Retinol-Binding Protein-Deficient Mice: Biochemical Basis for Impaired Vision†

Silke Vogel,‡ Roseann Piantedosi,‡ Sheila M. O'Byrne,§ Yuko Kako,‡ Loredana Quadro,|| Max E. Gottesman,§,|| Ira J. Goldberg,‡,§ and William S. Blaner*,‡,§

Department of Medicine, Institute of Human Nutrition, and Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received September 15, 2002; Revised Manuscript Received October 31, 2002

ABSTRACT: We reported previously that mice lacking plasma retinol-binding protein (RBP) are phenotypically normal except that they display impaired vision at the time of weaning. This visual defect is associated with greatly diminished eyecup levels of retinaldehyde and is reversible if the mutants are maintained for several months on a vitamin A-sufficient diet. Here we provide a biochemical basis for the visual phenotype of RBP-deficient mice. This phenotype does not result from inadequate milk total retinol levels since these are not different for RBP-deficient and wild-type mice. The eye, unlike all other tissues that have been examined, takes up dietary retinol very poorly. Moreover, compared to other tissues, the eye displays a strong preference for retinol uptake when retinol is delivered bound to RBP. The poor uptake of dietary retinol by the eye coupled with its marked ability to take up retinol from RBP, we propose, provides a basis for the impaired vision observed in weanling RBP-deficient mice. Further study of the mutants suggests that the impaired vision is reversible because the eyes of mutant mice slowly acquire sufficient retinol from the low levels of retinol present in their circulation either bound to albumin or present in lipoprotein fractions. Thus, the eye is unlike other tissues in the body in that it shows a very marked preference for acquiring retinol needed to support vision from the retinol−RBP complex and is unable to meet adequately its retinol need through uptake of recently absorbed dietary retinol. This provides an explanation for the impaired vision phenotype of RBP-deficient mice.

Higher organisms need retinoids (vitamin A and its analogues) to maintain many essential physiologic processes, including normal reproduction, normal immunity, normal growth and cellular differentiation, and normal vision (*1*−*4*). With the exception of vision, the actions of retinoids are mediated by the all-*trans* and 9-*cis* isomers of retinoic acid (*3*−*5*). These two retinoic acid isomers serve as ligands for the retinoic acid receptor (RAR)[1] and the retinoid X receptor (RXR) families of nuclear receptors, respectively (*3*−*5*). The three distinct RARs and three distinct RXRs are members of the steroid/thyroid/retinoid superfamily of ligand-dependent transcription factors (*3*−*5*). Through interactions with RARs and RXRs, all-*trans*- and 9-*cis*-retinoic acid have been proposed to influence expression of hundreds of distinct genes (*3*−*6*). In vision, 11-*cis*-retinaldehyde serves as the chromophore for the visual pigment rhodopsin (*2*).

All retinoids present within the body must be acquired from the diet (*7*, *8*). Following consumption of a vitamin A-rich meal, along with other dietary lipids, dietary retinoids (as retinyl esters) are packaged in chylomicrons. The chylomicrons undergo metabolism within the circulation and are converted to relatively triglyceride-poor chylomicron remnants through the action of lipoprotein lipase (LPL) (*9*). The majority of chylomicron remnant retinyl ester is cleared by the liver where the preponderance of the body's retinoid is stored in hepatic stellate cells (*7*, *8*). However, it has long been established that approximately 25−33% of postprandial retinoid is normally taken up by extrahepatic tissues (*10*).

To meet the body's need for retinoids, the liver secretes retinol bound to plasma retinol-binding protein (RBP) (*11*, *12*). RBP is the sole specific transport protein for retinol within the circulation, and in a fasting state, more than 95% of the retinoid present within the circulation exists as retinol bound to RBP (*11*−*13*). Circulating retinol is taken up by tissues and cells throughout the body and there undergoes oxidation to retinoic acid (*14*, *15*). Retinoic acid formed through oxidation of retinol is proposed to mediate essential retinoid-dependent functions within cells and tissues.

Earlier, we reported the targeted disruption of the mouse gene for RBP and the characteristics of RBP-deficient ($RBP^{-/-}$) mice (*16*). Our characterizations of $RBP^{-/-}$ mice demonstrated that the mutants display impaired vision at the time of weaning (19−21 days old) but that they are otherwise phenotypically normal. Moreover, we demonstrated that

† This work was supported by Grants R01 EY12858 and R01 DK52444 from the National Institutes of Health and Grant 2002-35200-1157 from the U.S. Department of Agriculture.

* To whom correspondence should be addressed: Department of Medicine, Hammer Health Sciences Bldg., Room 502, 701 W. 168th St., Columbia University, New York, NY 10032. Telephone: (212) 305-5429. Fax: (212) 305-2801. E-mail: wsb2@columbia.edu.

‡ Department of Medicine.

§ Institute of Human Nutrition.

|| Institute of Cancer Research of the College of Physicians and Surgeons.

[1] Abbreviations: RAR, retinoic acid receptor; RXR, retinoid X receptor; LPL, lipoprotein lipase; RBP, retinol-binding protein; $RBP^{-/-}$, retinol-binding protein-deficient mice; $RBP^{+/+}$, wild-type mice; PBS, 10 mM sodium phosphate (pH 7.4) containing 150 mM sodium chloride; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; RPE, retinal pigment epithelium; HPLC, high-performance liquid chromatography.

10.1021/bi0268551 CCC: $22.00
Published on Web 11/28/2002

when $RBP^{-/-}$ mice are maintained from the time of weaning on a control chow diet, they acquire normal vision by the time they reach 4−5 months of age (*16*). However, if these mice are maintained on a vitamin A-deficient diet from the time of weaning, they fail to acquire normal vision. On the basis of these data, we proposed that the essential physiologic function of RBP is to allow for the mobilization of retinol from hepatic stores, thus rendering the organism free of the obligate need to acquire vitamin A regularly from the diet (*16*). We further proposed that $RBP^{-/-}$ mice are phenotypically normal because their tissues are able to meet the essential need for retinoids from recently ingested dietary vitamin A present in chylomicrons or chylomicron remnants (*16*). However, this explanation for why the $RBP^{-/-}$ mice are phenotypically normal does not provide a basis for understanding the impaired vision phenotype of the $RBP^{-/-}$ mice at the time of weaning or why this phenotype is reversible. The studies presented here provide a biochemical basis for understanding the impaired vision phenotype of RBP-deficient mice.

EXPERIMENTAL PROCEDURES

Materials. [11,12-^{3}H]Retinol (49.3 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Rat albumin (fatty acid-free) and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC grade hexane, chloroform, methanol, methylene chloride, and benzene were purchased from Fisher Scientific Co. Hydroflor liquid scintillation cocktail was purchased from National Diagnostics (Atlanta, GA).

Mice. For our studies, we used mice lacking plasma RBP ($RBP^{-/-}$) and wild-type mice ($RBP^{+/+}$) from the same mixed genetic background (C57BL/6J × 129 mice) (Jackson Laboratories, Bar Harbor, ME). The generation and characteristics of the $RBP^{-/-}$ mice have been described by Quadro et al. (*16*, *17*). All mice used in our studies were maintained in a specific virus- and pathogen-free "barrier" facility prior to use in the studies. The animals were allowed ad libitum access to water and a standard rodent chow diet (Purina Products, Richmond, VA) except where indicated. The room housing the mice was maintained on a standard 12 h light and dark cycle.

