Transplacental delivery of retinoid: the role of retinol-binding protein and lipoprotein retinyl ester

Loredana Quadro,1,2 Leora Hamberger,1 Max E. Gottesman,1 Vittorio Colantuoni,2 Rajasekhar Ramakrishnan,3 and William S. Blaner4
1Institute of Cancer Research and Departments of 2Pediatrics and 3Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032; and 4Department of Biological and Environmental Sciences, University of Sannio, 82100 Benevento, Italy

Submitted 8 December 2003; accepted in final form 17 January 2004

Quadro, Loredana, Leora Hamberger, Max E. Gottesman, Vittorio Colantuoni, Rajasekhar Ramakrishnan, and William S. Blaner. Transplacental delivery of retinoid: the role of retinol-binding protein and lipoprotein retinyl ester. Am J Physiol Endocrinol Metab 286: E844–E851, 2004. First published January 21, 2004; 10.1152/ajpendo.00556.2003.—Retinoids are required for normal embryonic development. Both embryonic retinoid deficiency and excess result in congenital malformations. There is little understanding of the physiology underlying retinoid transfer from the maternal circulation to the embryo. We now report studies that explore this process using retinol-binding protein-deficient (RBP−/−) mice and mice that express human RBP on the RBP−/− background. Our studies establish that dietary retinoid, bound to lipoproteins, can serve as an important source for meeting tissue retinoid requirements during embryogenesis. Indeed, retinyl ester concentrations in the circulations of pregant RBP−/− mice are significantly elevated over those observed in wild-type mice, suggesting that lipoprotein retinyl esters may compensate for the absence of retinol-RBP during pregnancy. We also demonstrate, contrary to earlier proposals, that maternal RBP does not cross the placenta and cannot enter the fetal circulation. Overall, our data indicate that both retinol-RBP and retinyl esters bound to lipoproteins are able to provide sufficient retinoid to the embryo to allow for normal embryonic development.

The placenta. Two major retinoid species are present in the bloodstream. Retinol (vitamin A) bound to its specific transport protein, retinol-binding protein (RBP), is the predominant (95% or more) retinoid form in the fasting circulation (52). Postprandially, retinyl ester packaged in chylomicrons and chylomicron remnants can constitute a large percentage of the total retinoid present in the circulation (7, 58). Other retinoids are also present in the blood, albeit at much lower concentrations than retinol-RBP (7, 18). Retinoic acid is present in both the fasting and postprandial circulation, at concentrations that are 0.1–0.4% of those of retinol-RBP (7, 18). Retinyl esters are present in lipoprotein particles, primarily in very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) (20, 28). Finally, fully water-soluble glucuronides of both retinol and retinoic acid can also be found at extremely low levels in the circulation (2, 3, 7). The relative contributions made by each of these circulating retinoid forms to the bulk of retinoid transferred from the mother to the fetus are unknown.

As the sole specific transport protein for retinol, RBP has been proposed to play an important role in the delivery of retinoid from mother to fetus (46, 47, 52). However, the mechanisms and the physiology of maternal-fetal vitamin A transfer are not fully understood. Mice lacking RBP (RBP−/−) provide a valuable tool to investigate this aspect of retinoid physiology. The lack of RBP dramatically reduces serum retinol levels (12.5% of wild-type animals) and impairs visual function during the first months of life (42). Accumulation of hepatic retinoid stores is not impaired in RBP−/− mice. Indeed, the knockout mice accumulate retinol and retinyl ester in the liver at a higher rate compared with wild-type animals, presumably because they do not mobilize hepatic retinol bound to RBP (42). The good general health of these mice when they are maintained on a retinoid-sufficient diet indicates that they mostly rely on dietary retinoid to maintain normal physiological functions, including, evidently, reproduction and embryogenesis.

Here, we focus on the role played by RBP of maternal and embryonic origin in supplying developing tissues with adequate amounts of retinoid. We show that neither maternal nor fetal RBP crosses the placenta. Moreover, we show that RBP−/− mice maintained on a retinoid-sufficient diet rely on high circulating retinyl esters associated with the serum lipoprotein fractions to ensure normal fetal development.

