* **268:** 18859–18865.
57. Tosetti, F., F. Campelli, and G. Levi. 1999. Studies on the cellular uptake of retinol binding protein and retinol. *Exp. Cell Res.* **250:** 423–433.
58. van Bennekum, A. M., W. S. Blaner, I. Seifert-Bock, M. Moukides, A. Brower, and H. F. J. Hendriks. 1993. Retinol uptake from retinol binding protein (RBP) by liver parenchymal cells in vitro does not depend specifically from its binding to RBP. *Biochemistry.* **32:** 1727–1733.
59. Creek, K. J., C. S. Silverman-Jones, and L. M. De Luca. 1989. Comparison of the uptake and metabolism of retinol delivered to primary mouse keratinocytes either free or bound to rat serum retinol-binding protein. *J. Invest. Dermatol.* **92:** 283–289.
60. Blomhoff, R., T. Berg, and K. R. Norum. 1988. Transfer of retinol from parenchymal to stellate cells in liver is mediated by retinol-binding protein. *Proc. Natl. Acad. Sci. USA.* **85:** 3455–3458.
61. Blomhoff, R., M. H. Green, T. Berg, and K. R. Norum. 1990. Transport and storage of retinoid. *Science.* **250:** 399–404.
62. Blomhoff, R., and K. Wake. 1991. Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. *FASEB J.* **5:** 271–277.
63. Norum, K. R., and R. Blomhoff. 1992. McCollum Award Lecture, 1992. Retinoid absorption, transport, cellular uptake, and storage. *Am. J. Clin. Nutr.* **56:** 735–744.
64. Senoo, H., S. Smeland, L. Malaba, T. Bjerknes, E. Stang, N. Roos, T. Berg, K. R. Norum, and R. Blomhoff. 1993. Transfer of retinol-binding protein from HepG2 human hepatoma cells to cocultured rat stellate cells. *Proc. Natl. Acad. Sci. USA.* **90:** 3616–3620.
65. Vogel, S., R. Piantedosi, S. M. O'Byrne, Y. Kako, L. Quadro, M. E. Gottesman, I. J. Goldberg, and W. S. Blaner. 2002. Retinol-binding protein-deficient mice: biochemical basis for impaired vision. *Biochemistry.* **41:** 15360–15368.
66. Ghyselinck, N. B., C. Bavik, V. Sapin, M. Mark, D. Bonnier, C. Hindelang, A. Dierich, C. B. Nilsson, H. Hakansson, P. Sauvant, V. Azais-Braesco, M. Frasson, S. Picaud, and P. Chambon. 1999. Cellular retinol-binding protein I is essential for retinoid homeostasis. *EMBO J.* **18:** 4903–4914.