Plasma Clearance and Uptake of [^{3}H]Retinoid into Tissues. Three-month-old male $RBP^{+/+}$ and $RBP^{-/-}$ mice received an oral bolus of [^{3}H]retinol (10^6 cpm/100 μL) in peanut oil via gavage. Animals were sacrificed 1, 2, 4, and 10 h after the gavage and blood and tissues collected. Plasma samples were obtained after spinning the blood that had been collected into a tube containing EDTA, at 14000*g*. Dissected tissues were immediately placed in liquid nitrogen and stored at −70 °C until they were analyzed.

[^{3}H]Retinoid Levels in Plasma and Tissues. To assess [^{3}H]-retinoid concentrations in total plasma, 10 μL of each plasma sample was transferred to a scintillation vial and dissolved in 10 mL of Hydroflor liquid scintillation counting solution. To analyze tissue levels of [^{3}H]retinoids, tissues were weighed, homogenized in 3 volumes of phosphate-buffered saline (PBS) using a Polytron homogenizer (Brinkman Instruments, Westbury, NY), and extracted with a chloroform/methanol mixture (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. The ^{3}H counts per minute present in plasma and tissues samples was measured in a Beckman LS 1800 liquid scintillation counter. In a separate experiment, the contributions of [^{3}H]retinol and [^{3}H]retinyl esters to the total amount of [^{3}H]retinol present in the plasma were determined. For this purpose, plasma samples were extracted as previously described (*18*). Briefly, internal standard all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraen-1-ol (TMMP-ROH) (obtained as a gift from C. Eckhoff, Hofmann-LaRoche, Inc., Nutley, NJ) was added to each of the plasma samples. An equal volume of ethanol was added to the plasma to precipitate proteins, and the retinoids present in the mixture were subsequently extracted with 3 volumes of hexane. The hexane extracts were back-washed in 0.5 mL of water once and subsequently evaporated under a gentle stream of nitrogen. The lipid film was reconstituted in 110 μL of hexane and injected onto a normal phase high-performance liquid chromatography (HPLC) column connected in-line to both a Waters 996 photodiode array UV absorbance detector (PDA) and an in-line Berthold 506 radiation detector (EG&G Berthold, North Potomac, MD) for simultaneous analysis of retinoid mass and ^{3}H counts per minute (*19*). After analyses of the eluting retinoids by the PDA, samples were automatically mixed with scintillation fluid and assayed by the in-line radiation detector. Radiation profiles detected by the in-line radiation detector were superimposed with profiles generated by the PDA to allow for estimation of [^{3}H]retinyl ester and [^{3}H]retinol concentrations in plasma from the different strains of mice.

Lipoprotein Isolation and Determination of the Retinyl Ester Concentration in the Different Lipoprotein Fractions Present in Mouse Plasma. To assess the concentrations of retinoids in plasma lipoprotein fractions, male $RBP^{+/+}$ and $RBP^{-/-}$ mice received an oral bolus of [^{3}H]retinol (3×10^6 cpm/100 μL) in peanut oil and were than fasted overnight. Plasma samples from $RBP^{+/+}$ and $RBP^{-/-}$ mice were collected 20 h after administration of the [^{3}H]retinol dose and subsequently pooled. Lipoprotein fractions (VLDL, LDL, HDL, and 1.21 bottom fraction) were obtained by sequential centrifugation of the plasma pools following addition of solid KBr in a TL-100 centrifuge (Beckman) according to a literature procedure (*20*). An aliquot of each lipoprotein fraction was analyzed directly for total ^{3}H counts per minute levels using a Beckman LS 1800 LSC counter. The remainder of each fraction was extracted into hexane, as described above, for determination of retinol and retinyl ester concentrations, using retinyl acetate as the internal standard (*21*). After reconstitution in benzene, samples were analyzed by reverse phase HPLC. The HPLC mobile phase consisted of acetonitrile, methylene chloride, and methanol (70:15:15, v/v). The instruments used for the HPLC analysis consisted of a Waters 510 HPLC pump (Waters Associates, Milford, MA) operating at a flow rate of 1.8 mL/min, an Ultrasphere C18 column (5 μm, 4.6 mm × 25 cm) (Beckman Instruments), and a Waters 996 photodiode array detector. Retinol and retinyl esters were monitored at 325 nm and quantified using standard curves relating known mass ratios of retinol or retinyl esters to that of internal standard retinyl acetate.

Expression and Purification of Recombinant Mouse RBP. To obtain recombinant mouse RBP protein, a cDNA encoding mature mouse RBP (lacking the RBP signal peptide) was expressed in *Escherichia coli* using the PetVector expression system (Novagen, Madison, WI). A mouse RBP cDNA containing the RBP precursor sequence was obtained from Research Genetics (Huntsville, AL) and amplified by PCR to obtain a cDNA lacking the 17 amino acids of the RBP signal peptide. This product was subcloned into the pET13A vector (Novagen) at the *Nco*I and *Bgl*II restriction sites. The expression plasmid was sequenced in both directions by the Columbia University Comprehensive Cancer Center Core DNA Sequencing Facility to ensure that no mutations were present. Bl21(23) competent cells containing the mRBP plasmid were grown to an OD of approximately 0.6, and protein expression was induced with IPTG (final concentration of 0.4 mM) for 3 h at 30 °C. The *E. coli* cells expressing the mature mouse RBP were harvested, and the recombinant RBP was purified using a procedure similar to that described previously for recombinant human RBP from *E. coli* (*22*). Briefly, recombinant mouse RBP was solubilized from *E. coli* inclusion bodies of aggregated RBP using 5.0 M guanidinium chloride containing 10 mM dithiothreitol. Refolding of the RBP was carried out in the presence of 1 mM all-*trans*-retinol by diluting the denatured and reduced RBP into a redox refolding buffer consisting of 3 mM cysteine and 0.3 mM cystine at 4 °C. The solubilized and refolded RBP was purified to homogeneity by ion exchange chromatography on a 2.5 cm × 40 cm DEAE-cellulose column and gel filtration through a 1 cm × 40 cm Bio-Gel P-100 column.

The recombinant mouse RBP was incubated with [^{3}H]-retinol overnight at 4 °C to obtain the [^{3}H]retinol−RBP complex. Unbound [^{3}H]retinol was removed with dextran-activated charcoal (*23*). On the basis of the absorbance spectrum and the ratio of absorbance at 280 to 325 nm, we estimate that the recombinant RBP protein was approximately 80% saturated with [^{3}H]retinol with a specific activity of 6.67×10^4 ^{3}H cpm/μg of protein.

Clearance and Tissue Uptake of the Circulating Recombinant Mouse [^{3}H]Retinol−RBP Complex. For these turnover studies, 7.5 μg of recombinant mouse RBP (an amount of RBP that would be expected to be present in the circulations of wild-type mice) binding [^{3}H]retinol (0.5×10^6 ^{3}H cpm/7.5 μg of RBP in 50 μL of PBS) was injected into the right jugular vein of 3-month-old male RBP$^{+/+}$ and RBP$^{-/-}$ mice. Retro-orbital blood samples were taken 90 s after injection, and [^{3}H]retinoid levels in plasma at this time point were taken to represent for each mouse the total injected dose. To follow the turnover of the recombinant [^{3}H]retinol−RBP complex, blood samples were subsequently obtained 1, 3, and 5 h after injection. Five hours after injection, the mice were sacrificed, blood was collected, and tissues were excised and immediately placed in liquid nitrogen. An aliquot of the plasma was measured for total [^{3}H]retinoid levels by liquid scintillation counting as described above. Tissues were extracted and analyzed for total [^{3}H]retinoid as described above. A sensitive and specific radioimmunoassay was used to determine the recombinant RBP concentration present at each time point in the serum of RBP$^{-/-}$ mice (*24*).