RETINOIDS (vitamin A and its analogs) are required for normal embryonic development (11, 12, 33, 69). They are needed to ensure normal pattern formation in a number of organs and tissues, including hindbrain (21, 27, 37, 65), spinal cord (39, 51), eye (59), heart (16), kidney (4), lung (29), and limb buds (40, 54). Both retinoid deficiency and retinoid excess during development result in major embryonic defects (13, 31, 33, 55, 60). The features of embryonic retinoid deficiency syndrome include cleft face and palate, small or absent eyes, abnormalities in the urogenital system, abnormalities in the heart and large vessels, and malformation of the forelimbs (16, 22, 33, 56, 61, 66–67). Defects seen with embryonic retinoid excess overlap with those observed in retinoid deficiency and include malformations of the central nervous system, heart, thymus, urogenital system, and limbs and a number of abnormalities in craniofacial development (see review in Ref. 71).

To meet its requirement for retinoids, the developing mammalian embryo relies on circulating maternal retinoids that reach the fetus through the maternal-fetal barrier, i.e.,
PLACENTAL RETINOID TRANSFER

E845

MATERIALS AND METHODS

Mouse husbandry. Mice employed for these studies were from the same mixed genetic background as we have used in previous studies (41, 42, 58). Mice were maintained from the time of weaning on either a retinoid-sufficient diet (22–25 IU retinol/g of diet) or a retinoid-deficient diet (by lot analysis <0.22 IU retinol/g of diet). These diets were based on the AIN-93 formulation (44) and were purchased from Purina Labs. For all of our studies, both diet and water were available to the animals on an ad libitum basis. Mice were maintained on a 12:12-h dark-light cycle, with the period of darkness between 7:00 PM and 7:00 AM. All mice used for these studies were killed in the morning, ~9:30–11:30 AM. The animal experimentation described in this study was conducted in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals (35) and was approved by the Columbia University Institutional Committee on Animal Care.

HPLC analysis of retinoids. Reverse-phase HPLC analysis was performed as described (6, 62). Serum and tissues were flash-frozen in liquid N₂ after collection. For this analysis, tissues were homogenized in 10 volumes of PBS with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Retinoids present in the homogenates were extracted into hexane, as previously described (6, 62). The extracted retinoids were separated on a 4.6 × 250-mm Ultrasphere C₁₈ column (Beckman, Fullerton, CA) preceded by a C₁₈ guard column (Supelco, Bellefonte, PA), using 70% acetonitrile-15% methanol-15% methylene chloride as the running solvent flowing at 1.8 ml/min. Retinol and retinyl esters (retinyl palmitate, oleate, linoleate, stearate) were identified by comparing retention times and spectral data of experimental compounds with those of authentic standards. Concentrations of retinol and retinyl esters in the tissues were quantitated by comparing peak integrated areas for unknowns against those of experimental compounds with those of authentic standards. Loss during extraction was quantitated by comparing peak integrated areas for unknowns against those of authentic standards.

Isolation of lipoprotein fractions. Five lipoprotein fractions, consisting of a combined chylomicron and VLDL fraction, an intermediate-density lipoprotein (IDL) fraction, an LDL fraction, an HDL fraction, and a d > 1.21 g/ml bottom fraction were isolated by sequential ultracentrifugation of pools of serum obtained from wild-type and RBP⁻/⁻ mice, as described previously (10). The serum pools were constructed by mixing 400 μl from each of 15 age-matched female mice for each strain. Total retinol and retinyl ester levels for an aliquot of each serum pool were also determined by reverse-phase HPLC. Each pool was fractionated in duplicate. Retinol and retinyl ester levels for each of the five fractions were then assessed by reverse-phase HPLC, as described above.

RIA, Western blot, and Southern blot analysis. Analysis of tissue and blood RBP levels were carried out by RIA and/or Western blot. For these analyses, we employed monospecific antiserum against human or mouse RBP and procedures we reported earlier (5, 41, 42). Genotyping of mice was done by Southern blot analysis, as described earlier (41, 42).

RT-PCR analysis. Total RNA was extracted from mouse liver with TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. To ensure efficient removal of possible DNA contamination, RNA samples were further purified using RNeasy Mini Kit (Qiagen), according to the manufacturer’s protocol for RNA clean-up. Purified total RNA (1 μg) was converted to cDNA using SuperScript First-Strand Synthesis System for RT-PCR (In vitrogen, Carlsbad, CA). The reactions were primed with oligo(dt)12–18 primers, and the total volume of the reaction was 20 μl. The total RNA pool for each genotype was constructed by mixing identical amounts of total RNA prepared from six individual total RNA preparations for each genotype. Specific primers for CYP2C39 and β-actin and the PCR protocol were those reported by others (25). Ethidium bromide bands were quantitated by densitometric analysis.

