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Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes

(cholesterol/gene targeting/embryonic development/exencephalus)

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ABSTRACT **Apolipoprotein B is synthesized by the intestine and the liver in mammals, where it serves as the main structural component in the formation of chylomicrons and very low density lipoproteins, respectively. Apolipoprotein B is also expressed in mammalian fetal membranes. To examine the consequences of apolipoprotein B deficiency in mice, we used gene targeting in mouse embryonic stem cells to generate mice containing an insertional disruption of the 5′ region of the apolipoprotein B gene. Mice that were heterozygous for the disrupted apolipoprotein B allele had an ≈20% reduction in plasma cholesterol levels, markedly reduced plasma concentrations of the pre-β and β-migrating lipoproteins, and an ≈70% reduction in plasma apolipoprotein B levels. When fed a diet rich in fat and cholesterol, heterozygous mice were protected from diet-induced hypercholesterolemia; these mice, which constitute an animal model for hypobetalipoproteinemia, should be useful for studying the effects of decreased apolipoprotein B expression on atherogenesis. The breeding of heterozygous mice yielded no viable homozygous apolipoprotein B knockout mice. Most homozygous embryos were resorbed by midgestation (before gestational day 11.5); several embryos that survived until later in gestation exhibited exencephalus. The embryonic lethal phenotype was rescued by complementation with a human apolipoprotein B transgene—i.e., human apolipoprotein B transgenic mice that were homozygous for the murine apolipoprotein B knockout mutation were viable. Our findings indicate that apolipoprotein B plays an essential role in mouse embryonic development.**

The B apolipoproteins, apolipoprotein B48 (apo-B48) and apo-B100, serve as the main structural components of lipoproteins secreted by mammalian liver and intestine, where they are required for the synthesis of very low density lipoproteins (VLDL) and chylomicrons, respectively (1). Apo-B100, the full-length protein, is synthesized primarily by the liver. Apo-B48, which comprises the amino-terminal 48% of apo-B100, is produced by posttranscriptional editing of the apo-B mRNA, which converts the codon for Gln-2153 to a stop codon. Apo-B48 is synthesized in the intestine of all mammalian species thus far examined (2); some mammals, including mice, also have editing activity in the liver. In addition to hepatic and intestinal expression, apo-B expression has been observed in mammalian fetal membranes (3–5), although the physiologic purpose for apo-B expression in this tissue has remained obscure.

Currently, no animal models exist in which apo-B is absent. However, an apo-B-deficient mouse that synthesizes a truncated apo-B, apo-B70, was generated by Homanics *et al.* (6) by using gene targeting in mouse embryonic stem (ES) cells. These mice had reduced apo-B mRNA levels in the intestine and the liver and had reduced plasma levels of apo-B, cholesterol, and triacylglycerols. Unexpectedly, some apo-B70 homozygous mice exhibited exencephalus or hydrocephalus. Because these neurologic developmental abnormalities had not been observed in the human apo-B-deficiency states of familial abetalipoproteinemia or hypobetalipoproteinemia (7), it was unclear whether these abnormalities were a direct consequence of diminished apo-B expression in the embryos or were an indirect result of the multiple copies of the sequence-insertion vector that integrated into the apo-B locus (e.g., a toxic effect of the inserted sequences on the expression of a nearby gene).

To determine the physiologic consequences of an apo-B knockout in mice and to create an animal model for studying the effects of hypocholesterolemia on atherogenesis, we sought to knock out the mouse apo-B gene by using gene targeting in mouse ES cells. In this study, we report the generation of apo-B knockout mice by an insertional disruption of the 5′ region of the mouse apo-B gene, and we describe the phenotypic consequences of this disruption in heterozygotes and homozygotes.

MATERIALS AND METHODS

Construction of the Targeting Vector. A neomycin-resistance gene (*neo*) containing the RNA polymerase II promoter and a herpes simplex virus thymidine kinase (*tk*) polyadenylylation signal (provided by K. Thomas, University of Utah) was subcloned into the *Hin*dIII site of plasmid pBluescript SK II(+) (Stratagene). Next, a 6-kb *Sac* I fragment from pB2A30 (a plasmid clone encompassing the 5′ region of the mouse apo-B gene that was isolated from a mouse strain B10.A genomic library) that begins 44 bp 3′ of the transcriptional start site and encompasses exons 1–4 of the mouse apo-B gene was subcloned into the *Sac* I site of the vector. The vector was linearized at a unique *Sfi* I site located ≈1 kb upstream of the 3′ *Sac* I site.