Clearance of the [^{3}H]Retinol−Albumin Complex. [^{3}H]-Retinol (49.3 Ci/mmol) was bound to essentially fatty acid free rat serum albumin [0.1% (w/v) in PBS] (Sigma) and injected into the right jugular vein of RBP$^{+/+}$ and RBP$^{-/-}$ mice (10^6 cpm/50 μL). Retro-orbital blood samples were taken 90 s after the injection, and [^{3}H]retinoid concentrations present in plasma at this time point were taken to represent for each mouse its total dose. To follow the turnover of the [^{3}H]retinol−albumin complex, additional blood samples were obtained 1, 3, and 5 h after injection. The mice were sacrificed 5 h after injection, and tissues were dissected and immediately placed in liquid nitrogen. Plasma and tissue levels of [^{3}H]retinoids were measured as described above.

Adenovirus Transfections with Receptor-Related Protein (RAP). Recombinant adenovirus RAP was provided as a generous gift from J. Herz (University of Texas, Dallas, TX) (*25, 26*). The recombinant adenovirus was propagated and titrated on an Ad5 E1-transformed human embryonic kidney cell line as described previously (*27*). For the large-scale production of recombinant adenovirus stocks, 10 flasks (175 cm^2) of nearly confluent 911 monolayers were infected with adenovirus at a multiplicity of infection of 5−10 per cell. After 48−60 h, the nearly completely detached cells were harvested and collected in 1 mL of a PBS/1% horse serum mixture. Virus was released from the producer cells by three rounds of freezing and thawing and centrifugation. The CsCl was removed from the isolated virus by extensive dialysis against 25 mM Tris, 137 mM NaCl, 5 mM KCl, 0.73 mM Na_2PO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$ (pH 7.45). Virus stocks were routinely stored in 0.2% mouse serum albumin and 10% glycerol, flash-frozen in aliquots in liquid N_2, and stored at −80 °C. The titers of the stocks varied from 1 to 5×10^{10} plaque forming units/mL. For in vivo adenovirus transfection, on day zero 5×10^9 plaque forming units diluted in PBS to 200 μL was injected into the tail veins of the mice. Blood samples were drawn from the tails of fasted mice 2, 3, and 5 days after virus infection to assess for RAP production and plasma triglyceride levels.

Protocol for Dam Milk Collection and Milk Total Retinol Analysis. Female RBP$^{-/-}$ and RBP$^{+/+}$ mice were mated housing two females with one male. Mice were provided a commercial breeder chow diet containing 28 IU of retinol/g of diet prior to and during pregnancy and lactation. For milk collection, a dam was separated from her pups approximately 1 h before milk collection commenced but during this period was allowed free access to the breeder chow and water ad libitum. The pups were kept warm either by placing them under a lamp or with another nursing female in the same cage.

To collect milk, each mouse was weighed and then anesthetized via ip injection with a cocktail (1:1, v/v) of xylazine (100 mg/mL) and ketamine HCl (100 mg/mL) given at a dose of 100 μL/30 g of body weight. Approximately 10 min after the anesthetic was administered, a dose of oxytocin (10 units/mL; Pitocin, Parkedale Pharmaceuticals Inc., Rochester, MI) diluted 1:4 in PBS was injected into the tail vein (0.25 unit of oxytocin/mouse). Milk was collected using mild suction generated by a small pump approximately 5 min after the oxytocin injection. For this purpose, a standard 200 μL pipet tip was placed over the nipple and the tip was connected by a small tube to two inverted 1000 μL pipet tips which had holes drilled into them to allow us to control the intensity of suction from the vacuum pump. The collected milk was then rinsed from the tip into 1.5 mL microcentrifuge tubes containing 180 μL of 0.005 M EDTA. On average,

Table 1: Total Retinol Levels in Milk from Lactating Wild-Type and RBP-Deficient Mice[a]

days post partum	$RBP^{+/+}$	$RBP^{-/-}$
<5	10.0 ± 5.7 μM (4)	6.8 ± 3.6 μM (3)
5−10	15.1 μM	16.2 ± 4.7 μM (5)
11−15	9.7 μM	14.3 ± 0.3 μM (3)
>15	12.5 ± 4.4 μM (3)	9.9 ± 5.2 μM (5)
mean	11.0 ± 5.1 μM	11.0 ± 6.4 μM

[a] Mice were maintained ad libitum on a standard chow diet throughout the course of the study. Only retinyl esters (retinyl plamitate, stearate, oleate, linoleate, and myristate) were detected for all of the milk samples that were examined. Milk total retinol levels are given as individual values for milk samples collected during the indicated post partum period. The values in parentheses are the number of independent measures of milk total retinol levels carried out for each mouse strain during the different post partum periods.

approximately 15−20 μL of milk was collected over a 5 min period. Milk was immediately placed on ice and covered with foil to protect it from exposure to light. Subsequently, the milk sample was stored at −80 °C until it could be assayed for total retinol and triglyceride contents. For retinol and retinyl ester analysis, total lipids were extracted by the method of Folch et al. (*28*). After the lipid-containing $CHCl_3$ phase was evaporated to dryness under N_2, the lipids were redissolved in hexane and backwashed against 1.5 mL of H_2O. The hexane phase was then evaporated to dryness, and the resulting lipid extract was taken for analysis by reverse phase HPLC according to the procedures described above for determining the total retinol contents of lipoprotein fractions. A 1 μL aliquot of the milk was mixed with 10 μL of PBS and stored at −80 °C for triglyceride analysis. Triglycerides were analyzed using a kit (Sigma catalog number 344, Sigma Chemical Co.) according to the manufacturer's instructions.

RESULTS

$RBP^{-/-}$ mice display impaired vision at the time of weaning (at 19−21 days of age) but acquire normal vision within several months if they are maintained on a vitamin A-sufficient chow diet (*16*). In characterizing the impaired vision phenotype of $RBP^{-/-}$ mice, we reported that the phenotype is associated with eyecup retinal levels that are significantly lower in weanling $RBP^{-/-}$ mice than in weanling $RBP^{+/+}$ mice [10.7 ± 6.4 vs 57.3 ± 9.9 ng/pair eyecups (mean ± standard deviation)] (*16*). Consequently, we first asked whether this difference and the visual phenotype might arise because the levels of total retinol present in milk of $RBP^{-/-}$ dams are significantly lower than those in milk from $RBP^{+/+}$ dams. Milk total retinol levels for lactating $RBP^{-/-}$ and $RBP^{+/+}$ mice are given in Table 1. Although there is some variability in total retinol concentrations in milk, the levels present in milk from $RBP^{-/-}$ and $RBP^{+/+}$ mice are not significantly different throughout the entire weaning period. No difference in the triglyceride content of the milk was observed for $RBP^{+/+}$ and $RBP^{-/-}$ mice. Thus, these data provide no evidence that differences in milk total retinol concentrations account for the more than 5-fold difference in eyecup retinal concentrations observed for $RBP^{-/-}$ and $RBP^{+/+}$ mice and for the associated impaired vision phenotype of the mutant mice at the time of weaning.

