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Pathways of Vitamin A Delivery to the Embryo: Insights from a New Tunable Model of Embryonic Vitamin A Deficiency

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Circulating retinoids (vitamin A and its derivatives) are found predominantly as retinol bound to retinol-binding protein (RBP), which transports retinol from liver stores to target tissues, or as retinyl ester incorporated in lipoproteins of dietary origin. The transport of retinoids from maternal to fetal circulation is poorly understood, especially under conditions of inadequate dietary vitamin A intake. Here we present RBP$^{-/-}$ mice as a tunable model of embryonic vitamin A deficiency. This model has enabled us to analyze metabolic links between maternal nutrition and retinoid delivery to the fetus. Our data show that retinol-RBP is the primary contributor to fetal development, whereas retinyl ester are largely responsible for accumulation of fetal retinoid stores. Furthermore, these studies indicate the importance of embryonic RBP in distributing vitamin A to certain developing tissues under restrictive diets. We also show differences among developing tissues in their dependency on the embryonic retinol-RBP pathway. Finally, we demonstrate that accumulation of embryonic vitamin A stores does not depend on the expression of RBP in the fetal liver. (*Endocrinology* 146: 4479–4490, 2005)

VITAMIN A IS an essential lipid-soluble nutrient that is crucial for maintaining pregnancy and morphogenesis of most developing organs and tissues (1). Studies in rats first showed that offspring of dams bred on vitamin A-deficient diets died before or at birth and displayed a spectrum of malformations affecting the eye, skeletal system, thyroid and thymus, heart, lung, and branching organs (kidney, salivary and submaxillary gland) (2–7). These malformations are known collectively as vitamin A deficiency syndrome (VAD).

Vitamin A and its metabolites (retinol, retinyl ester, retinoic acid, and retinaldehyde) are naturally occurring retinoids. With the exception of the visual cycle, which requires retinaldehyde (8), the biologically active retinoid form is retinoic acid (9). It is a lipid soluble hormone that binds to and activates specific retinoid receptors [retinoic acid receptors (RARs), retinoid X receptors (RXRs)], which regulate the transcription of many target genes (10). Retinoid receptors are a large family containing at least 14 different proteins widely expressed in the adult and in the fetus (11, 12). Compound genetic inactivation of retinoid receptors (RAR/RXRα) in mouse generates phenotypes closely resembling VAD (13–17).

The mammalian fetus acquires vitamin A from the maternal circulation, in which retinoids are transported in association with carrier molecules. In the fasting state, the bulk of circulating vitamin A is in the form of retinol bound to retinol-binding protein (RBP), a 21-kDa lipocalin family member (18). RBP is the specific carrier for retinol in the circulation. It is primarily synthesized in the liver, from which it is secreted into the bloodstream. RBP binds one molecule of retinol and circulates as a 1:1 molar complex with another serum protein, transthyretin (19). The major function of RBP is to mobilize and transport retinol from liver to target tissues (18, 20). In the target tissues, metabolic enzymes convert retinol to retinoic acid, which then controls vitamin A signaling (9). Upon food ingestion, vitamin A is also transported as retinyl ester by chylomicrons, triglyceride-rich particles formed in the intestine (9). Once secreted into the bloodstream, most vitamin A carried in chylomicrons is taken up by the liver, in which the majority of vitamin A is stored in the form of retinyl ester (21). Transport of vitamin A from liver to target tissues requires hydrolysis of retinyl ester to retinol, binding to RBP and secretion of the holo-RBP complex into the bloodstream (18). Vitamin A may also be circulated via lipoproteins of hepatic origin, such as very low-density lipoprotein and low density protein, which transport vitamin A as retinyl ester (22, 23).

Retinol bound to RBP is thought to be the most physiologically important retinoid form transported from mother to fetus and within the fetus (18, 24). To reach the fetal circulation, maternal vitamin A must traverse the placenta. Within the rodent placenta, RBP is localized on the maternal side in the decidua basalis and the embryonic yolk sac endoderm from 7.0 d post coitum (dpc) (24). Starting from 11.5 dpc, RBP is also expressed in fetal liver (25). We recently demonstrated that maternal RBP does not cross the placenta (26). Thus, to

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Abbreviations: dpc, Days post coitum; E, embryonic day; lacZ, lacZ transgene; RAR, retinoic acid receptor; RBP, retinol-binding protein; RXR, retinoid X receptor; VAD, vitamin A deficiency syndrome.

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enter the fetal circulation, maternal retinol bound to RBP must be released at the maternal-fetal interface. Here it binds to RBP of the yolk sac origin and can be secreted into the fetal circulation (24, 26, 27).

These and other observations (28) suggest that loss of RBP would cause embryonic abnormalities typical of VAD. However, when maintained on a vitamin A-sufficient diet, mice lacking RBP ($RBP^{-/-}$) yield viable embryos that display only relatively mild and transient cardiac embryonic developmental anomalies (25, 29). The unremarkable phenotype of $RBP^{-/-}$ embryos reflects the existence of an alternative pathway(s) of vitamin A delivery to the fetus. In mice lacking retinol-RBP, high levels of retinyl ester incorporated in maternal circulating chylomicrons and/or very low-density lipoprotein particles provide the embryos with a sufficient amount of vitamin A to enable relatively normal development of most embryonic tissues, excluding heart (25, 26). These data suggest that, under normal maternal dietary vitamin A intake, postprandial vitamin A pathways can support normal development and accumulation of fetal vitamin A stores, independently of RBP expression in the embryo and in the mother.

The physiology of the transfer of vitamin A from mother to fetus under conditions of inadequate maternal vitamin A intake is poorly understood. Previous studies in rats established that the transplacental transport of vitamin A is regulated to maintain embryonic retinoid levels in the face of maternal vitamin A deficiency (30, 31). However, these regulatory mechanisms remain to be elucidated. The contributions of different maternal circulating retinoid forms to transplacental transfer during maternal dietary vitamin A deficiency are, for example, quite obscure. Moreover, neither the fetal response to a restricted maternal dietary regimen nor the limits of deficiency that the regulatory system can tolerate are known.

In this report, we use a novel tunable model of VAD to address these issues. We first show that, by simple manipulation of the dietary vitamin A intake of $RBP^{-/-}$ dams, we were able to generate a wide range of fetal malformations. According to the extent of maternal dietary vitamin A deprivation, these malformations varied from mild to severe symptoms of VAD to complete fetal resorption. In essence, this model system allows one to control the amount of vitamin A that reaches the developing embryonic tissues, thus generating VAD phenotypes of different severity. Moreover, it provided us with the opportunity to genetically dissect the maternal and fetal metabolic pathways (retinol-RBP *vs.* lipoprotein retinyl ester) that contribute to vitamin A delivery to developing tissues. We show that whereas the maternal retinol-RBP pathway is the primary contributor to fetal development, the retinyl ester pathway is largely responsible for the accumulation of fetal retinoid stores. Furthermore, we demonstrate that RBP of embryonic origin plays a key role in distributing vitamin A to the developing tissues in times of inadequate maternal vitamin A intake. We also show differences among developing tissues in their dependence on the embryonic retinol-RBP pathway. Finally, we demonstrate that accumulation of embryonic vitamin A stores does not depend on the expression of RBP in fetal liver.

Materials and Methods

Knockout and transgenic mice

$RBP^{-/-}$ were previously generated and described (29). Genotyping of the $RBP^{-/-}$ strain was performed accordingly. Mice doubly hemizygous for a lacZ transgene (lacZ), controlled by a retinoic acid response element in the RARβ2 promoter (32), were crossed with $RBP^{-/-}$ animals. We generated mice doubly hemizygous for the lacZ transgene in the RBP knockout background ($lacZ/RBP^{-/-}$), RBP wild-type background ($lacZ/RBP^{+/+}$), and RBP heterozygous background ($lacZ/RBP^{+/-}$). Detection of the lacZ transgene was performed as described (32). For timed matings, gestational d 0.5 post coitus [0.5 dpc or embryonic d 0.5 (E0.5)] was defined as the morning of the day when the vaginal plug was observed.