Statistical analysis. Retinyl ester values were not normally distributed; logarithms were taken to achieve normality before statistical analysis. Retinyl ester results are reported as geometric means, which are the antilogarithms of the means of log-transforms. One-way analysis of variance (ANOVA), followed by pairwise contrasts, was used to compare different genotypes and genotype/pregnancy combinations. When only two groups were to be compared, the procedure was reduced to the Student’s unpaired t-test.

RESULTS

Does RBP of maternal and/or fetal origin cross the placenta? To ask whether either maternal or embryonic RBP can cross the placenta, we took advantage of a mouse strain we previously generated, the hRBP⁻/⁻ mice (41, 42). This strain overexpresses human RBP (hRBP) under the control of the mouse muscle creatine kinase (MCK) promoter on the RBP⁻/⁻ background. The concentration of hRBP in the circulation of these mice is quite high [2.3 ± 0.4 mg/dl (41)] compared with the amount of mouse RBP normally present in wild-type mice. We have shown that, like endogenous RBP, hRBP protein is secreted in the circulation, binds retinol and mouse transthyretin (TTR), and delivers retinol to peripheral tissues, like endogenous RBP (41). hRBP⁻/⁻ females hemizygous for the transgene were mated with RBP⁻/⁻ males and monitored from noon of the day that a vaginal plug was detected [set as 0.5 days postcoitum (dpc)]. Fifty percent of the progeny of this cross will carry the transgene. The MCK promoter is active from 13.0 dpc (26). Hence, the embryos begin to express hRBP at this embryonic age. At 18.5 dpc, pregnant females were killed and embryos collected. Genomic DNA was extracted from maternal and embryonic tail clips to assess genotype by Southern blot analysis (41). Whole embryos were used to assess hRBP concentration by RIA (5, 41). Figure 1A shows that only embryos carrying the transgene have immunologically detectable levels of human RBP. This result indicates that hRBP present in the maternal circulation is not able to cross the placenta and enter into the fetal circulation. To assess whether fetal RBP can enter the maternal circulation, we mated RBP⁻/⁻ females with doubly hemizygous hRBP⁻/⁻ males. In this case, all embryos will carry the hRBP transgene. At 18.5 dpc, pregnant females from this cross were killed to collect maternal blood for Western blot analysis. Embryos were collected to confirm genotype by Southern blot and to confirm by RIA that these fetuses actually expressed RBP (data not shown). Western blot analysis was performed using a rabbit polyclonal anti-rat serum RBP that cross-reacts with both endogenous mouse RBP and exogenous human RBP (34). Figure 1B shows that no immunoreactive hRBP was detected in the blood of the pregnant RBP⁻/⁻ females. This result indicates that the placenta does not allow RBP of fetal origin to cross into the maternal circulation.

Why do RBP⁻/⁻ embryos reveal no gross external dimorphology? RBP is thought to play an essential role in the delivery of retinoid from the mother to the embryo (46, 47); yet when RBP⁻/⁻ females are maintained on a retinoid-sufficient diet, no differences are observed in their litter sizes compared with wild-type females (Table 1). Moreover, on the basis of their external features, RBP knockout embryos appear grossly phenotypically normal, indistinguishable from wild-type embryos throughout gestation/development (data not shown and Ref. 64). These observations suggest that, if the mother re-
Ce secrète un niveau de retinol suffisant, il y a suffisamment de retinol disponible dans le flux sanguin maternel pour toutes les besoins embryonnaires. Sur le plan de l’embryon, il n’y a pas de baisse significative des niveaux de retinol (Table 1) pendant la grossesse. Par conséquent, nous pouvons supposer que le retinol est suffisant pour le développement normal de l’embryon, malgré le fait qu’il commence la grossesse avec des niveaux plus bas de retinol, comme on s’y attendait.