Since low milk total retinol levels for RBP-deficient mice do not account for this phenotype, we asked whether differences exist in how the eye takes up postprandial retinol versus other tissues and whether this might provide a basis for explaining the vision phenotype of weanling $RBP^{-/-}$ mice. To address this question, we followed the plasma clearance and tissue uptake of the [^{3}H]retinoid 1, 2, 4, and 10 h after administration of the dose. Figure 1 shows [^{3}H]-retinoid levels in plasma, liver, eyecups, and skeletal muscle for the two mouse strains at the four different times after gavage. As seen in Figure 1A, the plasma clearance curves for the [^{3}H]retinol dose for the two mouse strains are identical 1 and 2 h after administration of the dose, but the plasma clearance curves diverge markedly after this time. Although plasma ^{3}H counts per minute levels for $RBP^{-/-}$ mice remain fairly constant after 1 h, in $RBP^{+/+}$ mice they continue to rise for 4 h. We suspected that this difference might reflect the uptake of chylomicron [^{3}H]retinoid by the livers of $RBP^{+/+}$ mice followed by resecretion of some of the retinoid as [^{3}H]retinol bound to RBP. Consequently, we analyzed [^{3}H]-retinol levels by HPLC in the circulations of $RBP^{-/-}$ and $RBP^{+/+}$ mice at three different times after dose administration. As can be seen in Table 2, 2 h after dose administration very little [^{3}H]retinol is present in the circulations of either $RBP^{+/+}$ or $RBP^{-/-}$ mice. However, at 4 h approximately 23% of the ^{3}H counts per minute present in the circulations of $RBP^{+/+}$ mice is present as [^{3}H]retinol, but only [^{3}H]retinyl ester is found in the circulations of $RBP^{-/-}$ mice. Thus, these data provide strong support for the suggestion that the $RBP^{+/+}$ mice are secreting newly absorbed [^{3}H]retinol bound to RBP back into the circulation.

The liver uptake curves for the treated mice (Figure 1B) also provide support for this same conclusion. Uptake of [^{3}H]-retinoid is identical for the two mouse strains 1 h after administration of the dose. For $RBP^{+/+}$ mice, hepatic ^{3}H counts per minute levels reach a peak at 4 h and then decline. However, for $RBP^{-/-}$ mice that lack hepatic RBP, liver ^{3}H counts per minute levels continue to rise through 2, 4, and 10 h. This difference in the hepatic accumulation of ^{3}H counts per minute can be most easily explained if [^{3}H]retinol is being secreted bound to RBP from the livers of $RBP^{+/+}$ but not $RBP^{-/-}$ mice.

Strikingly, the [^{3}H]retinoid uptake curves for eyecups of $RBP^{-/-}$ mice are very different from those of $RBP^{+/+}$ mice (Figure 1C). One and two hours after administration of the bolus dose of [^{3}H]retinol, very few ^{3}H counts per minute are taken up by eyecups for either of the two strains. However, starting at 2 h, corresponding to when the livers of $RBP^{+/+}$ mice begin to secrete [^{3}H]retinol bound to RBP, these uptake curves begin to markedly differ. Rates of uptake of [^{3}H]retinoid by the eyecups of $RBP^{-/-}$ mice remain very low 4 and 10 h after dose administration, whereas rates of uptake of ^{3}H counts per minute by eyecups from $RBP^{+/+}$ mice markedly increase between 2 and 4 h and again between 4 and 10 h. These data support the hypothesis that eyecups of $RBP^{+/+}$ mice are avidly taking up [^{3}H]retinol from RBP present in the circulation. For all other tissues that have been examined, including lung, skeletal muscle, heart, spleen, testis, and brain, the tissue [^{3}H]retinoid uptake curves were essentially identical for each of the two strains of mice. This is evidenced for skeletal muscle in Figure 1D.

Since our data indicated that the eye takes up very little postprandial or chylomicron [^{3}H]retinoid, we wondered whether uptake by the eye could be influenced by increasing

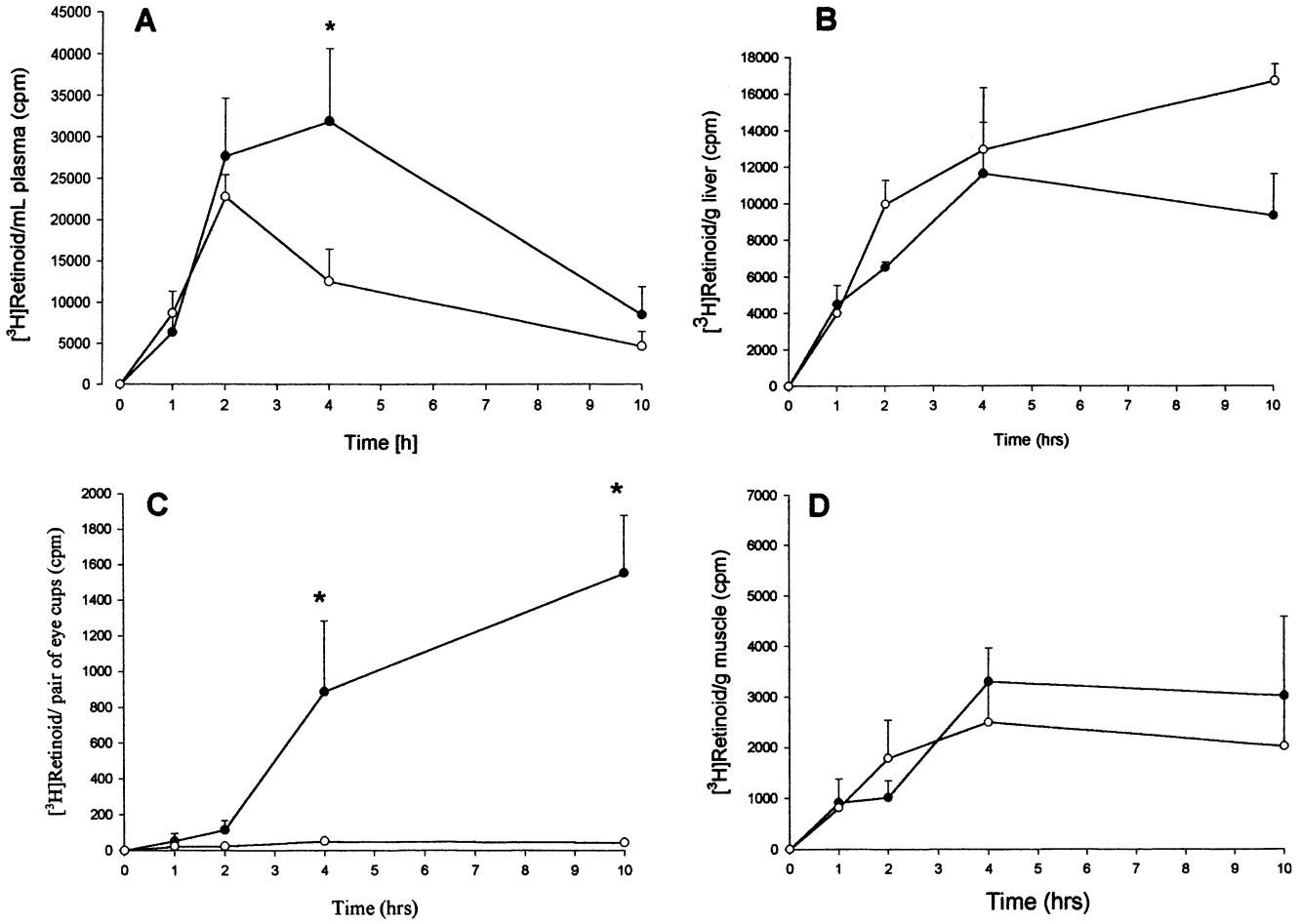

FIGURE 1: Comparison of plasma (A), liver (B), eye (C), and skeletal muscle (D) [^{3}H]retinoid levels 1, 2, 4, and 10 h after administration of a bolus dose of [^{3}H]retinol given by gavage in 0.1 mL of peanut oil to RBP$^{+/+}$ (●) and RBP$^{-/-}$ (○) mice. At each time, the values for each strain represent the mean ± standard deviation obtained for six mice. The data reported here reflect one representative experiment that was repeated three independent times giving the same results. Asterisks denote a p of <0.05; levels in RBP$^{+/+}$ mice are significantly different from those of RBP$^{-/-}$ mice.