Nutritional manipulation

Female mice used for this study were maintained on different dietary regimens from the time of weaning. These dietary regimens differ for the time span the mice were maintained either on a purified vitamin A-sufficient diet (22–25 IU retinol per gram of diet) or on a purified vitamin A-deficient diet (by lot analysis $<$ 0.22 IU retinolper gram of diet) throughout life, including gestation. These diets were based on the AIN-93 formulation (33) and were purchased from Purina Labs (W. F. Fisher & Son, Somerville, NJ). 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-h dark, 12-h light cycle with the period of darkness between 1900 and 0700 h. At 3 months of age, the female mice were mated and the time that a vaginal plug was detected was set 0.5 dpc. At different times of gestation, females were killed. Maternal serum, embryos, and/or newborns liver and lung were collected. Embryos and/or newborns genotype was assessed accordingly (29, 32). All mice used for these studies were killed in the morning, around 0930 and 1130 h. The animal experimentation described in this manuscript was conducted in accordance with the Natioanl Institutes of Health Guide for the Care and Use of Laboratory Animals (34) and were approved by the Columbia University Institutional Committee on Animal Care.

HPLC analysis of retinoids

Reverse-phase HPLC analysis was performed as described (29). Mouse serum and tissues were flash frozen in liquid N_2 after collection. For this analysis, tissues were homogenized in 10 volumes of PBS using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Retinoids present in the homogenates were extracted into hexane as previously described (29). The extracted retinoids were separated on a 4.6 $\times$ 250 mm Ultrasphere C_{18} column (Beckman, Fullerton, CA) preceded by a C_{18} guard column (Supelco Inc., Bellefonte, PA), using 70% acetonitrile, 15% methanol, and 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 known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of internal standard retinyl acetate added immediately afterhomogenization of the tissues.

Whole-mount detection of β-galactosidase activity

Embryos were dissected in PBS to avoid any potential outside source of retinoids. Subsequently they were prefixed in a solution containing 4% paraformaldehyde in phosphate buffer (pH 7.4), freshly made, for 60 min on ice. Next, they were rinsed with 100 mM phosphate buffer (pH7.4), 5 mM EGTA, 2 mM $MgCl_2$ twice (5 and 20 min) and with 100 mM phosphate buffer (pH7.4), 2 mM $MgCl_2$, 0.01% sodium deoxycolate, 0.02% Nonidet-P40 twice for 5 min and stained at 37 C for 4 h to overnight in a solution containing this buffer and 1 mg/ml X-gal, 1 mM spermidine HCL, 5 mM $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$-$6H_20$. The next day embryos were postfixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 10–20 min and stored in PBS at 4 C.

Hematoxylin and eosin staining

Embryos were fixed and embedded in paraffin according to standard procedures. Paraffin blocks were sectioned at 6 μm and stained with hematoxylin and eosin according to standard procedures.

Alcian blue and alzarin red staining

Newborns were killed and then skinned, fixed in 95% ethanol, and stored at 4 C until use. Staining for cartilage and bone was performed by using alcian blue and alzarin red as described (35).

Statistical analysis

Retinyl ester values were not normally distributed; logarithms were taken to achieve normality before statistical analysis. Retinyl esters are reported as geometric means (which are the antilogarithms of the means of the log transforms). All data were analyzed for statistically significant differences using standard procedures consisting of Student's unpaired *t* test or ANOVA. $P < 0.05$ was used to establish statistical significance between groups of samples.

Results

RBP$^{-/-}$ mice are a tunable model of VAD

External embryonic malformations. RBP$^{-/-}$ mice are highly dependent on dietary vitamin A to support pregnancy and fetal development (26). This phenotype enabled us to examine the effects of dietary vitamin A deprivation and impairment of the retinol-RBP pathway on embryonic development and, by extension, on transplacental transfer of retinoid in a mammalian model system. Groups of RBP$^{-/-}$ and control (RBP$^{+/+}$ or RBP$^{+/-}$) females were maintained on different regimens of dietary vitamin A deprivation up to approximately 3 months of age, when they were mated with RBP$^{+/-}$ males. These dietary regimens are schematically represented in Fig. 1. Females included in group I were maintained on a purified vitamin A-sufficient diet (22–25 IU/g) throughout life and gestation. Females in group II were fed a vitamin A-deficient diet (<0.22 IU/g) from the time that a vaginal plug was detected (set 0.5 dpc, the onset of gestation). In groups III–V, females were maintained on a vitamin A-deficient diet for increasing number of weeks before plug. Females in group VI were deprived of dietary vitamin A from weaning (21 d) and then maintained on a vitamin A-deficient diet throughout gestation.

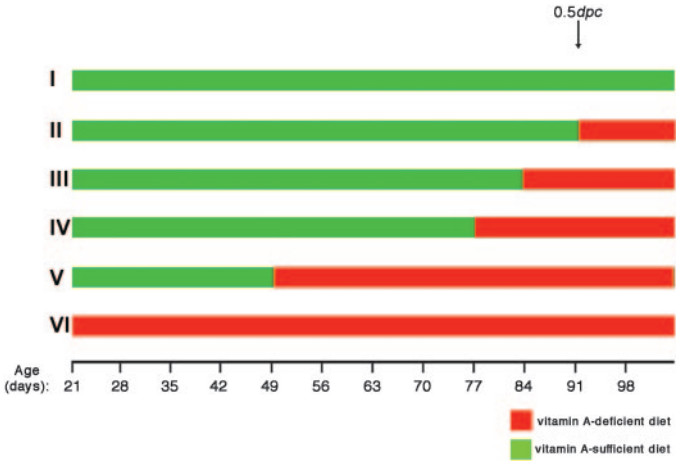

FIG. 1. Schematic representation of different regimens of maternal dietary vitamin A deprivation. The *different colors* of the *bars* indicate the type of diet [*green*, vitamin A-sufficient diet (22–25 IU/g); *red*, vitamin A-deficient diet (<0.22 IU/g)] on which RBP$^{-/-}$ and controls (RBP$^{+/+}$ or $^{+/-}$) dams were maintained over a 3-month period, starting at weaning (21 d) and including gestation. Beginning of gestation was set at 0.5 dpc, the time that a vaginal plug was detected, and it is indicated by an *arrow* on the *top right* of the figure. The *numbers at the bottom* of the figure indicate the age of the mice expressed in days. Each dietary regimen is identified by a *Roman numeral* on the *left side* of the panel (from I to VI).

Dams from each of these groups were killed at 14.5 dpc. Embryos were dissected, genotyped, and analyzed for external gross morphology and by histology. Embryos developing from RBP$^{+/+}$ and RBP$^{+/-}$ dams maintained on any of the dietary regimens described above or developing from RBP$^{-/-}$ dams on a vitamin A-sufficient diet (group I as in Fig. 1) were grossly phenotypically normal. In contrast, malformations were observed in embryos developing from RBP$^{-/-}$ dams maintained on a vitamin A-deficient diet. The severity of these malformations increased with the length of dietary vitamin A deprivation. It also depended on presence or absence of RBP in the fetus.

Gross morphological analysis revealed that dietary vitamin A deprivation of RBP$^{-/-}$ dams during pregnancy (group II as in Fig. 1) affected the development of embryos lacking RBP but not the development of heterozygous littermate embryos (RBP$^{+/-}$) (Table 1). The growth of E14.5 RBP$^{-/-}$ embryos from RBP$^{-/-}$ dams was generally retarded, compared with their RBP$^{+/-}$ littermates. Moreover, these RBP$^{-/-}$ embryos displayed small eyes (Fig. 2F) and peripheral edema, an indication of cardiac insufficiency [(14); Fig. 2F]. This embryonic phenotype resembles that described in RXRα knockout and RXRα/RAR double mutants (14, 15), in which vitamin A signaling is disrupted by inactivation of the retinoid receptors. In contrast, RBP$^{+/-}$ littermate embryos were grossly phenotypically normal (Fig. 2B).

Extending dietary vitamin A deprivation of RBP$^{-/-}$ dams to 1 or 2 wk before the onset of gestation (groups III and IV as in Fig. 1) also generated malformed RBP$^{-/-}$ embryos. However, their gross external morphology was undistinguishable from that of malformed RBP$^{-/-}$ embryos generated from group II RBP$^{-/-}$ dams (data not shown).