ES Cell Culture and Generation of Chimeric Mice. Culture and electroporation of AB1 ES cells (provided by A. Bradley, Baylor College of Medicine, Houston) were as described (8, 9). For Southern blotting, DNA prepared from G418-resistant ES cell clones grown on 24-well plates (10) was digested with *Eco*RI and size-fractionated by agarose gel electrophoresis; the DNA was then transferred and crosslinked to a nylon membrane. The blots were probed with a ^{32}P-labeled, 1.1-kb *Eco*RI–*Hin*dIII fragment located 5′ of the vector sequences

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Abbreviations: apo-, apolipoprotein; ES, embryonic stem; VLDL, very low density lipoprotein(s); LDL, low density lipoprotein(s); HDL, high density lipoprotein(s); IDL, intermediate density lipoprotein(s).

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(Fig. 1*A*). ES cells (10–15) from targeted clones were microinjected into C57BL/6 blastocysts as described (11). Male chimeric offspring were bred with C57BL/6 and 129/Sv female mice to achieve germline transmission.

Plasma Lipid and Lipoprotein Analyses. Lipid measurements were performed on 3- to 6-week-old mice (129/Sv × C57BL/6) consuming either a chow diet (PicoLab Mouse Chow 20, no. 5058, Purina) or a high-fat synthetic diet containing 50% sucrose, 18.45% butter, 1.25% cholesterol, and 0.5% sodium cholate (ICN). Plasma cholesterol and triacylglycerol levels were measured from 4-hr fasted mice [as recommended (12)] with enzymatic kits (Spectrum Cholesterol, Abbott; Triglycerides/GB, Boehringer-Mannheim). High density lipoprotein (HDL) cholesterol was measured after a polyethylene glycol precipitation of the apo-B-containing lipoproteins (13). Fast protein liquid chromatography (FPLC; Pharmacia) was performed with pooled plasma samples (50 μl) from four to six mice as described (14). Plasma lipoproteins were analyzed by agarose gel electrophoresis (15). In some instances, the separated lipoproteins were transferred to nitrocellulose membranes for immunoblotting with an ^{125}I-labeled rabbit antibody specific for rat apo-B (provided by R. Davis, San Diego State University). Bands on immunoblots were quantified with a gel scanner (AMBIS Systems).

Analysis of Embryos. Embryos were harvested from timed matings of heterozygote intercrosses (16). Genotyping of embryos was performed by Southern blotting of DNA isolated from fetal membranes.

For immunoblotting, fetal membranes were homogenized in 200 μl of a homogenization buffer (17). Protein samples (≈30 μg) were subjected to SDS/PAGE in 3–12% gradient gels, and the size-fractionated proteins were transferred to nitrocellulose membranes for immunoblotting. To detect mouse apo-B, a rabbit antiserum specific for mouse apo-B (provided by H. Hobbs, University of Texas Southwestern Medical Center) was used; the binding of this antiserum was detected with ^{125}I-labeled goat anti-rabbit IgG. To detect human apo-B, the monoclonal antibody 1D1 (18) conjugated to horseradish peroxidase was used; the binding of this antibody was detected with an enhanced chemiluminescence (ECL) kit (Amersham).

RESULTS

Targeting of the Mouse Apo-B Gene in ES Cells. To disrupt the 5′ region of the 29-exon mouse apo-B gene, we used a sequence-insertion vector containing ≈6 kb of the mouse apo-B gene (Fig. 1*A*). The insertion of this vector by homologous recombination is predicted to interrupt the coding sequence after exon 4 and result in the synthesis of a truncated apo-B protein that would be <2% of the full-length apo-B100. Although the insertion of this vector into the apo-B locus results in a downstream duplication of a portion of exon 1 and exons 2–4, all mRNA splicing events from the endogenous exon 4 to exons within the vector result in frameshifts. Furthermore, because the vector sequences lack the 5′ portion of exon 1 (including the transcriptional start site), the initiation of transcription from the duplicated (vector) sequences is not possible.