Table 2: Relative Distribution of Retinol and Retinyl Ester in Plasma at Different Times after Administration of an Oral Dose of [^{3}H]Retinol to Wild-Type and RBP-Deficient Mice[a]

time (h)	n	RBP$^{+/+}$	RBP$^{-/-}$
2	4	98.7 ± 2.8%	100%
4	4	76.9 ± 19%	100%
10	4	0%	0%

[a] Values represent the percentage of the total [^{3}H]retinol (retinol and retinyl ester) present in the circulation as [^{3}H]retinyl ester and are given as means ± standard deviation.

the half-life of chylomicron [^{3}H]retinoid in the circulation through overexpression of receptor-related protein (RAP) in the liver. RAP expression interferes with chylomicron uptake by tissues (*8*, *25*−*27*). To establish if we could increase the rate of eye uptake of chylomicron retinoid by increasing the half-life of chylomicrons in the circulation, we infected RBP$^{-/-}$ and RBP$^{+/+}$ mice with an adenovirus construct that brings about overexpression of RAP in mouse liver (*26*−*28*). Since RAP is a protein that binds to members of the LDL receptor superfamily, including the LDL receptor-related protein (LRP) and the VLDL receptor, it blocks receptor-mediated uptake of chylomicron remnants by the liver and other tissues (*25*−*27*). Although RAP expression slowed the clearance of chylomicron [^{3}H]retinoid from the circulation (Figure 2A), RAP expression did not alter the uptake of postprandial [^{3}H]retinoid by the eye (Figure 2B).

The studies reported in Figure 1 strongly suggest that the eye has a marked preference for acquiring retinol from RBP. Consequently, we directly investigated this possibility. For this purpose, homogeneously purified mouse holo-RBP labeled with [^{3}H]retinol (Figure 3) was injected intravenously into RBP$^{-/-}$ and RBP$^{+/+}$ mice and the uptake of [^{3}H]retinol by tissues was followed over a 5 h time interval. Figure 4 shows the plasma clearance curves for injected [^{3}H]retinol bound to RBP for RBP$^{-/-}$ and RBP$^{+/+}$ mice. As seen in Figure 4A, there are no differences in the rates of [^{3}H]retinol clearance from the circulations of the two strains. One hour after the injection, 50% of the initial dose has been eliminated from the plasma of both RBP$^{-/-}$ and RBP$^{+/+}$ mice. This rate of clearance is very similar to that we observed when we intravenously injected a dose of purified human RBP that had been purified from serum into wild-type and transthyretin-deficient mice (*29*). Since RBP$^{-/-}$ mice lack any endogenous RBP, it was possible with a radioimmunoassay to measure the rate of plasma clearance of RBP from the circulations of the RBP$^{-/-}$ mice. As seen in Figure 4B, the plasma clearance curves for [^{3}H]retinol and recombinant mouse RBP are fully overlapping in RBP$^{-/-}$ mice. This may suggest that retinol and RBP are being cleared from the

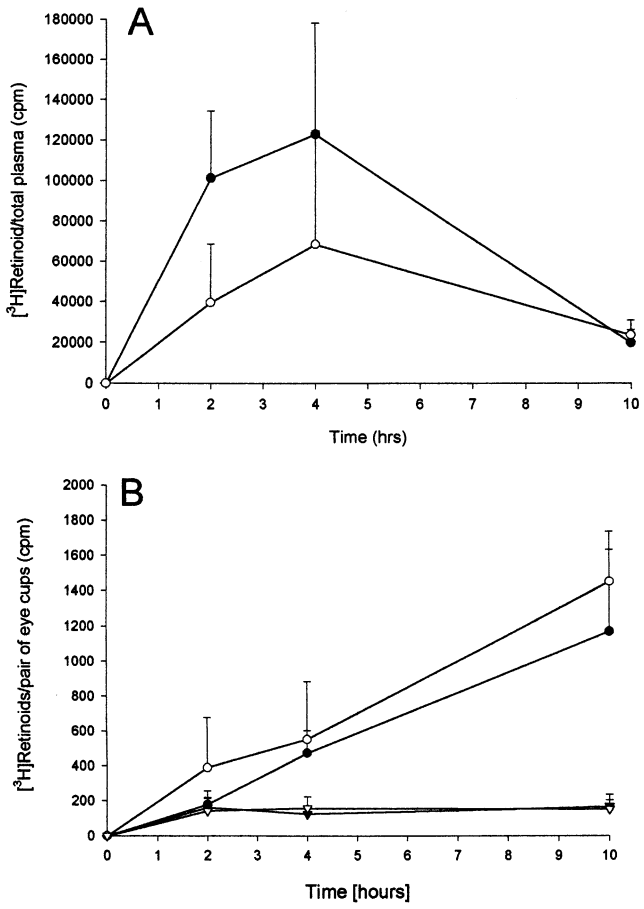

FIGURE 2: Effect of RAP expression on the clearance of [^{3}H]-retinoid given as an oral dose of [^{3}H]retinol in peanut oil. (A) Plasma clearance curves for [^{3}H]retinoid from mice infected with an adenovirus construct bringing about RAP expression (●) or with an empty adenovirus construct (○). (B) Uptake of [^{3}H]retinoid by RBP$^{+/+}$ mice expressing (●) or not expressing (○) RAP and by RBP$^{-/-}$ mice expressing (▼) or not expressing (▽) RAP. The error bars indicate one standard deviation.

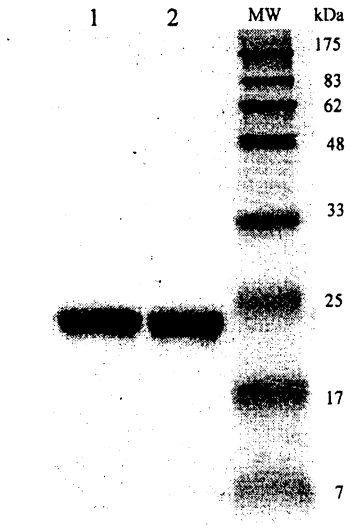

FIGURE 3: Coomassie-stained SDS−PAGE of purified recombinant mouse RBP. Lanes 1 and 2 were loaded with 25 μg of purified recombinant mouse RBP. The lane marked MW shows the migration of molecular weight standards whose mass is given in kilodaltons by the numbers on the right of the lane.

circulation as the retinol−RBP complex. The tissue distribution of [^{3}H]retinol 5 h after its intravenous injection as the retinol−RBP complex into RBP$^{-/-}$ and RBP$^{+/+}$ mice is shown in Figure 5. When these data are normalized for tissue weight, aside from the kidneys, the eye takes up the largest amount of [^{3}H]retinol of any tissue that has been examined, exceeding some tissues by 9-fold (heart) to 26-fold (skeletal muscle) (Figure 5). Interestingly, we observed a small but statistically significant difference ($p < 0.05$) between the two strains with regard to the total amount of ^{3}H counts per minute taken up by the liver and eyecups. On average, the livers and eyecups of RBP$^{+/+}$ mice took up more of the ^{3}H counts per minute than these tissues from RBP$^{-/-}$ mice. One explanation for these small but real differences may be that the lower rate of uptake of [^{3}H]retinol by these two tissues in RBP$^{-/-}$ mice could arise through competition between the liver and eye and other tissues that have never previously seen the [^{3}H]retinol−RBP complex. The amount of [^{3}H]-retinol taken up by other tissues was not statistically different for RBP$^{-/-}$ and RBP$^{+/+}$ mice.