More severe malformations were observed in E14.5 RBP$^{-/-}$ embryos developed from RBP$^{-/-}$ dams on a vitamin A-deficient diet for more than 5 wk before plug formation (group V as in Fig. 1). These embryos were significantly smaller than control embryos. Their other phenotypes included eyes reduced in size or sometimes completely undetectable and abnormal midfacial regions and forelimbs (Fig. 2G). Some of these embryos (2%) also showed exencephaly (exteriorized brain). These malformed RBP$^{-/-}$ embryos resemble those described in VAD (3, 4, 6, 7, 36) and mice bearing retinoid receptor knockouts (16, 17). In contrast, their RBP$^{+/-}$ littermates were grossly phenotypically normal (Fig. 2C). Note also that the number of resorptions in RBP$^{-/-}$ dams increases with the extent of the dietary vitamin A deprivation and is elevated, compared with the number of resorptions in controls dams under similar dietary regimens (Table 1).

Gross morphological analysis of embryos from females deprived of dietary vitamin A from weaning (group VI as in Fig. 1) showed that RBP$^{+/+}$ and RBP$^{+/-}$ dams supported

TABLE 1. Effects of different regimens of maternal dietary vitamin A intake on embryonic development

Dams			Embryos (E12.5–14.5)						
			Normal			Abnormal			Resorbed[a]
Dietary regimen	Genotype	n	+/+	+/−	−/−	+/+	+/−	−/−	
Group I	+/+	7	24	11					3
	−/−	12		40	60				5
Group II	+/+ or +/−	10	14	40	15				5
	−/−	14		38				52	26
Group V	+/+ or +/−	10	36	9	12				5
	−/−	17		35				32	55
Group VI	+/+ or +/−	12	40	25	17				9
	−/−	10			0		20		36 (29)[b]

Three-month-old pregnant RBP knockout, heterozygous, and wild-type females were maintained under different regimens of dietary vitamin A intake as in Fig. 1 (group I, II, V, and VI). The females were mated with RBP heterozygous males. Dams were killed at midgestation and embryos collected and analyzed for their external gross morphology. We define as normal wild-type embryos from wild-type dams bred on a vitamin A-sufficient diet (group I). Embryos classified as abnormal display reduced size, white appearance, and small or apparent absence of eye. They also showed one or more of the following features: peripheral edema, abnormal midfacial region, abnormal limbs. Embryos classified as resorbed were retarded or completely resorbed (no embryo was observed at the time of the dissection). n, Number of dams analyzed per each group. +/+, RBP wild-type; +/−, RBP heterozygous; −/−, RBP knockout.

[a] All different genotypes are included in this category.

[b] Twenty-nine of 36 were −/− embryos and they were totally resorbed. 0 indicates that embryos were expected but not obtained.

normal fetal development, regardless of the genotype of the embryo (Table 1). In contrast, no $RBP^{-/-}$ embryos from $RBP^{-/-}$ dams were found at 14.5 dpc. Analysis of offspring from $RBP^{-/-}$ dams from group VI at 11.5 dpc revealed that $RBP^{-/-}$ embryos were smaller and retarded and had not undergone the axial rotation that occurs approximately at 8.5 dpc in wild-type embryos (37). The heart was dilated and the neural tube remained open. The frontonasal region was truncated and only the first brachial arch was distinguishable (Fig. 2, H–K). This phenotype resembles that of mutant embryos lacking retinaldehyde dehydrogenase 2, an enzyme required for embryonic retinoic acid synthesis. These embryos die most likely as a consequence of circulatory or heart abnormalities (38). In contrast, $RBP^{+/-}$ littermate embryos continued to develop, but gross morphological analysis reveals abnormalities (Table 1). Figure 2D shows eye malformations in an E11.5 $RBP^{+/-}$ embryo.

Defects in organogenesis. Histological analysis confirms that the severity of the VAD malformations correlated with the extent of maternal dietary vitamin A deprivation. These malformations closely resembled those seen in dietary studies in rats and retinoid receptor knockout mice and included de-

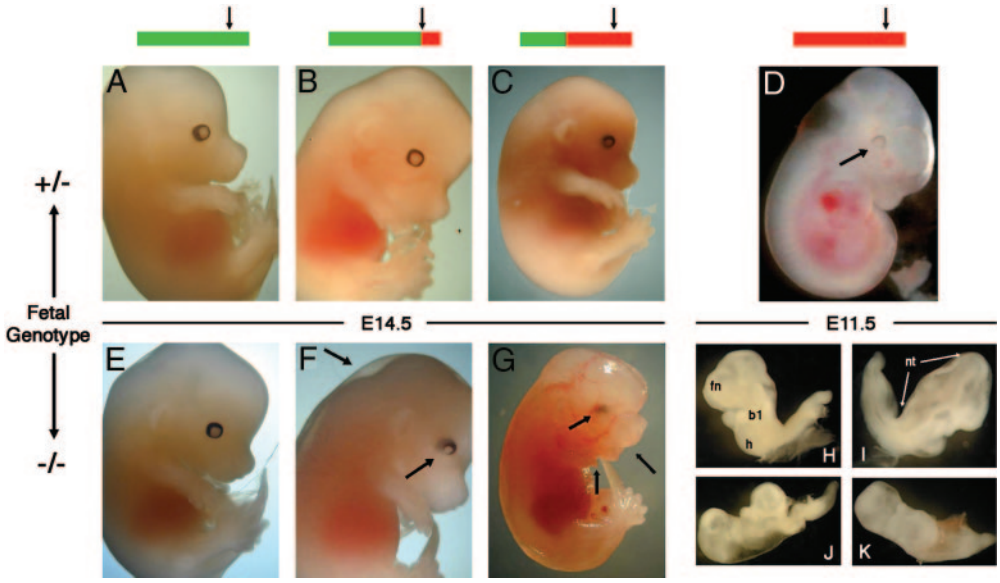

FIG. 2. Gross morphology of embryos from $RBP^{-/-}$ dams maintained on different regimens of dietary vitamin A deprivation. The *color code* of the *bars on the top of the panels* indicates the type of maternal dietary regimen according to the scheme in Fig. 1. The *arrow on top of each bar* indicates the beginning of gestation (0.5 dpc). Images of littermate embryos $RBP^{+/-}$ ($^{+/-}$) and $RBP^{-/-}$ ($^{-/-}$) are shown in A and E, B and F, C and G, and in D–K. Embryos in A–C and E–G were collected at 14.5 dpc. Embryos in D and H–K were collected at 11.5 dpc. In H–K the same embryo is shown. $RBP^{-/-}$ females were mated with $RBP^{+/-}$ males. The *arrows* (F) point to abnormal eye (reduced pigmentation in the ventral region) and peripheral edema. The *arrow* (D) points to abnormal eye. The *arrows* (G) point to abnormal eye and abnormal midfacial region (snout foreshortened and divided by a sagittal median cleft, prolabium absent, maxillary process bearing whiskers separated by a larger than normal distance). fn, Frontonasal; h, heart; b1, first branchial arch; nt, open neural tube. The same magnification was used for A and E, B and F, and C and G.

fects in the respiratory, urinary, and female genital tracts (Fig. 3). In the respiratory tract, vitamin A deficiency results in left lung agenesis caused by failure to form the primary bronchus, lung hypoplasia, absence of the esophageal/tracheal septum, and abnormal patterning of tracheal cartilagenous rings (3, 16, 17, 39). In $RBP^{-/-}$ embryos from $RBP^{-/-}$