Approximately 450 G418-resistant ES cell clones were screened by Southern blotting and 18 targeted clones were identified by the presence of a 12.9-kb *Eco*RI restriction fragment (Fig. 1*B*); the targeting frequency was ≈1 in 25. [We used the identical apo-B sequences to construct a series of sequence-replacement vectors in which exon 2 was interrupted with a *neo* gene. Screening of >1,200 G418- and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil-resistant ES cell clones identified none that were targeted. However, using isogenic DNA, another investigator (Li-Shin Huang, personal communication) has used a sequence-replacement vector to knock out the mouse apo-B gene.] Targeted clones used for blastocyst injection were verified by Southern blotting using a 3′ flanking probe and a *neo* probe (data not shown). Several of the targeted clones were microinjected into C57BL/6 blas-

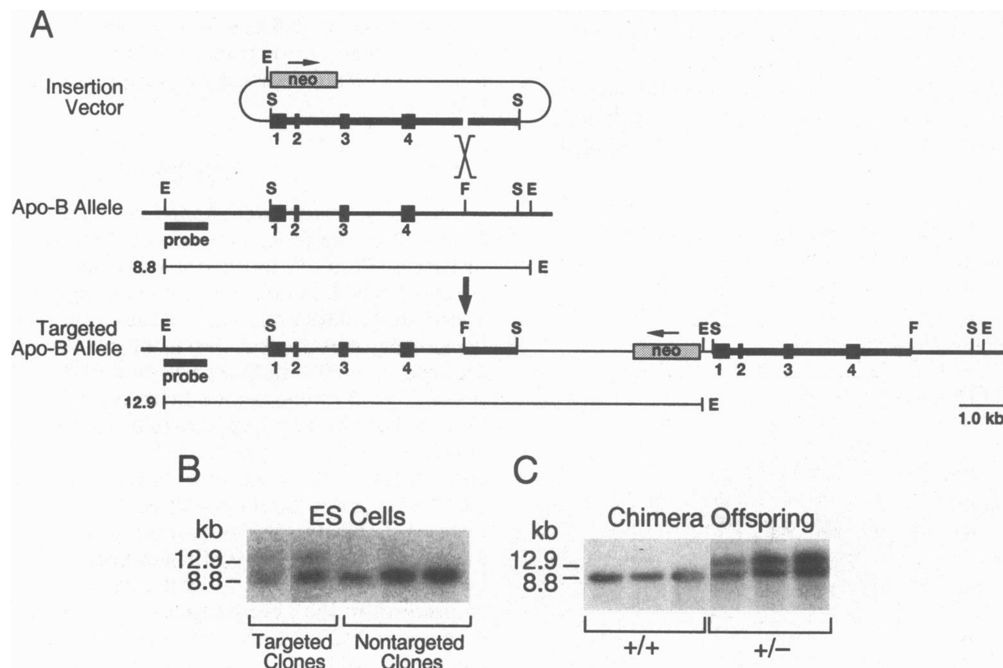

FIG. 1. Targeted disruption of the apo-B gene in mouse ES cells and mice. (*A*) Scheme for disrupting the mouse apo-B allele, using a sequence-insertion vector. The homologous recombination of the *Sfi* I-linearized vector with the cognate apo-B allele disrupts the allele by interrupting the apo-B coding sequence after exon 4. Restriction sites: E, *Eco*RI; S, *Sac* I; F, *Sfi* I. (*B* and *C*) Southern blot analysis demonstrating targeted ES cell clones and mice that are heterozygous for the apo-B knockout mutation. Genomic DNA (≈10 μg) was digested with *Eco*RI. The 1.1-kb *Eco*RI–*Hin*dIII fragment used as a probe detects an 8.8-kb *Eco*RI restriction fragment in a wild-type allele and a 12.9-kb fragment in a targeted allele.

tocysts, and one yielded germline-transmitting chimeras (Fig. 1*C*).

Apo-B Knockout Heterozygotes. Mice that were heterozygous for the apo-B gene disruption appeared normal. Analysis of plasma samples of heterozygous mice by agarose gel electrophoresis revealed that they had reduced plasma concentrations of the β- and pre-β-migrating lipoproteins (Fig. 2*A*). Although this was a consistent finding, some variability in the degree of reduction of these lipoproteins was observed. Immunoblots of the agarose gels with an ^{125}I-labeled apo-B-specific antibody revealed that heterozygotes had markedly diminished apo-B concentrations ($\approx$70% reduction as revealed by gel scanning) in the plasma β- and pre-β-migrating lipoproteins (Fig. 2*B*).