Since RBP$^{-/-}$ mice do eventually acquire normal vision when maintained on a chow diet and since this is accompanied by increases in eyecup retinaldehyde and retinol levels (*16*), we wondered how, aside from some postprandial uptake, the eye acquires retinol in the absence of RBP. We previously reported that the low concentrations of retinol present in the circulations of RBP$^{-/-}$ mice are associated with a protein with a weight of approximately 70 000, a weight similar to that of albumin, a protein that binds retinol in vitro (*16*). Hence, we explored whether [^{3}H]retinol bound to albumin might specifically be directed to or taken up by the eyes of RBP$^{-/-}$ mice. However, as seen in Figure 6, the eyes of both RBP$^{-/-}$ and RBP$^{+/+}$ mice accumulate [^{3}H]retinol poorly from intravenously injected [^{3}H]retinol bound to fatty acid free rat serum albumin. Thus, relative to other tissues, retinol bound to albumin is not selectively taken up by the eye.

In earlier studies of transthyretin-deficient mice (*29*), we demonstrated that retinyl esters are present in VLDL, LDL, and HDL in the fasting circulations of both wild-type and transthyretin-deficient mice. Although the levels of retinyl esters present in VLDL, LDL, and HDL are relatively low compared to the amount of the retinol−RBP complex present in the circulations of wild-type mice, such retinyl ester concentrations could be significant and physiologically important to RBP$^{-/-}$ mice. Thus, we measured retinyl ester levels for pools of plasma collected from fasting RBP$^{-/-}$ and RBP$^{+/+}$ mice (Table 3). The levels given in Table 3 are very similar to those reported for other fasting mice (*29*). On the basis of these and previous measures, there appears to be no upregulation in the retinyl ester content of lipoproteins present in the circulations of RBP$^{-/-}$ mice.

DISCUSSION

One possible explanation that could explain the vision phenotype observed in RBP deficiency is that milk total retinol levels may be greatly diminished in RBP$^{-/-}$ mice. If this were the case, RBP$^{-/-}$ mice would acquire very little retinol during the weaning period, and this could account for the low retinal and retinol levels observed in eyecups of weanling RBP$^{-/-}$ mice and for the impaired vision of the mutants. Our data, however, indicate that this is not the case. Mean milk total retinol levels were not different for RBP$^{-/-}$

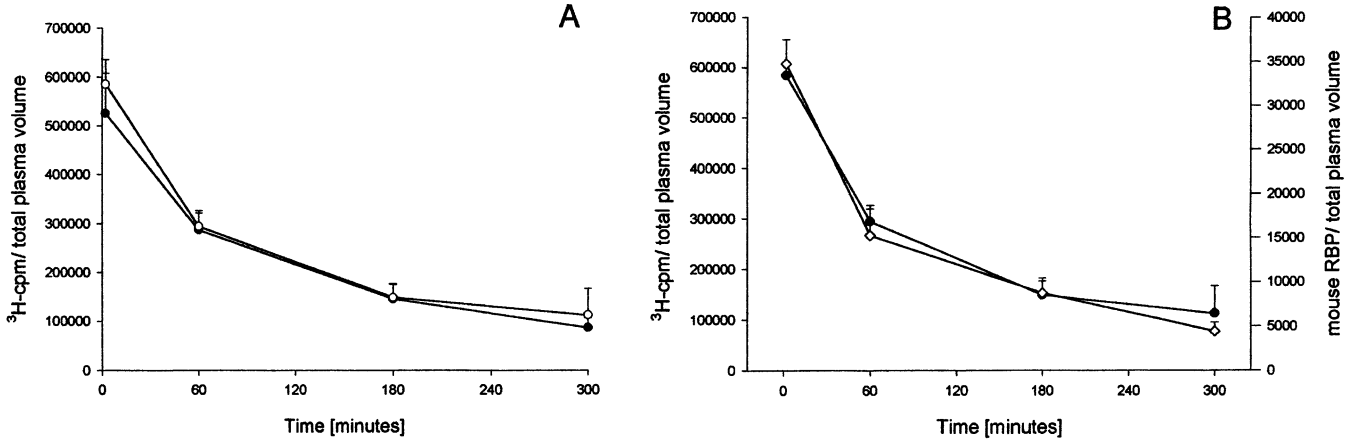

FIGURE 4: Clearance of the [^{3}H]retinol−RBP complex from the circulation. (A) Plasma clearance of [^{3}H]retinol in RBP$^{+/+}$ (●) and RBP$^{-/-}$ (○) mice. Circulating levels measured 90 s after dose injection into the jugular vein were taken to be the initial plasma levels. (B) Comparison of the rate of plasma clearance of [^{3}H]retinol (●) and recombinant mouse RBP (◇) in RBP$^{-/-}$ mice. The error bars indicate one standard deviation.

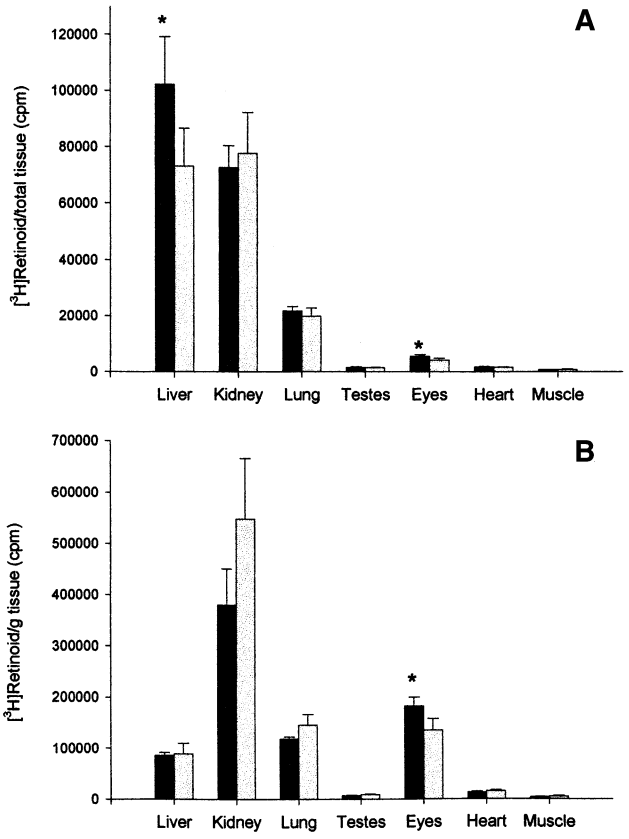

FIGURE 5: Bar graph of the levels of [^{3}H]retinoid in tissues in RBP$^{+/+}$ (black bars) and RBP$^{-/-}$ (gray bars) mice 5 h after injection of the recombinant [^{3}H]retinol−RBP complex into the jugular vein of RBP$^{+/+}$ and RBP$^{-/-}$ mice. Tissue levels are expressed both as ^{3}H counts per minute per total tissue (A) and as ^{3}H counts per minute per gram of tissue (B). Means ± standard deviations for four mice from each strain are shown (asterisks denote a p of <0.05).