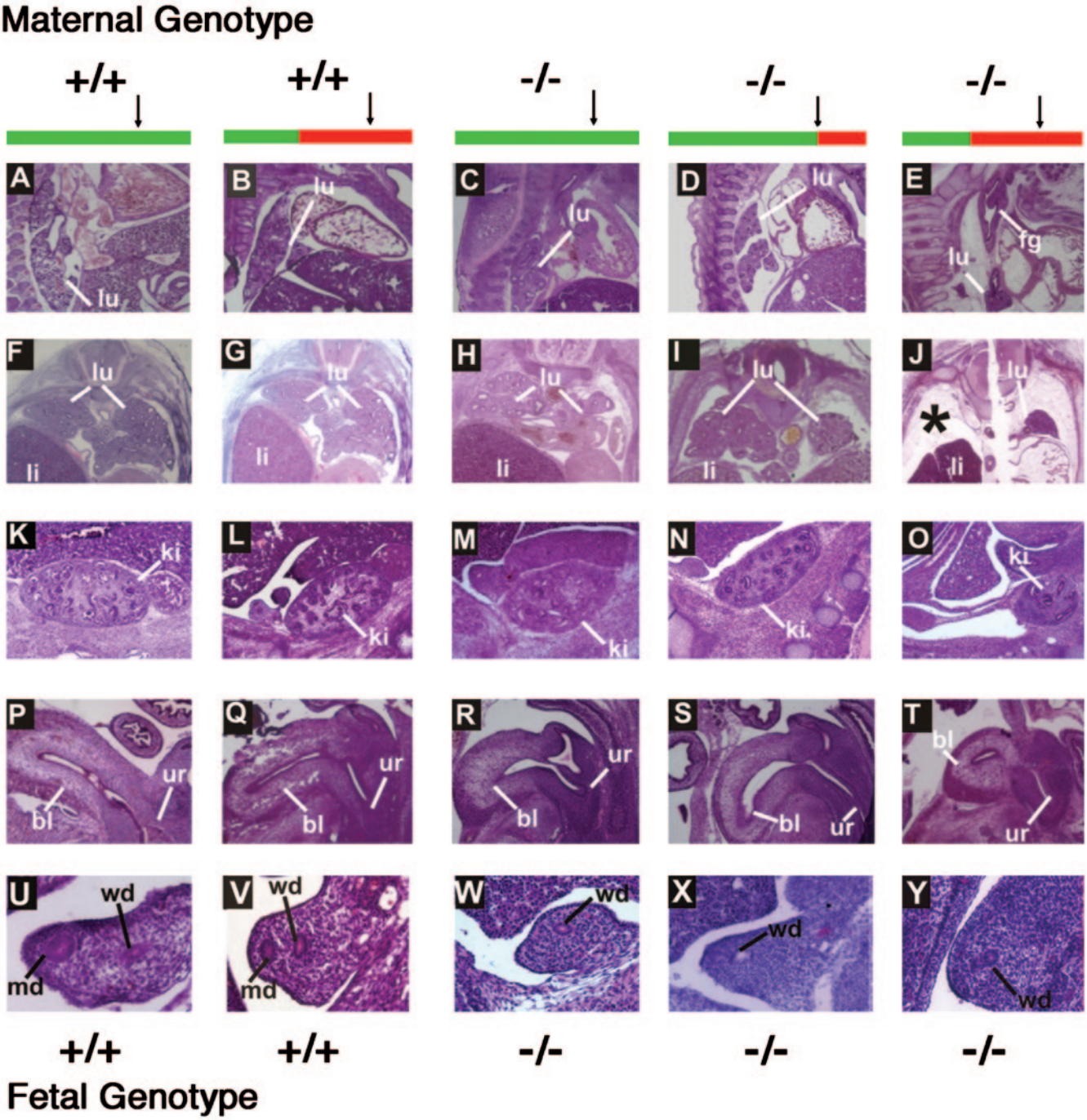

FIG. 3. Malformations induced by maternal dietary vitamin A deprivation in $RBP^{-/-}$ embryos. Shown are sections from E14.5 $RBP^{+/+}$ embryos from $RBP^{+/+}$ dams on a vitamin A-sufficient diet (A, F, K, P, and U) or a vitamin A-deficient diet for more than 5 wk before mating (B, G, L, Q, and V), and E14.5 $RBP^{-/-}$ embryos from $RBP^{-/-}$ dams on a vitamin A-sufficient diet (C, H, M, R, and W) or a vitamin A-deficient diet from 0.5 dpc (D, I, N, S, and X) or for more than 5 wk before mating (E, J, O, T, and Y). The *color code* of the *bars on the top of the panels* indicates the type of maternal dietary regimen according to the scheme in Fig. 1. The *arrow on top of each bar* indicates the beginning of gestation (0.5 dpc). The panels show formation of the lungs and trachea (A–E); transverse section showing the lobes of the lungs (F–J); formation of the kidney (K–O); formation of the bladder and urethra (P–T); and formation of the Mullerian duct (U–Y). *Asterisk* (J) indicates the lack of left lung. Bl, bladder; fg, foregut; ki, kidney; li, liver; lu, lungs; md, Mullerian duct; ur, urethra; wd, Wolffian duct; $^{+/+}$, RBP wild-type; $^{-/-}$, RBP knockout. Magnifications: A–J, ×40; K–T, ×100, U–Y, ×200.

dams bred on vitamin A-sufficient diet (group I as in Fig. 1), lung and tracheal formation was indistinguishable from controls ($RBP^{+/+}$ embryos from $RBP^{+/+}$ dams bred on vitamin A-sufficient or -deficient diet). The esophageal/tracheal septum formed, regularly spaced cartilage condensations were visible in the mesenchyme lining the trachea, and the lungs contained four lobes, three on the right and one on the left (Fig. 3, A–C and F–H). In $RBP^{-/-}$ embryos from $RBP^{-/-}$ dams maintained on vitamin A-deficient diet for more than 5 wk before onset of gestation (group V as in Fig. 1), the esophageal/tracheal septum failed to form, the mesenchyme lining the trachea was undifferentiated, the left lung failed to form, and the right lung was hypoplastic (Fig. 3, E and J). Malformations were also present in $RBP^{-/-}$ embryos from $RBP^{-/-}$ dams kept on vitamin A-deficient diet only during gestation (group II as in Fig. 1), but they were of a less severe nature. The esophagus and trachea separated and condensations that form the tracheal rings were present, but there spacing was irregular (data not shown). In addition, the lungs were smaller than in controls, suggesting that their formation was impaired (Fig. 3, D and I). Agenesis of the left lung is linked to failure in budding of the primary bronchus, and the overall size reduction (hypoplasia) is likely to be due to later defects during lung morphogenesis. Thus, severe vitamin A deficiency induced defects at both stages, whereas mild deficiency induced defects that were primarily at stages after lung budding.

Previous studies demonstrated that impaired vitamin A signaling results in malformations of the kidneys and ureters, including renal hypoplasia and ectopically ending ureters that join the lower urinary tract outside the bladder (3, 17, 40–42). We did not detect morphological abnormalities in the metanephric kidneys of embryos from control dams or $RBP^{-/-}$ embryos from $RBP^{-/-}$ dams bred on vitamin A-deficient diet from 0.5 dpc (group II as in Fig. 1) (Fig. 3, K–N). However, in $RBP^{-/-}$ embryos from $RBP^{-/-}$ dams maintained on vitamin A-deficient diet for more than 5 wk before mating (group V as in Fig. 1), renal development was severely disrupted (Fig. 3O). Fetal kidneys were small and hypoplastic, containing few nephrons and tubules and were in a pelvic rather than lumbar position (data not shown). This is a pattern of abnormalities nearly identical with those reported in VAD and retinoid receptor knockout mice (5, 13, 15, 17). In addition, severe VAD in this group also disturbed formation of the bladder and urethra, which were present but much smaller than wild-type counterparts (Fig. 3T).

Mullerian ducts are epithelial tubes that form alongside the Wolffian ducts, primordia of the male genital tract. During sexual differentiation, which occurs at about E15, Mullerian ducts regress in males and persist in females, differentiating into the uterus and upper portion of the vagina. VAD and retinoid receptor knockout animals display either complete or partial agenesis of the Mullerian ducts, resulting in agenesis of the uterus or both the uterus and upper vagina, respectively (3). In wild-type females at E14.5, sexual differentiation has not yet begun, and both Mullerian ducts and Wolffian ducts are present (Fig. 3, U and V). An interesting finding was that differentiation of the Mullerian ducts was aberrant in $RBP^{-/-}$ embryos, regardless of the maternal diet. Defects ranged from delayed formation in female embryos from $RBP^{-/-}$ dams bred on vitamin A-sufficient diet to absence or hypoplasia in female embryos from $RBP^{-/-}$ dams maintained on a vitamin A-sufficient diet during pregnancy or for more than 5 wk before onset of gestation (Fig. 3, W–Y).

Our analysis revealed a number of other phenotypes that display a similar sensitivity to VAD, including malformations of the eyes, heart, uterus, and vagina and other tissues (data not shown), all of which have been described in VAD studies in rodents and retinoid receptor knockout mice (5, 13, 15, 17, 43).