Analysis of plasma lipid levels of mice consuming a chow diet demonstrated that the heterozygotes had a 19% reduction in the total plasma cholesterol level as compared with wild-type mice (81 ± 18 vs. 104 ± 17 mg/dl, $P < 0.001$) (Fig. 3*A*). This decrease could be largely accounted for by a 21% reduction in the HDL cholesterol level (60 ± 18 vs. 76 ± 21 mg/dl in wild-type mice, $P < 0.001$). The non-HDL cholesterol level was also lower in the heterozygotes (22 ± 8 vs. 27 ± 12 mg/dl in the wild-type mice, $P = 0.057$); this decrease presumably contributed to the decrease in the total plasma cholesterol level, although to a lesser degree. Plasma triacylglycerol levels did not differ in the heterozygous and wild-type mice. Plasma lipoprotein analysis by FPLC confirmed the decrease of cholesterol in the HDL-sized lipoproteins in the heterozygotes (Fig. 3*B*). In the VLDL-, IDL-, and LDL-sized lipoproteins, both heterozygous and wild-type mice had low levels of cholesterol, and no differences were discernible.

To determine whether disrupting one apo-B allele would have a protective effect against diet-induced hypercholesterolemia, we fed female heterozygous and wild-type mice a diet rich in fat and cholesterol. As expected, wild-type mice approximately doubled their plasma cholesterol (from 104 ± 17 to 229 ± 29 mg/dl) after 6 weeks of high-fat feeding (Fig. 3*A*). Although the plasma cholesterol level also increased in the heterozygotes (from 81 ± 18 to 152 ± 15 mg/dl), their total plasma cholesterol levels on the high-fat diet were 34% lower than those of the wild-type mice. Plasma lipoprotein analysis by FPLC showed that the disrupted apo-B allele largely prevented the diet-induced increase in the apo-B-containing lipoproteins (VLDL, IDL, and LDL) that normally occurs in mice consuming a high-fat diet (Fig. 3*B*).

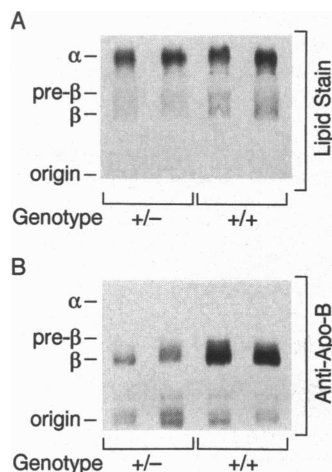

FIG. 2. Agarose gel electrophoresis of plasma lipoproteins from apo-B knockout heterozygotes and wild-type mice. (*A*) Agarose gel stained for lipids with Fat Red 7B. Aliquots of mouse plasma (2.5 μl) were used for electrophoresis. (*B*) Anti-apo-B immunoblot of an identical agarose gel. Immunoblotting was performed with an ^{125}I-labeled rabbit antibody specific for rat apo-B. The less dense bands at the origin represent nonspecific binding of the antibody.

Apo-B Knockout Homozygotes. Apo-B knockout heterozygotes were intercrossed to determine the phenotype of homozygotes. Of the 179 mice screened at age 3 weeks by Southern blotting, no homozygotes were identified (Table 1). Two of three pups that were found dead at birth proved to be homozygotes: one had a cranial defect and an empty cranial cavity (consistent with exencephalus and subsequent cannibalization by the mother), and one was grossly normal. The apo-B knockout mutation was also established in a pure 129/Sv genetic background, and intercrosses of heterozygotes in this inbred strain also yielded no viable homozygotes (data not shown).

To determine the approximate stage of embryonic development that was affected by the apo-B knockout mutation, the genotypes of 208 embryos (gestational days 9.5 to 20) from heterozygous intercrosses were analyzed. When compared with the expected Mendelian ratio, the number of homozygous embryos that appeared to be viable was reduced at the 10.5-day time point and afterward (Table 1). At gestational day 9.5, the total number of homozygous embryos was close to the predicted number, but all the homozygous embryos were either runted or appeared to be nonviable and in the process of resorption. The few viable homozygous embryos that we observed after gestational day 10.5 appeared to be exencephalic (Fig. 4). Immunoblots of fetal membranes from 10.5-day homozygous embryos demonstrated no detectable apo-B protein (Fig. 5*A*).