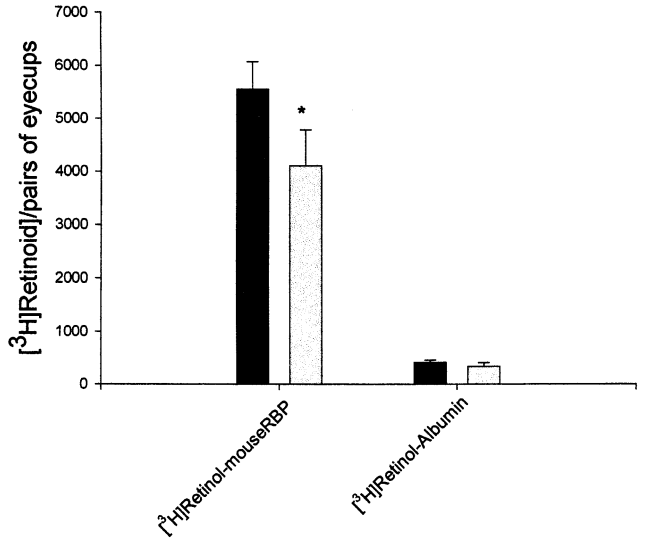

FIGURE 6: Comparison of [^{3}H]retinol uptake by the eyecups of RBP$^{+/+}$ (gray bars) and RBP$^{-/-}$ (black bars) mice when the [^{3}H]-retinol is provided either bound to recombinant mouse RBP or bound to rat albumin. Levels are expressed as the mean ± standard deviation for four mice from each strain. Equal amounts of [^{3}H]-retinol either bound to recombinant RBP or rat albumin were injected into the mice.

and RBP$^{+/+}$ mice (see Table 1). Our data rather indicate that the eye, unlike other tissues in the body, takes up postprandial retinol very poorly. Because of this, the eye must rely primarily on retinol bound to RBP as its primary means for acquiring the retinoid needed for normal visual pigment formation. Our investigations support this conclusion and demonstrate that the eye avidly takes up retinol when it is delivered bound to RBP. Thus, we propose that the eyes of RBP$^{-/-}$ mice inefficiently and only slowly acquire retinol from the circulation. Ultimately, it is this poor uptake of postprandial retinol, compared to that in other tissues, that is the biochemical lesion that underlies the impaired vision phenotype of these mutant mice.

If RBP$^{-/-}$ mice are maintained on a vitamin A-sufficient chow diet, the eyes and other peripheral tissues have several potential RBP-independent sources for acquiring retinol: postprandially as retinyl ester bound to chylomicrons or their remnants, as retinyl ester bound to VLDL, LDL, and/or HDL, or as retinol bound to albumin (*16*). Surprisingly, the literature regarding how the retinal pigment epithelium (RPE) and retina acquire lipids from VLDL, LDL, and HDL particles is relatively sparse (*30*−*32*). It is not fully established how much of the cholesterol and triglyceride that is present in the RPE and retina is derived from lipoprotein sources. Our data suggest that the RPE and the retina take

Table 3: Retinyl Ester Concentrations in Lipoprotein Fractions for Fasted Wild-Type and RBP-Deficient Mice[a]

fraction	[retinyl ester] (μM)		
	$RBP^{+/+}$	$RBP^{-/-}$	$RBP^{-/-}$
VLDL	0.059	0.053	0.099
LDL	0.026	0.038	0.036
HDL	0.015	0.023	0.016
$d \geq 1.21$ bottom	nd[b]	nd[b]	nd[b]

[a] Concentrations of retinyl esters were determined for pooled plasma samples constructed by taking equal volumes of plasma from 10 3-month-old male mice of each strain. Retinol was present only in the $d \geq 1.21$ g/mL fraction and not in the VLDL, LDL, and HDL fractions. Two separate pools were prepared for RBP-deficient mice (from a total of 20 mice). For routine measurement of retinyl ester concentrations using this HPLC protocol, the within assay coefficient of variation is 5% and the between assay coefficient of variation is 7%. [b] Not detected.

up little if any chylomicron or chylomicron remnant lipid. In addition, the low levels of retinol bound to albumin that are present in the circulations of $RBP^{-/-}$ mice receiving a vitamin A-sufficient diet (*16*) do not seem to be specifically targeted to the eye. On the basis of these considerations, we believe that all three retinol sources probably play a physiologically significant role in allowing the eye to slowly accumulate sufficient retinoid to allow for normal vision, if the mutants are maintained on a vitamin A-sufficient diet, by 4−5 months of age.

The ability of the eyes of both $RBP^{-/-}$ and $RBP^{+/+}$ mice to take up [^{3}H]retinol from the circulation when it is present as the [^{3}H]retinol−RBP complex is very striking. The rate of uptake of [^{3}H]retinol by the eye, when normalized per gram of tissue, markedly exceeds that of all of the other tissues we examined except for the kidney. This raises the question of what is special about the eye that provides it with such great avidity for retinol delivered bound to RBP. Cell surface receptors for RBP are reported to be present on the basal surfaces of human and bovine RPE cells (*8*, *12*, *13*, *33*, *34*). However, cell surface receptors for RBP have been described for many cells and tissues throughout the body (*8*, *12*, *13*). There also is no agreement in the literature regarding the existence of such RBP receptors since no credible reports of the cloning of such a receptor have been published (*8*, *13*). Nevertheless, the simplest interpretation of our observations would be to invoke the hypothesis that RPE cells possess a specific mechanism that allows them to recognize and take up retinol when it arrives bound to RBP.

It is interesting that milk total retinol levels for $RBP^{-/-}$ mice are essentially the same as those measured for $RBP^{+/+}$ mice. The literature indicates that postprandial vitamin A is an important source of the total retinol content of rat milk (*35*−*37*) and that the amount of total retinol present in rat milk can vary in response to the content of vitamin A in the diet (*35*−*37*). Kinetic modeling studies of the incorporation of retinyl ester into rat milk predicted, for a healthy rat receiving a control vitamin A-sufficient, that approximately 50% of the total retinol present in the milk is derived from the retinol−RBP complex and the remainder is derived from postprandial retinol (*35*−*37*). It is clear from our studies in $RBP^{-/-}$ mice that RBP is not required for incorporating retinol (as retinyl ester) into milk. This suggests that the total retinol present in $RBP^{-/-}$ milk is derived directly from recently ingested retinol. Viewed more globally, these observations regarding milk retinol content are in sharp contrast to those we made regarding the eye and vision. Retinoid needs for maintaining normal vision are met primarily through the delivery of retinol bound to RBP, whereas retinoid needs for maintaining normal lactation are met substantially and directly through recent dietary intake. The significance of why two essential physiologic processes, vision and lactation, should rely to such different degrees on alternative pathways for retinol delivery is not obvious at present.

In summary, we have demonstrated that the eye takes up postprandial retinol very poorly compared to other tissues in the body. Because tissues other than the eye are able to obtain sufficient vitamin A to meet their needs from recently ingested postprandial retinol, $RBP^{-/-}$ mice maintained on a vitamin A-sufficient diet are phenotypically normal except for their vision. Because the eye takes up postprandial retinol very poorly, $RBP^{-/-}$ mice show an impaired vision phenotype that normalizes as the eye slowly accumulates sufficient retinoid to allow normal vision. This distinction between the eye and the remainder of the tissues in the body, we suggest, accounts for why $RBP^{-/-}$ mice are phenotypically normal except for impaired vision that is observed at the time of weaning.