Viability and skeletal anomalies of the malformed embryos. We observed that $RBP^{-/-}$ dams maintained on any of the vitamin A-deficient dietary regimens described above completed parturition successfully. Whereas heterozygous newborns from $RBP^{-/-}$ dams in groups II-V survived to adulthood on a vitamin A-sufficient diet, the knockout newborns from $RBP^{-/-}$ dams in groups II-V and the heterozygous from $RBP^{-/-}$ dams in group VI died shortly after umbilical separation. Within a few minutes, the newborn showed signs of respiratory distress and shortly turned cyanotic and died. Animals with the most severe external malformations had the briefest life spans.

Alcian blue and alzarin red staining was performed to analyze the skeletal patterning of these newborns (35). We observed malformation that varied from retarded ossification ($RBP^{-/-}$ embryos from $RBP^{-/-}$ dams in group II; data not shown) to severe abnormalities ($RBP^{-/-}$ embryos from $RBP^{-/-}$ dams in group V), similar to those described in animals bearing compound retinoid receptor knockouts (16). Figure 4 shows skeletal abnormalities in $RBP^{-/-}$ newborn from $RBP^{-/-}$ dams maintained on a vitamin A-deficient diet for more than 5 wk before mating (group V). Severe defects were observed in the midfacial region and rostral cranial base, consistent with the loss of midfacial structures described above at E14.5 and with their phenotype at birth (Fig. 4G). Most of the skeletal elements normally derived from the frontonasal mesectoderm (16) were deficient or absent. The medial portions of the frontal and nasal bones were lacking. The nasal capsule, and nasal septum, and and lamina cribriform could not be identified. The incisive (or premaxillar) and vomer bones were reduced in size. Many of the first pharyngeal arch-derived skeletal elements (44) were malformed. Maxillary, palatine, and alisphenoid bones were malformed or absent. The mandibular bone appeared abnormal. The skull vault caudal to the frontal region lacked supraoccipital bones and part of the parietal bone and showed reduction of the interparietal and exoccipital bones. Figure 4 also shows abnormalities of the axial skeleton that affected primarily the cervical region. Reduced size of the C1 and fusion of the neural arches of C2, C3, and C4 are shown. The sternum was malformed and distorted and the number of sternebrae was reduced. Malformation of the appendicular skeleton similar to those described in animals bearing retinoid receptor knockout (16) were present (data not shown).

Overall these data indicate that that fetal offspring from $RBP^{-/-}$ dams display a wide range of embryonic VAD phenotypes, dependent on the time span of maternal dietary vitamin A deprivation. Thus, $RBP^{-/-}$ mice represent a tunable model system for studying vitamin A deficiency-

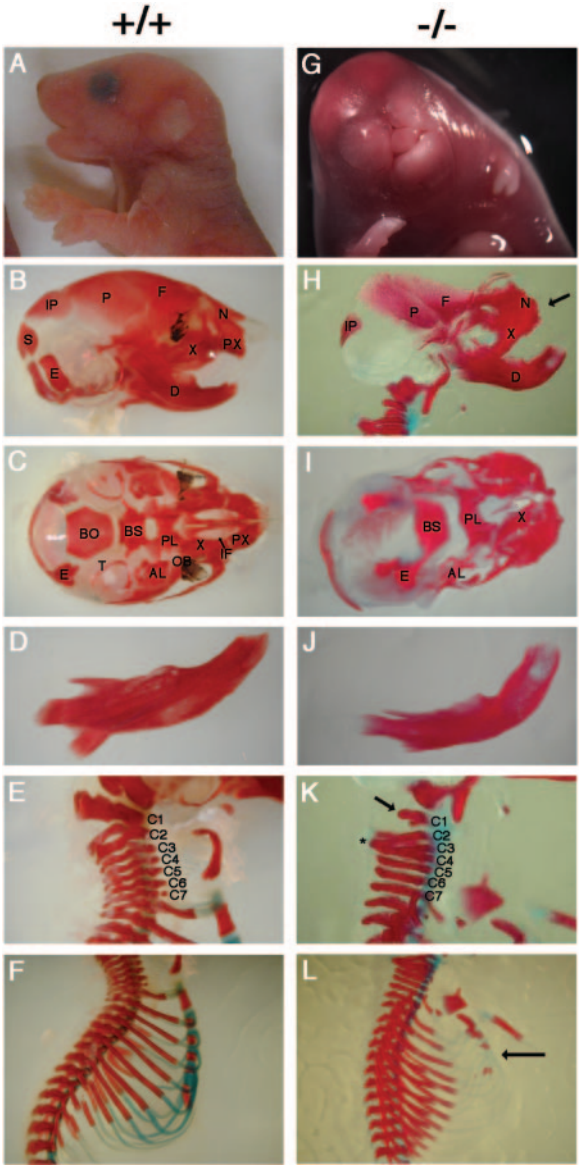

FIG. 4. Comparison of the craniofacial and axial skelton between 18.5 dpc wild-type and RBP knockout fetuses. Wild-type ($^{+/+}$) and RBP knockout ($^{-/-}$) fetuses were from dams, respectively, wild-type and knockout, maintained on a vitamin A-deficient diet for more than 5 wk before plug and throughout pregnancy. A and G, Gross morphology of the head and thoracic upper region. Note cleft face and palate, absence of the eyes, and abnormal forelimbs in RBP$^{-/-}$ fetuses. B and H, Lateral views of the skull. Note the complete lack of the cranial vault in the RBP$^{-/-}$ skull. C and I, Ventral views of the cranial base. Note the ossified fusion between the basioccipital and the exoccipital bones. D and J, Dentary (mandibular) bone. E and K, Lateral views of cervical region. F and L, Lateral views of thoracic region. AL, Alisphenoid; BO, basioccipital bone; BS, basisphenoid bone; D, mandibular (dentary) bone; E, exoccipital bone; F, frontal bone; IF, incisive foramen; IP, interparietal bone; N, nasal bone; OB, orbitosphenoid; P, parietal bone; PL, palatine bone; PX, incisive (premaxillary) bone; S, supraoccipital bone; T, timpanic bone; X, maxillary bone; C1 to C7, first to seventh cervical vertebrae. The *arrow* points to cleft (H) and to a reduction of the neural arch of the first cervical vertebra (K). The *asterisk* indicates fusion between the neural arches of the second and third and third and fourth cervical vertebrae. The *arrow* indicates the abnormal sternum (sternum distorted, reduced number of sternebrae, incompletely closed) (L). The same magnification was used for A and G, B and H, C and I, D and J, E and K, and F and L.

induced defects in organogenesis, which become increasingly severe as the extent of maternal dietary vitamin A deprivation increases. These data also indicate that, up to a certain limit, embryonic RBP can prevent malformations induced by maternal dietary vitamin A deficiency. In all cases, embryonic RBP rescues the embryos from early lethality.

Embryonic retinoic acid distribution

The variation in sensitivities to vitamin A deficiency observed in fetal eye and limbs (Fig. 2, F and G) suggests that some tissues are able to acquire, store, or metabolize vitamin A more efficiently than others. To address this question, we next determined how maternal dietary vitamin A deprivation affected the embryonic distribution of retinoic acid in superficial tissues such as eye, limb, skin, and spinal cord. We took advantage of a retinoic acid reporter mouse strain. In this strain, expression of a lacZ transgene is controlled by the retinoic acid response element of the RARβ2 promoter (32). LacZ expression in these animals is dependent on the local availability of retinoic acid. Impaired vitamin A signaling or vitamin A deficiency down-regulates RARβ2 promoter activity and thus reduces expression of the lacZ reporter (45). Vitamin A signaling in these superficial embryonic tissues containing retinoic acid is revealed by whole-mount staining for β-galactosidase with X-gal (32, 46). We crossed RBP$^{-/-}$ and lacZ transgenic mice and we generated RBP$^{+/-}$ progeny carrying two copies of the lacZ transgene (lacZ/RBP$^{+/-}$). These animals were crossed with RBP$^{-/-}$ and control (RBP$^{+/+}$ or RBP$^{+/-}$) females maintained on different regimens of dietary vitamin A deprivation, as described above. Figure 5 shows whole-mount X-gal staining of embryos from RBP$^{-/-}$ dams maintained on a vitamin A-sufficient diet (group I as in Fig. 1), a vitamin A-deficient diet during gestation (group II as in Fig. 1), or a vitamin A-deficient diet from weaning (group VI as in Fig. 1). E14.5 malformed RBP$^{-/-}$ embryos from RBP$^{-/-}$ dams on vitamin A-deficient diet from 0.5 dpc (Fig. 5, M–O) revealed a general and significant down-regulation of transgene activity (trunk, limb, craniofacial region, forebrain areas) when compared with RBP$^{+/-}$ littermates (Fig. 5, D–F) or E14.5 embryos from RBP$^{-/-}$ dams maintained on a vitamin A-sufficient diet (Fig. 5, A–C and J–L). A more marked down-regulation of transgene activity was observed in E14.5 malformed RBP$^{+/-}$ embryos from RBP$^{-/-}$ dams maintained on a vitamin A-deficient diet from weaning (Fig. 5, G–I).