Table 1. Total retinol levels in liver and lung for newborns from wild-type and RBP-deficient females

<table>
<thead>
<tr>
<th>Maternal Genotype</th>
<th>Newborn Genotype</th>
<th>Litter</th>
<th>Maternal Genotype</th>
<th>Total Retinol Levels, µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>+/- or +/-</td>
<td></td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>-/+-</td>
<td>+/-</td>
<td></td>
<td>-/+</td>
<td></td>
</tr>
<tr>
<td>--/--</td>
<td>+/-</td>
<td></td>
<td>--/--</td>
<td></td>
</tr>
<tr>
<td>+/-</td>
<td>+/- or +/-</td>
<td></td>
<td>+/-</td>
<td></td>
</tr>
<tr>
<td>-/+-</td>
<td>+/-</td>
<td></td>
<td>-/+</td>
<td></td>
</tr>
<tr>
<td>--/--</td>
<td>+/-</td>
<td></td>
<td>--/--</td>
<td></td>
</tr>
</tbody>
</table>

Litters from 3-mo-old females were analyzed. Newborn pups were taken immediately at birth and never allowed to suckle. Total retinol (retinyl ester) levels were determined by reverse-phase HPLC. Values are means ± SD. RBP, retinol-binding protein; +/-, wild-type mice; -/--, heterozygous RBP-deficient mice; --/--, RBP-deficient mice. *P = 0.01 and †P = 0.002 vs. newborns from wild-type mothers.

Table 2. Serum retinol and retinyl ester levels in pregnant and nonpregnant wild-type and RBP-deficient mice maintained on a retinoid-sufficient diet

<table>
<thead>
<tr>
<th>Female Genotype</th>
<th>Serum Retinol (µg/dl)</th>
<th>Serum Retinyl Ester Levels (µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpregnant</td>
<td>15.5 ± 2.0</td>
<td>2.6 (0.5–9.6)</td>
</tr>
<tr>
<td>Pregnant</td>
<td>5.8 ± 0.8*</td>
<td>1.9 (1.1–6.7)</td>
</tr>
<tr>
<td>--/--</td>
<td>1.6 ± 0.5</td>
<td>11.2 (1.4–55.2)*</td>
</tr>
<tr>
<td>Nonpregnant</td>
<td>1.5 ± 0.3</td>
<td>3.5 (0.7–12.8)†</td>
</tr>
</tbody>
</table>

Nonfasted pregnant females were killed at midgestation. Retinol and retinyl ester levels were determined by reverse-phase HPLC. *P = 0.0005 and †P < 0.05 vs. corresponding nonpregnant females; ∗P < 0.01 vs. nonpregnant wild-type mice.
Table 3. Serum retinol and retinyl ester levels in fasted wild-type and RBP-deficient mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Retinol Levels, µg/dl</th>
<th>Retinyl ester content, geometric mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>25.0±3.4</td>
<td>1.03 (0.6–1.8)</td>
</tr>
<tr>
<td>--/--</td>
<td>2.0±0.7</td>
<td>1.79 (1.2–2.3)</td>
</tr>
</tbody>
</table>

Retinol and retinyl ester levels were determined by reverse-phase HPLC. Five male mice at 4 mo. of age were analyzed per group. Mice were fasted overnight before they were killed.

Residual retinol in the circulation of the knockout mice, which is bound to albumin (42), does not change in response to pregnancy. This suggests a specific preference of the placenta for retinol delivered bound to RBP. However, unlike wild-type mice, RBP−/− mothers have high initial circulating retinyl ester levels that drop during pregnancy (Table 2). We take this observation to suggest that RBP−/− mice utilize this retinyl ester to ensure normal embryonic development.

The finding of high retinyl ester levels in the circulation of RBP−/− mice was unexpected, since we previously demonstrated that retinyl ester transport in the circulation of fasted RBP−/− mice is not upregulated (58). Indeed, fasting serum levels of retinyl ester associated with lipoprotein particles are similar and very low in both wild and RBP−/− mice maintained on a retinoid-sufficient diet (42, 58). Table 3 shows serum retinol and retinyl ester levels in fasted age- and sex-matched RBP−/− and wild-type mice and confirms our previous data (58). Recall that the data in Table 2 are derived from animals in a nonfasted state. It is known that, after consumption of a retinoid-containing meal, the circulation can contain relatively high concentrations of retinyl ester associated with postprandial lipoprotein particles (57). To verify that the increased serum retinyl ester levels in the knockout mice arose from increased circulating lipoprotein and/or upregulation of the retinyl ester content of serum lipoproteins, we collected pools of serum from continuously fed age- and sex-matched RBP−/− and wild-type mice and maintained on a retinoid-sufficient diet throughout life. The pools were fractionated into different lipoprotein fractions (chylomicrons/VLDL, IDL, LDL, HDL, and >1.21 bottom fractions) and analyzed by reverse-phase HPLC to determine their retinyl ester contents. These data, shown in Table 4, confirm that fed RBP−/− mice have higher retinyl ester content associated with the chylomicron/VLDL fraction than age- and sex-matched wild-type mice. Moreover, measurement of triglyceride and cholesterol concentrations, the main lipid components of circulating lipoproteins (15, 49), in the same pools of serum showed higher circulating triglyceride levels compared with wild-type animals (molar ratio of triglyceride/cholesterol: 9.1 in RBP−/− mice and 2.4 in wild-type mice for serum pools constructed from equal volumes of serum from 15 mice).