To determine whether the embryonic lethal phenotype could be rescued by a human apo-B transgene, we crossed the apo-B knockout heterozygotes with human apo-B transgenic mice (19). Mice that were both hemizygous for the human apo-B transgene and heterozygous for the apo-B knockout mutation were generated and intercrossed. Southern blot analysis of the offspring revealed many human apo-B transgenic mice that were homozygous for the knockout mutation. Thus, the human apo-B transgene prevented the lethal developmental abnormalities caused by the absence of murine apo-B. Immunoblots of 10.5-day fetal membranes isolated from matings of human apo-B transgenic mice demonstrated that the human apo-B100 transgene was expressed in fetal membranes (Fig. 5*B*).

DISCUSSION

In this study, we used a sequence-insertion vector to disrupt the 5′ region of the mouse apo-B gene and found that this gene disruption resulted in phenotypic consequences in both heterozygotes and homozygotes. In heterozygotes, disrupting one apo-B allele had significant consequences on several aspects of lipoprotein metabolism. First, the plasma apo-B levels were reduced by $\approx$70% in heterozygotes. This >50% reduction in plasma apo-B levels was not unexpected, however, inasmuch as human hypobetalipoproteinemia subjects frequently have apo-B levels that are reduced by 60–80% (7). Total plasma cholesterol levels were also reduced in the heterozygotes, albeit to a lesser extent ($\approx$20%). Because the mouse carries most of its plasma cholesterol on non-apo-B-containing particles (i.e., HDL), this result was also not unexpected. However, the finding that most of the decrease in the total plasma cholesterol in the heterozygotes could be accounted for by a decrease in the HDL cholesterol was surprising. Although the metabolism of HDL and that of apo-B-containing particles are known to be closely interrelated (1), the mechanism for the reduction in the HDL cholesterol in the heterozygotes is not understood. A similar decrease in plasma HDL cholesterol levels was observed in apo-B70 homozygous mice (6).

When the heterozygotes were fed a diet rich in fat and cholesterol, protection against developing diet-induced hyper-