These data indicate that depriving RBP$^{-/-}$ dams of dietary vitamin A reduces the amount of vitamin A delivered from the maternal circulation to the fetus. As a consequence, less vitamin A is available to the developing tissues to be converted to retinoic acid to maintain normal development.

Maternal vs. fetal contribution to transplacental delivery of vitamin A

At the time the females were killed, maternal serum was also collected. The levels of retinol and retinyl ester in the circulation of pregnant (midgestation) and nonpregnant wild-type and knockout females in groups II and VI was measured by reverse-phase HPLC (Table 2). Nonpregnant females under similar dietary regimen served as controls.

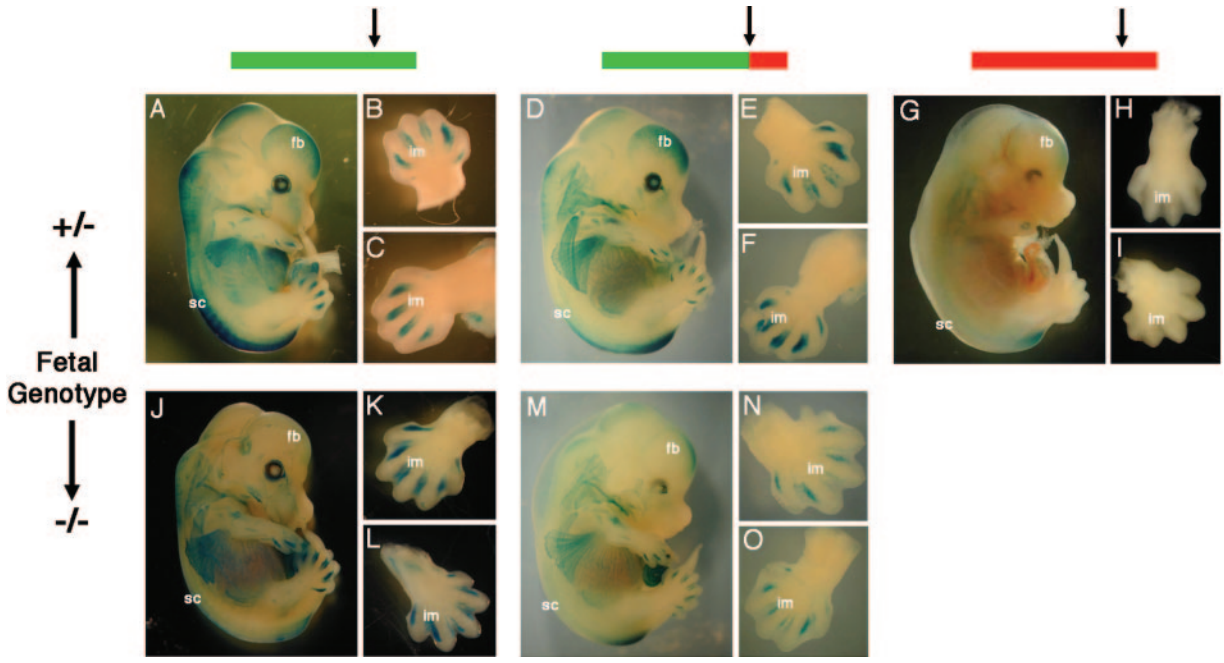

FIG. 5. Whole-mount X-gal staining of embryos from RBP$^{-/-}$ dams maintained under different regimens of dietary vitamin A deprivation. The *color code* of the *bars on the top of the panels* indicates the type of maternal dietary regimen according to the scheme in Fig. 1. The *arrow on top of each bar* indicates the beginning of gestation (0.5 dpc). Embryos were collected at E14.5 from RBP$^{-/-}$ females mated with lacZ/RBP$^{+/-}$ males. A, D, G, J, and M, Lateral view. B, E, H, K, and N, Forelimb. C, F, I, L, and O, Hindlimb. im, Interdigital mesenchime; fb, forebrain; sc, spinal cord. $^{+/-}$, RBP heterozygous; $^{-/-}$, RBP knockout. Images of littermate embryos are shown in A–C and J–L and D-F and M–O.

First, circulating retinol levels in nonpregnant wild-type females maintained on a vitamin A-deficient diet were unaffected by dietary vitamin A deprivation (Table 2) (29). During pregnancy their serum retinol dropped (in both groups II and VI), reflecting, at least in part, useof maternal circulating retinol by the fetus (26, 47). RBP$^{-/-}$ females on a vitamin A-deficient diet have very low serum retinol levels (26, 29), which do not decrease when dams are deprived of dietary vitamin A during pregnancy (group II). The concentration of serum retinyl ester is reduced in both wild-type and knockout group II females, as expected from animals fed a vitamin A-deficient diet. Taken together, these data confirm that RBP$^{-/-}$ dams deprived of dietary vitamin A during pregnancy deliver less vitamin A to the fetus. Interestingly, the low serum retinol levels in RBP$^{-/-}$ females were further reduced during pregnancy when the dams experienced severe dietary vitamin A deprivation (group VI). Serum retinyl ester could not be detected in wild-type and knockout dams maintained on a vitamin A-deficient diet from weaning (group VI). Overall, these data indicate that wild-type females maintain adequate levels of serum retinol, even under a severe regimen of dietary vitamin A deprivation (group VI). These retinol concentrations support normal embryonic development, regardless of the fetal genotype. In contrast, very little retinol crosses the placenta of the knockout dams under this regimen. These retinoid levels are insufficient to

TABLE 2. Serum retinol and retinyl ester levels in RBP wild-type and knockout pregnant and nonpregnant females under restricted regimens of dietary vitamin A intake

Maternal genotype	Serum retinol and retinyl ester levels (μg/dl)						
	Dams (group II)				Dams (group VI)		
	Retinol (mean ± SD)	n	Retinyl ester geometric mean (range)	n	Retinol (mean ± SD)	Retinyl ester geometric mean (range)	n$^{\alpha}$
+/+							
Nonpregnant	20.4 ± 6.1	12	1.1 (0.5–2.0)	8	19.8 ± 7.3	n.d.	8
Pregnant	4.9 ± 1.5^{b}	10	1.4 (0.5–3.5)	10	4.4 ± 1.4^{b}	n.d.	9
−/−							
Nonpregnant	0.5 ± 0.2	8	1.3 (0.7–4.3)	6	0.7 ± 0.4	n.d.	9
Pregnant	0.7 ± 0.1	8	1.5 (1.0–3.8)	8	0.3 ± 0.2^{c}	n.d.	10

Knockout and wild-type females in group II were maintained on a vitamin A-deficient diet during gestation; females in group VI were maintained on a vitamin A-deficient diet from weaning (see Fig. 1). Pregnant females were killed at midgestation. Age-matched nonpregnant knockout and wild-type females maintained on a vitamin A-deficient diet for a similar length of time served as control. Retinol and retinyl ester levels determined by reverse-phase HPLC. Statistical analysis by unpaired Student's *t* test. +/+, RBP wild-type; −/−, RBP knockout; n.d., nondetectable (<0.1 μg/dl). n, Number of 3-month-old female mice analyzed per group. n$^{\alpha}$, the number of 3-month-old females in group VI is the same for retinol mean and retinyl ester mean.

^{b} $P < 0.001$ and ^{c} $P = 0.01$ *vs.* the corresponding nonpregnant group.

support either normal development of heterozyogous fetuses or survival of knockout fetuses.