How does dietary deprivation of retinoid affect stores in RBP−/− mice? To investigate further the dependence of RBP−/− mice on dietary retinoid intake, we maintained RBP−/− and wild-type mice on a retinoid-deficient diet from the time of weaning (21 days) for ≤6 mo and followed time-dependent changes in total retinol levels in liver, lung, and serum. We observed that total retinol levels in liver and lung decline over time in wild-type and RBP−/− mice (Fig. 2). However, after 4 mo of dietary retinoid deprivation, the residual retinol in the circulation of RBP−/− mice is very low (Table 5), but not undetectable, as it is after 1 wk on a retinoid-deficient diet (42). In contrast, wild-type serum retinol levels are significantly lower only after 6 mo of dietary retinoid deprivation. Note that, for both strains, serum retinyl ester levels are below the limit of detection when the mice are kept on a retinoid-deficient diet (Table 5). We also observed that RBP−/− mice maintained on a retinoid-deficient diet from weaning show no evident signs of illness, even ≤7 or 8 mo of age. Around this age, however, the mice do develop observable symptoms of retinoid deficiency, including cloudy eyes, loss of body fat, matted fur, a hunchback posture, and premature death (32). This suggests that, even when deprived of dietary retinoid, RBP−/− mice can meet, at least up to a certain point, their tissue retinoid requirements by mobilizing retinoids through RBP-independent pathways.

To understand better the observation that during dietary retinoid deprivation hepatic retinoid levels decline for RBP−/− mice at the same rate as for wild-type mice, we carried out preliminary gene array studies using RNA from the livers of RBP−/− and wild-type mice maintained on a retinoid-sufficient diet. We chose to carry out these investigations in mice receiving a retinoid-sufficient diet because we were interested in understanding whether gene expression patterns were different in livers at the start of retinoid deprivation. We observed and confirmed by RT-PCR that expression of cytochrome CYP2C39 was elevated in RBP−/− mice (Fig. 3). This hepatic enzyme has recently been reported to catalyze the oxidative metabolism of retinoids (1). No other cytochromes or other enzymes reported to catalyze retinoid metabolism were found to display elevated expression patterns in livers of RBP−/− mice.

Table 4. Retinol and retinyl ester content of lipoprotein fractions isolated from plasma pools for wild-type and RBP-deficient mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Retinol, µg/dl</th>
<th>RE, µg/dl</th>
<th>%RE</th>
<th>Chylomicron/VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
<th>&gt;1.21 bottom</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>23.1</td>
<td>6.4</td>
<td>22</td>
<td>58</td>
<td>18</td>
<td>17</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>--/--</td>
<td>1.7</td>
<td>31.4</td>
<td>95</td>
<td>84</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Pools were constructed by mixing 400 µl of serum from each of 15 nonfasted age-matched female mice for each strain. Serum retinol and retinyl ester (RE) levels were determined for an aliquot of each serum pool by reverse-phase HPLC. The remainder of the serum pools underwent sequential ultracentrifugation to separate lipoprotein fractions, as described in MATERIALS AND METHODS. Each pool was fractionated in duplicate. Retinol and RE levels for each of the 6 fractions were assessed by HPLC. IDL, intermediate-density lipoprotein. Values are means obtained from duplicated fractions.
and colleagues (Takahashi et al., Ref. 55) proposed in the 1970s that maternal retinol-RBP crosses the placenta to deliver needed retinoid to the embryo. However, with the advent of molecular techniques in the 1980s, it became clear that both placental and embryonic tissues express RBP mRNA (52, 53). Hence, whether the maternal retinol-RBP complex actually crossed the placenta was questioned (52, 53). Our studies employing hRBP–/− mice that express hRBP [hRBP shares 83% identity with mRBP (52)] in the RBP-null background conclusively demonstrate that maternal RBP does not cross the placenta and enter the developing embryo. These studies also show convincingly that RBP of fetal origin is unable to traverse the placenta and enter the maternal circulation. Thus, for retinol bound to RBP in the maternal circulation to be transferred to the fetus, it must dissociate from maternal RBP, traverse the placenta, and enter the fetal circulation through a mechanism that is independent of maternal RBP.