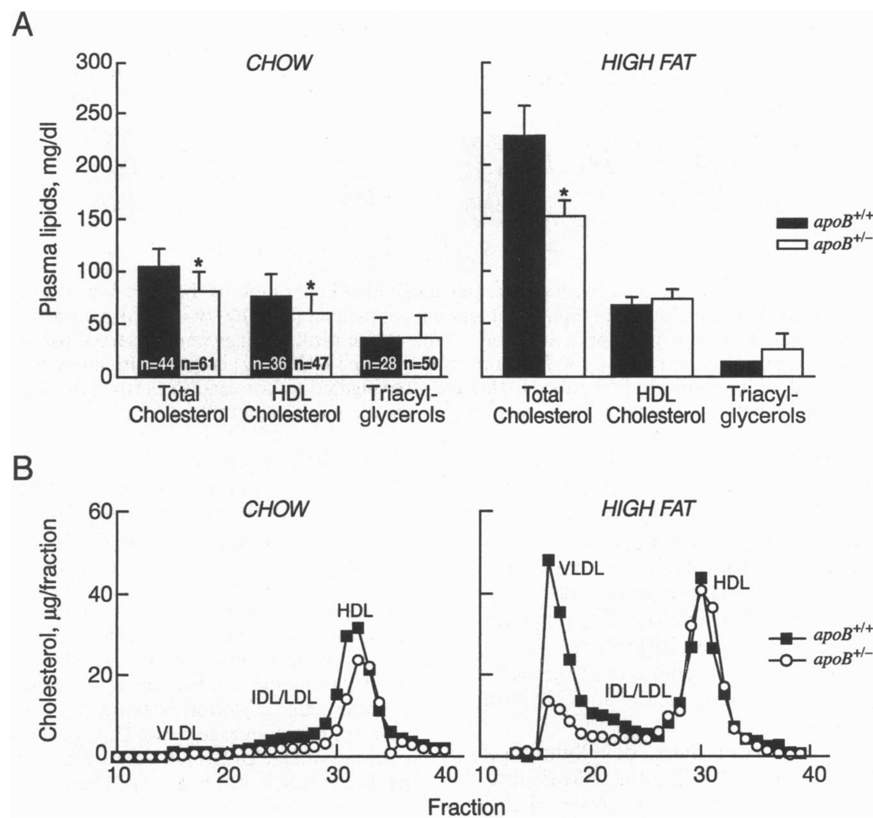

FIG. 3. Plasma lipid analyses of apo-B knockout heterozygotes ($apoB^{+/-}$) and wild-type ($apoB^{+/+}$) mice consuming either a chow or a high-fat diet. (*A*) Plasma lipid concentrations. Significant decreases ($P < 0.001$, unpaired t test) between the heterozygous and wild-type mice are denoted by asterisks. For the high-fat experiments, six female mice were analyzed for each genotype. (*B*) FPLC profiles of the plasma lipoproteins. For each profile, samples (50 μl) of plasma from four to six fasted female mice were pooled and subjected to gel filtration by FPLC, and the cholesterol content of each fraction was determined. IDL, intermediate density lipoprotein; LDL, low density lipoprotein.

cholesterolemia was observed (Fig. 3). The mechanism(s) involved in this protective effect is not understood. One possibility is that decreased apo-B synthesis in the intestine results in decreased cholesterol absorption, which in turn could cause an upregulation of hepatic LDL receptors and enhanced clearance of apo-B-containing lipoproteins from the plasma. Alternatively, the diminished hypercholesterolemic response could simply relate to decreased synthesis of apo-B-containing lipoproteins by the liver. Whatever the mechanism, our findings demonstrate that the diminished expression of a protein involved in lipoprotein synthesis can partially prevent the hypercholesterolemic response to a high-fat diet. Similar findings have been reported in mice with genetic alterations designed to accelerate lipoprotein clearance (20, 21).

Previously, Homanics *et al.* (6) generated gene-targeted mice that synthesized low levels of a truncated apo-B, apo-B70; the apo-B70 homozygous mice frequently exhibited hydrocephalus or exencephalus, suggesting a possible role for apo-B in mouse development. In the present study, we observed a phenotype for apo-B knockout mice that appeared similar to, but more severe than, that observed in the apo-B70 homozygous mice: apo-B knockout homozygous mice were not viable, and most homozygous embryos underwent resorption by 11.5

Table 1. Results of apo-B heterozygote matings

Age	No. of embryos or pups	Genotype +/+	+/−	−/−*
Prenatal				
9.5 days	73	21	34	18 (11)†
10.5 days‡	69	15	43	11 (6)†
11.5–13.5 days‡	40	13	23	4 (1)†
15.5–20 days	26	5	20	1 (1)
Postnatal				
Dead at birth	3	1	0	2§
3 weeks	179	59	120	0

DNA was isolated from fetal membranes or tail tips and analyzed by Southern blotting as described in *Materials and Methods.*

*For embryos, both the total number and the number of these that appeared to be viable (in parentheses) are shown.

†Two −/− resorptions were also identified.

‡$P < 0.01$ for the difference between the number of viable embryos in each genotype and a 1:2:1 (+/+:+/−:−/−) distribution (χ^2 analysis).

§One pup was exencephalic and one was grossly normal.

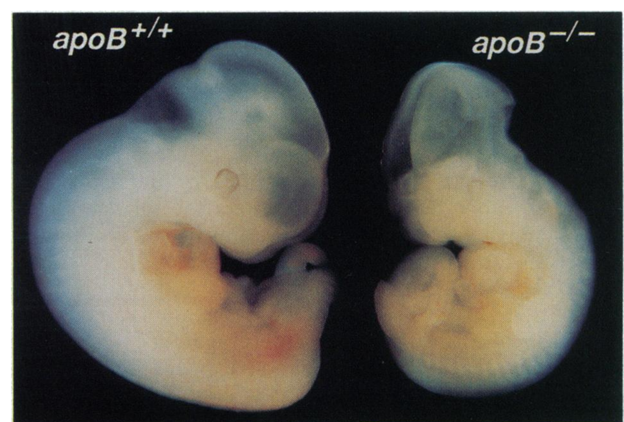

FIG. 4. Homozygous apo-B knockout embryo (11.5 days of gestation) demonstrating exencephalus. Embryos were harvested from intercrosses of heterozygous apo-B knockout mice.