To estimate the concentration of fetal retinoid stores under various maternal dietary conditions, we measured vitamin A levels in liver and lung of newborn from groups II and VI dams (Table 3). We previously reported that liver and lung retinoid stores of newborn from $RBP^{-/-}$ dams maintained on a vitamin A-sufficient diet were equivalent to those of newborn from wild-type dams on a similar dietary regimen, regardless of the expression of RBP in the embryonic liver (26). These levels are also similar to those reported in Table 3 for newborn from wild-type dams in group II. In contrast, vitamin A stores of newborn from $RBP^{-/-}$ group II dams were significantly reduced. Importantly, this reduction was independent of fetal genotype or phenotype (Table 3).

Table 3 also shows total retinol levels in liver and lung of newborn from dams deprived of dietary vitamin A from weaning (group VI). Interestingly, total retinol levels in the stores of newborn from wild-type dams were significantly reduced, compared with those of newborn from wild-type dams in group II. Despite these low stores, newborn from wild-type dams were normal, regardless of their genotype. $RBP^{-/-}$ embryos from $RBP^{+/-}$ dams deprived of dietary vitamin A from weaning were also normal (data not shown). Note that total retinol levels in the stores of heterozygous newborns from $RBP^{-/-}$ dams in group VI were also very low. However, even though these newborn express RBP, they were malformed (Table 1).

Taken together, our data suggest that maternal circulating retinyl ester is the major source of fetal retinoid stores. Accumulation of these stores is independent of RBP expression in embryonic liver.

Discussion

The requirement of vitamin A for normal embryonic development is well established. However, the regulation of retinoid delivery to developing tissues is still poorly understood, especially under conditions of inadequate maternal dietary vitamin A intake. This issue is highly relevant to human health. According to the World Health Organization, vitamin A deficiency is a significant public health problem (48). Maternal subclinical vitamin A deficiency is endemic in low-income countries and is often associated with maternal mortality; congenital abnormalities such as ocular, cardiac, and urogenital defects; and low birth weight (49–51). Populations of developing countries and nutritionally disadvantaged populations in the industrialized world are also particularly at risk of developing subclinical vitamin A deficiency (52–54).

Vitamin A is found in the circulation as retinol bound to RBP and retinyl ester incorporated in lipoprotein of dietary origin. In this paper, we determine the contributions of these different maternal circulating retinoids to transplacental retinoid transfer under various regimens of maternal dietary vitamin A intake. We also probe the limits of vitamin A deficiency that the maternal-fetal system can tolerate before embryonic defects and lethality ensue. We accomplished this by using a novel mouse model of VAD. We demonstrated that simple modification of the dietary retinoid intake of $RBP^{-/-}$ dams generates a wide range of fetal malformations in $RBP^{-/-}$ embryos. Similar to retinaldehyde dehydrogenase 2 mutant mice and rat VAD models, $RBP^{-/-}$ embryos from $RBP^{-/-}$ dams subjected to the most severe dietary restrictions did not survive after 8.5–9.5 dpc, (Figs. 2 and 6C). However, when the extent of maternal dietary vitamin A deprivation was reduced, $RBP^{-/-}$ embryos survived up to term and showed a gradation in the severity of their VAD phenotypes (Figs. 2 and 3). The same dietary regimens had no effect on fetal development in $RBP^{+/+}$ or $RBP^{+/-}$ dams (Table 1). Moreover, with the exception of delayed Mullerian duct formation, all the VAD embryonic phenotypes were suppressed when $RBP^{-/-}$ dams were maintained on a vitamin A-sufficient diet from 0.5 dpc, regardless of the dietary history of the females before plug (data not shown). The delayed Mullerian duct formation suggests that development of this organ may require high vitamin A concentrations. The delay does not, however, affect the subsequent fertility of these females (Table 1). Thus, $RBP^{-/-}$ mice constitute a novel, tunable model of VAD, which enables one to investigate the role of vitamin A during embryonic development.

This model might be especially useful to extensively study vitamin A effects during later stages of organogenesis. After the early identification of VAD, studies of knockout mice with mutations in retinoid receptor genes (13–17) or genes coding for retinoic acid synthesizing or degrading enzymes

TABLE 3. Total retinol levels in liver and lung of newborns from RBP wild-type and knockout dams under restricted regimens of dietary vitamin A intake

Dams genotype	Newborns genotype	n	Newborns from dams in group II			n	Newborns from dams in group VI		
			Litter size	Total retinol levels			Litter size	Total retinol levels	
				Liver (μg/g)	Lung (μg/g)			Liver (μg/g)	Lung (μg/g)
+/+	+/+ or +/−	7	5 ± 2	7.6 ± 1.9	1.1 ± 0.4	4	7 ± 1	0.9 ± 0.4	0.2 ± 0.1
−/−	+/−	10	4 ± 2	4.8 ± 2.6[a]	0.6 ± 0.3[a]	3	4 ± 1	0.2 ± 0.1[a]	0.3 ± 0.1
−/−	−/−	7	7 ± 3	3.0 ± 0.6[b]	0.3 ± 0.1[b]	0	–	–	–

Three-month-old knockout and wild-type females were maintained on a vitamin A-deficient diet during pregnancy or from weaning (group II and VI as in Fig. 1). Wild-type females were mated with RBP heterozygous males. RBP knockout females were mated either with wild-type or with RBP knockout males. Retinol and retinyl ester levels in newborns tissues were determined by reverse-phase HPLC. Total retinol (retinol + retinyl ester) concentration is expressed as mean ± SD. Statistical analysis by Student's unpaired *t* test. +/+, RBP wild-type; +/−, RBP heterozygous; −/−, RBP knockout. n, Number of dams analyzed per each group. 0 indicates that no RBP−/− dams were analyzed in group VI since no newborns were expected. *Dashed lines* indicates that data are not available. The lower limit of detection of retinoid in our assay is 0.1 μg/g.

[a] $P < 0.03$ and [b] $P < 0.0003$ *vs.* the corresponding group of newborns from wild-type dams.

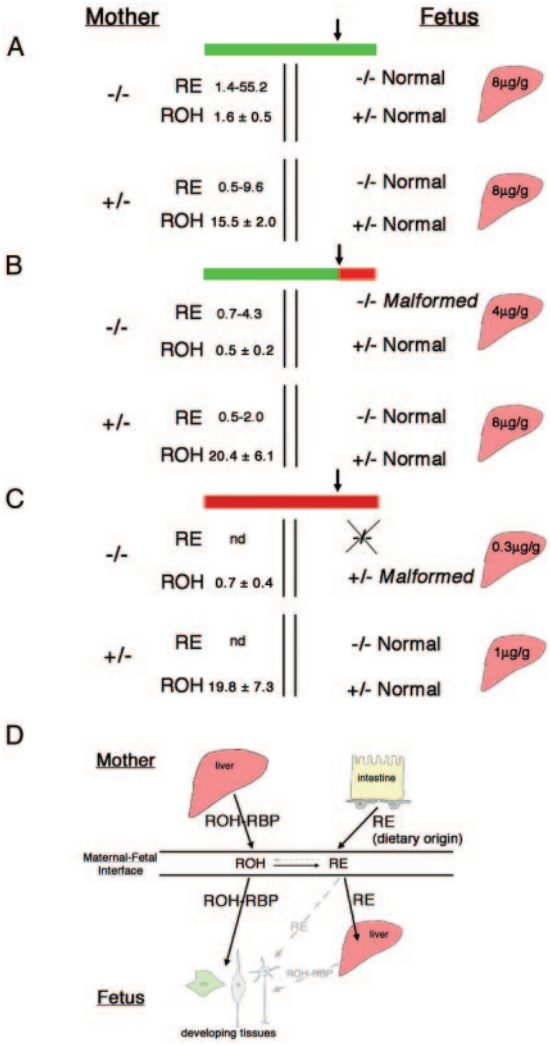

FIG. 6. Summary of the results and proposed model. A–C, Summary of the results of this study. The *color code* of the *bars on the top of the panels* indicates the type of maternal dietary regimen according to the scheme in Fig. 1. The *arrow on top of each bar* indicates the beginning of gestation (0.5 dpc). The *numbers on the left side of the panels* indicate the concentration of retinyl ester (RE; expressed as a geometric mean) and retinol (ROH; expressed as mean ± SD) circulating in the maternal bloodstream, according to the data reported in Table 2 and in a previous publication (26). nd, Nondetectable. The data refer to the levels of circulating retinoids at the beginning of the pregnancy (nonpregnant females). Note that no differences were observed in the levels of circulating retinoids levels between wild-type and heterozygous dams (data not shown). The *numbers on the right side of the panels* indicate the mean concentration of total retinol (retinol + retinyl ester) in the liver of the offspring (newborns), according to the data reported in Table 3. The genotype of dams and newborns ($^{-/-}$, RBP knockout; $^{+/-}$, RBP heterozygous) and the phenotype of the newborns are also indicated. D, Summary of our proposed model. It shows the pathways of vitamin A delivery from the maternal circulation to the developing tissues in wild-type animals. *Dashed gray arrows* indicate hypothetical pathways that might contribute to transplacental vitamin A transfer. The maternal-fetal interface in rodents includes tissues such as deciduas basalis and the yolk sac, which also express RBP (24).