We have proposed that the failure of in vivo RBP ablation to recapitulate severe retinoid-deficient phenotypes during embryogenesis may reflect compensation by maternal dietary retinoids (64). Here we demonstrate that RBP–/− mice continuously fed a retinoid-sufficient diet rely on high serum retinyl ester concentrations, associated with the chylomicron/VLDL lipoprotein fraction, to ensure adequate transfer of retinoid across the placenta and to allow normal development of the fetus (Tables 1, 2, and 4). This, we propose, is the basis for the viability and fertility of RBP–/− mice maintained on a retinoid-sufficient diet. We do not yet understand the biochemical basis for this upregulation in retinyl ester content of the chylomicron/VLDL fraction. It seems unlikely that it is due to poor postprandial clearance of the chylomicron retinyl ester, since we observed normal levels of retinyl ester in fasted mice. Moreover, preliminary data from gavage studies (data not shown) argue against this hypothesis. We are currently undertaking experiments to establish whether well-studied lipoprotein-related proteins such as the LDL receptor (28, 38), lipoprotein lipase (19), or endothelial lipase (24, 43, 70), are involved in placental uptake of retinoid from lipoprotein particles.

Two lines of evidence establish that retinol is needed to support embryogenesis. First, the disruption of the mouse gene for retinaldehyde dehydrogenase type 2 (RALDH-2) results in embryonic lethality arising from a number of developmental defects such as neural tube defect (9, 54). Second, maternal dietary retinoid is needed to support pregnancy. Mice that express hRBP [hRBP shares 27.1% identity with mRBP (52)] in the RBP-null background exhibit embryonic lethality recapitulating severe retinoid-deficient phenotypes during embryogenesis. These findings, together with our studies of RBP–/− mice, demonstrate that retinol is necessary for normal development, thereby satisfying a long-standing proposal that maternal vitamin A is essential for normal embryogenesis (11, 12, 33, 39, 66). All-trans- and 9-cis-retinoic acids are the active retinoid forms that regulate expression of retinoid-responsive genes (11, 30). Both act through the retinoic acid receptor (RAR) and retinoid X receptor (RXR) classes of ligand-dependent transcription factors (11, 30). RXRs and RXRs regulate transcription of a number of developmentally important genes, thus influencing the pattern formation of many organs and tissues. Although it is well established that retinoic acid and its receptors are needed to ensure normal embryogenesis, there is little biochemical understanding of the factors and processes that facilitate and control transfer of retinoids from the maternal circulation to the embryo. The studies described in this report were undertaken to provide a better understanding of these.

As the sole specific transport protein for retinol, RBP has long been proposed to play an important role in the delivery of retinoid from mother to fetus (46, 47, 52). On the basis of immunological studies of RBP protein distribution, Goodman and colleagues (Takahashi et al., Ref. 55) proposed in the 1970s that maternal retinol-RBP crosses the placenta to deliver needed retinoid to the embryo. However, with the advent of molecular techniques in the 1980s, it became clear that both placental and embryonic tissues express RBP mRNA (52, 53). Hence, whether the maternal retinol-RBP complex actually crossed the placenta was questioned (52, 53). Our studies employing hRBP–/− mice that express hRBP [hRBP shares 83% identity with mRBP (52)] in the RBP-null background conclusively demonstrate that maternal RBP does not cross the placenta and enter the developing embryo. These studies also show convincingly that RBP of fetal origin is unable to traverse the placenta and enter the maternal circulation. Thus, for retinol bound to RBP in the maternal circulation to be transferred to the fetus, it must dissociate from maternal RBP, traverse the placenta, and enter the fetal circulation through a mechanism that is independent of maternal RBP.