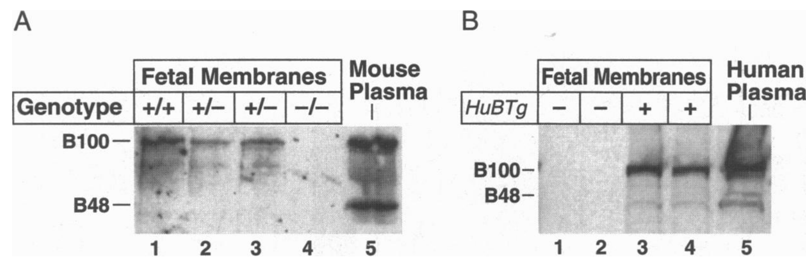

FIG. 5. Apo-B immunoblots of 10.5-day fetal mouse membranes. (*A*) apo-B knockout embryos. Fetal membrane homogenates were prepared as described in *Materials and Methods*. Immunoblotting of equivalent amounts of protein (≈30 μg) was performed with a rabbit antiserum specific for mouse apo-B. The results from one homozygous embryo are shown (lane 4); identical findings were observed for other homozygous embryos. Lane 5 shows the result for 1 μl of normal mouse plasma. (*B*) Human apo-B transgenic (*HuBTg*) embryos. Immunoblotting of equivalent amounts of protein (≈20 μg) was performed with a monoclonal antibody, 1D1 (18), conjugated to horseradish peroxidase. Lane 5 shows the result for 1 μl of a 1:5 dilution of human plasma, with an exposure time ≈1/10th that for the fetal membranes.

days of gestation, demonstrating clearly that apo-B is essential for mouse embryonic development. The phenotype of homozygous embryos was variable, however; some homozygous embryos survived until the later stages of gestation, although most of these appeared to have exencephalus. The reason for the phenotypic variability in our apo-B knockout embryos is unclear; possible explanations include stochastic differences in nutrient delivery to individual embryos, differences in the genetic background among embryos, or genetic heterogeneity in the knockout phenotype, which is theoretically possible with sequence-insertion vectors (22).

In contrast to our apo-B knockout mice, developmental abnormalities have not been observed in human apo-B deficiency syndromes. What could account for this apparent difference in the phenotypes of apo-B deficiency in mice and humans? One possible explanation relates to developmental differences between the two species. In both species, apo-B is expressed in the yolk sac (3, 5); however, the yolk sac plays a different developmental role in the two species. In the mouse, the yolk sac becomes part of the membranes that surround the developing embryo and contact the maternal tissues early in gestation, whereas in human embryos the yolk sac is a less prominent organ that becomes an appendage attached near the allantoic mesoderm. Thus, it is possible that apo-B produced by the yolk sac in the mouse might play a more important role in facilitating the delivery of lipids or fat-soluble vitamins to the developing embryo early in gestation.

If mouse embryos lacking apo-B are deficient in a critical nutrient, there are reasons to consider vitamin E as a possibly deficient nutrient. First, vitamin E is normally transported in the circulation by apo-B-containing lipoproteins (23). Second, the phenotype of vitamin E deficiency in pregnant rats, which includes fetal resorption and embryonic exencephalus (24–26), is similar to that in our apo-B-deficient mice. This phenotypic similarity led Homanics *et al.* (6) to postulate that vitamin E deficiency may be responsible for the developmental abnormalities in their apo-B70 mice. Indeed, in preliminary experiments, α-tocopherol was undetectable in several 9.5- to 10.5-day homozygous embryos (R.V.F. and H. Kayden, unpublished observations). Although such a defect in vitamin E transport represents an attractive hypothesis that can be further tested, other possible explanations for the embryonic lethal phenotype exist. For example, apo-B may be required for the transfer of other lipid-soluble nutrients (e.g., cholesterol) to the developing embryo. Alternatively, it is conceivable that apo-B expression in some other embryonic tissues may be critical for normal mouse development.

The finding that the embryonic lethal phenotype could be rescued by genetic complementation with a human apo-B transgene has several important implications. First, it provides genetic evidence that the observed phenotype is due to apo-B deficiency. Second, it demonstrates that the human apo-B transgene, which is expressed in the adult mouse liver but not in the adult mouse intestine (19), is expressed in the tissues needed for normal mouse development [presumably the yolk sac (Fig. 5*B*)]. Finally, the offspring generated by this rescue experiment provide a mouse model in which human apo-B is expressed in the liver but not in the intestine.

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