(38, 55, 56) yielded significant insights into the molecular mechanisms of retinoid action during embryonic development. Rat models of maternal nutritional deprivation also demonstrated a crucial role of vitamin A in early events of embryonic development, including heart formation, neural crest cell differentiation, eye morphogenesis, and formation of the nervous system (43, 57, 58). An absolute vitamin A requirement in early embryogenesis was also demonstrated in an avian system. In this model, embryos deprived of vitamin A or its precursors from the beginning of fertilization develop gross abnormalities in the cardiovascular and central nervous system and trunk and die by d 3.5–4 of embryonic life (59). However, with these models, there are very few reports of embryos malformed that survived after midgestation.

Our studies also demonstrate, for the first time, the importance of RBP of fetal origin in maintaining embryonic organogenesis in the face of maternal vitamin A deficiency. Except under the most severe conditions of maternal dietary vitamin A deprivation, $RBP^{+/-}$ embryos developed normally, whereas their $RBP^{-/-}$ littermates did not. And, even under severe dietary vitamin A deprivation (Fig. 6C), $RBP^{+/-}$ embryos, although malformed, survived until birth, whereas their $RBP^{-/-}$ littermates were resorbed.

Vitamin A that reaches the fetus is used both to support embryonic development and accumulate vitamin A stores (47). We show that accumulation of hepatic stores is independent of fetal RBP genotype. Vitamin A levels in the livers of littermate $RBP^{-/-}$ and $RBP^{+/-}$ newborn were identical (Fig. 6, A and B), even when the $RBP^{-/-}$ fetus was malformed (Fig. 6B). In this respect, fetal hepatic retinoid metabolism mimics that of adult liver, *i.e.* RBP is required for neither storage of vitamin A nor its intrahepatic transport (60). These data imply that the principal role of fetal RBP is to distribute limiting vitamin A to target tissues.

These experiments also indicate that some developing tissues and organs, such as the Mullerian duct and the eye, for example, are more sensitive to reductions of vitamin A delivery across the maternal-fetal interface. As a consequence, these tissues must rely heavily on retinol-RBP, which represents an efficient and stable pathway of vitamin A delivery (20). Biochemical evidence suggests that a cell surface receptor mediates uptake of retinol bound to RBP in adult retinal pigment epithelium (61) and embryonic tissue [yolk sac; (62)]. To date, however, the cloning of such a receptor has not been described.

Maternal dietary vitamin A deprivation influences both the amount and the type of retinoid circulating in the maternal bloodstream of wild-type and $RBP^{-/-}$ strains. In wild-type or heterozygous dams on a vitamin A-deficient diet, circulating retinyl ester progressively declined to undetectable levels with prolonged dietary vitamin A deprivation. In contrast, maternal serum retinol-RBP levels were unaffected by these diets (Fig. 6, A–C). Despite normal levels of retinol-RBP, heterozygous dams maintained on a vitamin A-deficient diet since weaning generated newborns that although morphologically normal, had significantly reduced hepatic retinoid stores (1 μg/g *vs.* 8 μg/g; Fig. 6C *vs.* 6A), regardless of their genotype. These findings imply that embryonic development predominantly relies on the maternal retinol-RBP pathway, whereas accumulation of fetal retinoid stores uses principally retinyl ester (Fig. 6D). It makes perfect sense that embryonic development, which is a crucial biological function, relies on a delivery system (etinol-RBP) that is not de-

pendent on dietary vitamin A availability. In the wild, the amount of dietary vitamin A can vary dramatically in different seasons. Recall that vision, another crucial function in higher vertebrates, relies principally on retinol-RBP rather than retinyl ester delivery (63).

Maternal retinol-RBP is so effective in maintaining normal embryonic development because it ensures delivery of adequate and steady amount of retinoid at the maternal-fetal interface, despite dietary vitamin A deprivation (Fig. 6, A–C). In $RBP^{+/-}$ embryos, maternal retinol may be transferred to fetal RBP at this interface. From here, retinol-RBP can be delivered directly to developing tissues. We cannot exclude the possibility that some retinol is esterified in the placenta (64). This retinyl ester might be released into the fetal circulation incorporated in lipoprotein (65) to be delivered to developing tissues (Fig. 6D). We argue that maternal retinyl ester may contribute to embryonic development in wild-type mice but that this contribution is not crucial. Indeed, when maternal circulating retinyl ester levels are undetectable (Fig. 6C), newborns from wild-type dams are phenotypically normal, regardless of their genotype. However, the retinoid stores of these newborns are significantly reduced. In the case of RBP-deficient embryos from heterozygous mothers, we speculate that retinol from the maternal circulation might be esterified in the placenta (64). The resultant retinyl ester might be released into the fetal circulation incorporated in lipoprotein (65) to be delivered to developing tissues or liver for storage. Some retinol might circulate in $RBP^{-/-}$ fetuses bound to albumin, as it does in adult $RBP^{-/-}$ mice (29). Although individually less efficient, these two pathways together overcome the lack of fetal RBP.

In the absence of maternal RBP, circulating retinyl ester, which is found at significantly elevated levels, represents the main pathway available for embryonic development and accumulation of fetal retinoid stores (26). We cannot exclude the possibility that residual retinol, bound to albumin in the serum of $RBP^{-/-}$ dams (29) crosses the maternal-fetal interface and is released into the fetal circulation bound to albumin or in $RBP^{-/-}$ embryos or bound to RBP in $RBP^{+/-}$ embryos. However, the exact contribution of this pathway to embryonic development remains to be established. In the case of $RBP^{+/-}$ fetuses, as it is in adults, we also expect that hepatic RBP mobilizes stored vitamin A to deliver retinol to target tissues (20). Hydrolysis of retinyl ester at the maternal-fetal interface has never been reported. If it occurred, retinol from this source might bind to RBP synthesized in the yolk sac to be released into the fetal circulation.

$RBP^{-/-}$ embryos rely on less efficient pathways of vitamin A delivery to developing tissues (retinyl ester and retinol-albumin) than wild-type embryos. Thus, when circulating retinyl ester levels in $RBP^{-/-}$ dams decrease on dietary vitamin A deprivation from the beginning of gestation, only the development of knockout embryos was impaired (Fig. 6B). We propose that embryonic RBP promotes: 1) more efficient uptake of retinol from the maternal circulation; 2) efficient delivery to tissues that acquire retinoid principally from retinol-RBP, especially when maternal circulating retinoid levels are limiting; and 3) mobilization of hepatic vitamin A stores. Under the most restrictive dietary regimen, maternal circulating retinoid in $RBP^{-/-}$ dams is so limiting that fetal RBP can overcome embryonic lethality but cannot ensure proper embryonic development (Fig. 6C). As expected, hepatic vitamin A levels of these $RBP^{+/-}$ newborns were extremely low (Table 3 and Fig. 6C).

Thus, $RBP^{-/-}$ dams severely deprived of dietary vitamin A (Fig. 6C) mimic the status of a vitamin A-deficient pregnant woman, characterized by lack of circulating retinyl ester of dietary origin and very low levels of serum retinol. By adjusting the extent of dietary vitamin A deprivation, we have established the first model system to analyze the metabolic links between maternal nutrition and incidence and type of developmental abnormalities.

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