We have proposed that the failure of in vivo RBP ablation to recapitulate severe retinoid-deficient phenotypes during embryogenesis may reflect compensation by maternal dietary retinoids (64). Here we demonstrate that RBP–/− mice continuously fed a retinoid-sufficient diet rely on high serum retinyl ester concentrations, associated with the chylomicron/VLDL lipoprotein fraction, to ensure adequate transfer of retinoid across the placenta and to allow normal development of the fetus (Tables 1, 2, and 4). This, we propose, is the basis for the viability and fertility of RBP–/− mice maintained on a retinoid-sufficient diet. We do not yet understand the biochemical basis for this upregulation in retinyl ester content of the chylomicron/VLDL fraction. It seems unlikely that it is due to poor postprandial clearance of the chylomicron retinyl ester, since we observed normal levels of retinyl ester in fasted mice. Moreover, preliminary data from gavage studies (data not shown) argue against this hypothesis. We are currently undertaking experiments to establish whether well-studied lipoprotein-related proteins such as the LDL receptor (28, 38), lipoprotein lipase (19), or endothelial lipase (24, 43, 70), are involved in placental uptake of retinoid from lipoprotein particles.

Two lines of evidence establish that retinol is needed to support embryogenesis. First, the disruption of the mouse gene for retinaldehyde dehydrogenase type 2 (RALDH-2) results in embryonic lethality arising from a number of developmental

## Table 5. Serum retinol and retinyl ester levels in wild-type and RBP-deficient mice at weaning and after dietary retinoid deprivation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Age, days</th>
<th>Diet</th>
<th>Retinol Levels, μg/dl</th>
<th>Retinyl ester Levels, μg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>4</td>
<td>21</td>
<td>Vit A+</td>
<td>27.1 ± 4.0</td>
<td>0.2–4.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>120</td>
<td>Vit A−</td>
<td>19.7 ± 10.7</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>180</td>
<td>Vit A−</td>
<td>5.5 ± 5.4</td>
<td>ND</td>
</tr>
<tr>
<td>−/−</td>
<td>6</td>
<td>21</td>
<td>Vit A+</td>
<td>3.9 ± 2.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>120</td>
<td>Vit A−</td>
<td>1.0 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>180</td>
<td>Vit A−</td>
<td>1.5 ± 0.5†</td>
<td>ND</td>
</tr>
</tbody>
</table>

Retinol and retinyl ester levels were determined by reverse-phase HPLC. n: No. of male mice analyzed. Vit A+ = retinoid-sufficient diet. Vit A− = retinoid-deficient diet. ND: nondetectable. *P < 0.0005 and †P = 0.01 vs. mice receiving retinoid-sufficient diet.
Fig. 3. Increased expression of CYP2C39 mRNA in liver of RBP<sup>−/−</sup> mice maintained on a retinoid-sufficient diet. A: detection by RT-PCR of CYP2C39 mRNA in livers of wild-type (+/+ ) and RBP-deficient (−/−) mice maintained on a retinoid-sufficient diet. cDNA was synthesized from a pool of 6 age- and sex-matched mice for each genotype. The RNA pool was constructed by mixing the same amount of RNA for each sample prepared from each genotype. The cDNA was amplified by PCR using sequence-specific primer pairs for mouse CYP2C39 and mouse β-actin according to a published protocol (25). The expected size of the bands is 285 bp (CYP2C39) and 410 bp (mouse β-actin). PCR products (5 µl) were electrophoresed on a 1% agarose gel for analysis with ethidium bromide staining. Quantitation of the ethidium bromide bands was performed by densitometric analysis. M, molecular weight marker: puc18 HinfI digest. B: relative expression levels of CYP2C39 gene in livers of wild-type (+/+ ) and RBP-deficient (−/−) mice maintained on a retinoid-sufficient diet, normalized to the endogenous reference (β-actin) after densitometric analysis.