REFERENCES

1. Goodman, D. S. (1984) Vitamin A and retinoids in health and disease, *N. Engl. J. Med. 310*, 1023−1031.
2. Saari, J. C. (1994) Retinoids in photosensitive systems, in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) pp 351−386, Raven Press, New York.
3. Hofmann, C., and Eichele, G. (1984) Retinoids in development, in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) pp 387−442, Raven Press, New York.
4. Gudas, L. J., Sporn, M. B., and Roberts, A. B. (1994) Cellular biology and biochemistry of the retinoids, in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) pp 443−520, Raven Press, New York.
5. Mangelsdorf, D. M., Umesono, K., and Evans, R. M. (1994) The retinoid receptors, in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) pp 319−350, Raven Press, New York.
6. Clagett-Dame, M., and DeLuca, H. F. (2002) The role of vitamin A in mammalian reproduction and embryonic development, *Annu. Rev. Nutr. 22*, 347−381.
7. Blaner, W. S., and Olson, J. A. (1994) Retinol and retinoic acid metabolism, in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) pp 229−255, Raven Press, New York.
8. Vogel, S., Gamble, M. V., and Blaner, W. S. (1999) Retinoid Uptake, Metabolism and Transport, in *Retinoids: The Biochemical and Molecular Basis of Vitamin A and Retinoid Action, Handbook of Experimental Pharmacology* (Nau, H., and Blaner, W. S., Eds.) Vol. 139, pp 31−96, Springer-Verlag, Heidelberg, Germany.
9. Cooper, A. D. (1997) Hepatic uptake of chylomicron remnants, *J. Lipid Res. 38*, 2173−2192.
10. Goodman, D. S., Huang, H. S., and Shiratori, T. (1965) Mechanism of the biosynthesis of vitamin A from β-carotene, *J. Lipid Res. 6*, 390−396.
11. Goodman, D. S. (1984) Plasma retinol-binding protein, in *The Retinoids* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) Vol. 2, pp 41−88, Academic Press, Orlando, FL.
12. Soprano, D. R., and Blaner, W. S. (1994) Plasma retinol-binding protein, in *The Retinoids: Biology, Chemistry, and Medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., Eds.) pp 257−282, Raven Press, New York.
13. Gamble, M. V., and Blaner, W. S. (1999) Factors Affecting Blood Retinol Levels of Vitamin A, in *Vitamin A and Retinoids: An*

Update of Biological Aspects and Clinical Applications (MCBU) (Livrea, M. A., Ed.) pp 1−18, Birkhauser Publishing Ltd., Basel, Switzerland.
14. Napoli, J. L. (1999) Retinoic acid: its biosynthesis and metabolism, *Prog. Nucleic Acid Res. Mol. Biol. 63*, 139−188.
15. Duester, G. (1996) Involvement of alcohol dehydrogenase, short-chain dehydrogenase/reductase, aldehyde dehydrogenase, and cytochrome P450 in the control of retinoid signaling by activation of retinoic acid synthesis, *Biochemistry 35*, 12221−12227.
16. Quadro, L., Blaner, W. S., Salchow, D. J., Vogel, S., Piantedosi, R., Gouras, P., Freeman, S., Cosma, M. P., Colantuoni, V., and Gottesman, M. E. (1999) Visual defect and impaired retinoid availability in mice lacking retinol-binding protein, *EMBO J. 18*, 4633−4644.
17. Quadro, L., Blaner, W. S., Hamberger, L., van Gelder, R., Vogel, S., Piantedosi, R., Gouras, P., Colantuoni, V., and Gottesman, M. E. (2002) Muscle expression of human retinol-binding protein (RBP): Suppression of the visual defect of RBP knockout mice, *J. Biol. Chem. 277*, 30191−30197.
18. Paik, J., During, A., Harrison, E. H., Mendelsohn, C. L., Lai, K., and Blaner, W. S. (2001) Expression and characterization of a murine enzyme able to cleave β-carotene, *J. Biol. Chem. 276*, 32160−32168.
19. Kurlandsky, S. B., Gamble, M. V., Ramakrishnan, R., and Blaner, W. S. (1995) Plasma delivery of retinoic acid to tissues in the rat, *J. Biol. Chem. 270*, 17850−17857.
20. Bronzert, T. J., and Brewer, H. B., Jr. (1977) New micromethod for measuring cholesterol in plasma lipoprotein fractions, *Clin. Chem. 23*, 2089−2098.
21. Yamada, M., Blaner, W. S., Soprano, D. R., Dixon, J. L., Kjeldbye, H. M., and Goodman, D. S. (1987) Biochemical characteristics of isolated rat liver stellate cells, *Hepatology 7*, 1224−1229.
22. Xie, Y., Lashuel, H. A., Miroy, G. J., Dikler, S., and Kelly, J. W. (1998) Recombinant human retinol-binding protein refolding, native disulfide formation, and characterization, *Protein Expression Purif. 14*, 31−37.
23. Grippo, J. F., and Sherman, M. I. (1990) Retinoid-binding proteins in embryonal carcinomal cells, *Methods Enzymol. 189*, 148−155.
24. Blaner, W. S. (1990) Radioimmunoassays for retinol-binding protein, cellular retinol-binding protein, and cellular retinoic acid-binding protein, *Methods Enzymol. 189*, 270−281.
25. Willnow, T. E., Sheng, Z., Ishibashi, S., and Herz, J. (1994) Inhibition of hepatic chylomicron remnant uptake by gene transfer of a receptor antagonist, *Science 264*, 1471−1474.
26. Herz, J., and Gerard, R. D. (1993) Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice, *Proc. Natl. Acad. Sci. U.S.A. 90*, 2812−2816.
27. Jong, M. C., Dahlmans, V. E. H., van Gorp, P. J. J., van Dijk, K. W., Breuer, M. L., Hofker, M. H., and Havekes, L. M. (1996) In the absence of the low-density lipoprotein receptor, human apolipoprotein C1 overexpression in transgenic mice inhibits the hepatic uptake of very low-density lipoproteins via a receptor-associated protein-sensitive pathway, *J. Clin. Invest. 98*, 2259−2267.
28. Folch, J., Lees, M., and Sloane Stanley, G. H. (1956) A simple method for the isolation and purification of total lipids from animal tissues, *J. Biol. Chem. 226*, 497−509.
29. van Bennekum, A. M., Wei, S., Gamble, M. V., Vogel, S., Piantedosi, R., Gottesman, M. E., Episkopou, V., and Blaner, W. S. (2001) Biochemical basis for the depressed serum retinol levels in transthyretin-deficient mice, *J. Biol. Chem. 276*, 1107−1113.
30. Hayes, K. C., Lindsey, S., Stephan, Z. F., and Brecker, D. (1989) Retinal pigment epithelium possesses both LDL and scavenger receptor activity, *Invest. Ophthalmol. Visual Sci. 30*, 225−232.
31. Wang, N., and Anderson, R. E. (1993) Transport of 22:6n-3 in the plasma and uptake into retinal pigment epithelium and retina, *Exp. Eye Res. 57*, 225−233.
32. Noske, U. M., Schmidt-Erfurth, U., Meyer, C., and Diddens, H. (1998) Lipid metabolism in retinal pigment epithelium. Possible significance of lipoprotein receptors, *Ophthalmologe 95*, 814−819.
33. Heller, M., and Bok, D. (1976) A specific receptor for retinol binding protein as detected by the binding of human and bovine retinol binding protein to pigment epithelial cells, *Am. J. Ophthalmol. 81*, 93−97.
34. Pfeffer, B. A., Clark, V. M., Flannery, J. G., and Bok, D. (1986) Membrane receptors for retinol-binding protein in cultured human retinal pigment epithelium, *Invest. Ophthalmol. Visual Sci. 27*, 1031−1040.
35. Pasatiempo, A. M. G., and Ross, A. C. (1990) Effects of food or nutrient restriction on milk vitamin A transfer and neonatal vitamin A stores in the rat, *Br. J. Nutr. 63*, 351−362.
36. Green, M. H., Balmer Green, J., Akohoue, S. A., and Kelley, S. K. (2001) Vitamin A intake affects the contribution of chylomicrons vs. retinol-binding protein to milk vitamin A in lactating rats, *J. Nutr. 131*, 1279−1282.
37. Green, M. H., Snyder, R. W., Akohoue, S. A., and Balmer Green, J. (2001) Increased rat mammary tissue vitamin A associated with increased vitamin A intake during lactation is maintained after lactation, *J. Nutr. 131*, 1544−1547.

BI0268551