abnormalities consistent with those observed in retinoid deficiency (36, 37). Because RALDH-2 is one of two enzymes needed to catalyze the two oxidative steps needed for the formation of retinoic acid from retinol, retinol must be required to support normal embryogenesis. Second, Wellik and DeLuca (63) have reported nutritional studies demonstrating that retinol is required to support normal embryogenesis and that retinoid acid administration cannot supplant this requirement for retinol (63). Nevertheless, retinoid acid is present in low concentrations in the circulation, typically 2–3 orders of magnitude lower than the concentration of retinol (7). We observed that the concentrations of all-trans-retinoic acid in the serum pools of wild-type and RBP<sup>−/−</sup> mice are similar and very low (wild type, 0.7–1.5 ng/ml; RBP<sup>−/−</sup>, 0.6–1.8 ng/ml). Similarly, we noted that 13-cis-retinoic acid levels are not elevated in RBP<sup>−/−</sup> mice (data not shown), and we were not able to detect 9-cis-retinoic acid in pooled sera from either wild-type or RBP<sup>−/−</sup> mice. Thus circulating retinoic acid concentrations are not upregulated to compensate for the absence of RBP. On the basis of these considerations, we propose that circulating retinoic acid does not compensate for the loss of RBP during embryogenesis in RBP-deficient mice.

In this report, we also show that depriving RBP<sup>−/−</sup> mice of dietary retinoid at the time of weaning does not have an immediately lethal or other deleterious effect on these mice. The mice remain healthy for ≤7 or 8 mo of age, presumably by using the relatively small amount of retinoid that they accumulate in tissue stores during the suckling period. In fact, we show that liver and lung retinoid levels decline over time in RBP<sup>−/−</sup> mice and that very low but detectable amounts of retinol circulate, even after an extensive period of dietary retinoid deprivation. This retinol, which is not bound to RBP, might represent one of the retinoid forms that are utilized to support normal retinoid-dependent functions in RBP<sup>−/−</sup> mice. Because the liver of RBP<sup>−/−</sup> mice is unable to efficiently mobilize retinol through RBP (42), we had assumed that these mice would retain hepatic retinol more tenaciously than heterozygous or wild-type mice, and lose retinoid more slowly when challenged with a retinoid-deficient diet. However, this proved not to be the case. Inherent in our assumption was the notion that the liver does not catabolize its retinoid stores to any significant degree and that elimination occurs primarily in peripheral tissues (7). This assumption appears to be incorrect, because hepatic retinoid stores decline at similar rates in RBP<sup>−/−</sup> and wild-type mice, even though RBP<sup>−/−</sup> mice cannot mobilize retinol bound to RBP. This suggests that the liver does, to a significant extent, turn over its retinoid stores without secreting these stores as retinol bound to RBP. Recently, Andreola et al. (1) reported that the cytochrome CYP2C39 catalyzes the oxidative or catabolic metabolism of retinoids in the liver. Expression of CYP2C39 is markedly downregulated in aryl hydrocarbon receptor-deficient (AHR<sup>−/−</sup>) mice. These investigators proposed that the reduction in CYP2C39 expression accounts for the elevated retinyl ester stores observed in the AHR<sup>−/−</sup> mice. Our data indicate that CYP2C39 expression is markedly upregulated in the livers of RBP<sup>−/−</sup> mice. By use of the same line of logic as used by Andreola et al., it seems reasonable to propose that elevation of CYP2C39 expression in liver of RBP<sup>−/−</sup> mice may account for turnover of hepatic retinoid reserves. Possibly, CYP2C39 expression is upregulated in response to the higher total retinol concentrations present in the livers of RBP<sup>−/−</sup> mice (41, 42).

It is clear from our data that the pathways responsible for delivery of retinoids from the mother to the embryo are complex and overlapping. Although retinol bound to RBP certainly accounts for much of the retinoid that is delivered from the mother to the embryo, the normal sizes observed for litters from RBP-deficient dams and the general good health of their pups indicate that retinol-RBP is not the only source for retinoids that reach the embryo. Our studies establish that retinyl esters in lipoprotein particles can be a significant source for retinoid that is utilized by the fetus to support embryogenesis. These data are consistent with several reports from the early 1990s showing that VLDL and LDL can be taken up by trophoblasts (8, 9, 23). We previously proposed, on the basis of our study of the RBP<sup>−/−</sup> mice, that dietary retinoid is an important source through which tissues acquire needed retinoid, and we have suggested that this postprandial pathway is
likely the primordial delivery pathway through which tissues acquire retinoid (41, 42, 58). RBP-dependent delivery of retinol to tissues allows for hepatic retinoid stores and, consequently, buffers against dietary retinoid insufficiency. The studies reported in this manuscript support our contention that dietary retinoid is an important source through which tissues can acquire retinoid needed to regulate gene expression by establishing the significance of this pathway in supporting embryogenesis.

GRANTS

This work was supported by National Institutes of Health Grants EY-12858 and DK-52444.

REFERENCES
