A Gene-targeted Mouse Model for Familial Hypobetalipoproteinemia

LOW LEVELS OF APOLIPOPROTEIN B mRNA IN ASSOCIATION WITH A NONSENSE MUTATION IN EXON 26 OF THE APOLIPOPROTEIN B GENE

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Familial hypobetalipoproteinemia, a syndrome characterized by abnormally low plasma levels of low density lipoprotein cholesterol, is caused by mutations in the apolipoprotein (apo) B gene that interfere with the synthesis of a full-length apoB100. In many cases of familial hypobetalipoproteinemia, nonsense or frameshift mutations result in the synthesis of a truncated apoB protein. To understand why these mutations result in low plasma cholesterol levels, we used gene targeting in mouse embryonic stem cells to introduce a nonsense mutation (N1785Stop) into exon 26 of the mouse Apob gene. The sole product of this mutant Apob allele was a truncated apoB, apoB39. Mice homozygous for this “apoB39-only” (Apob39) allele had low plasma levels of apoB39 and markedly reduced plasma levels of very low density lipoprotein and low density lipoprotein cholesterol when fed a high fat diet. Analysis of liver and intestinal RNA from heterozygous apoB39-only mice revealed that the Apob39 mRNA levels were 60–70% lower than those from the wild-type allele. Interestingly, apoB39 was not cleared as rapidly from the plasma as apoB48. The apoB39-only mice provide new insights into the mechanisms of familial hypobetalipoproteinemia and the structural features of apoB that are important for lipoprotein metabolism.

Familial hypobetalipoproteinemia (FHb),1 an autosomal codominant disorder characterized by low plasma cholesterol levels (1), is caused by mutations in the apolipoprotein (apo) B gene that interfere with the synthesis of a full-length apoB100. Most of the FHb mutations described to date have been nonsense or frameshift mutations within exon 26 of the apoB gene (1), which result in the synthesis of truncated forms of apoB. These truncated apoB proteins are invariably present in low concentrations in the plasma. Human FHb heterozygotes typically have moderately low levels of low density lipoprotein (LDL) cholesterol (<40 mg/dl) and are asymptomatic. FHb homozygotes have extremely low LDL cholesterol levels, typically less than 5 mg/dl, and a variable clinical phenotype, ranging from a complete absence of symptoms to a rather severe clinical phenotype with intestinal fat malabsorption, vitamin E deficiency, and neurological disease (1).

To understand metabolic mechanisms underlying FHb, our laboratory has used gene targeting in mouse embryonic stem (ES) cells to introduce nonsense mutations into exon 26 of the mouse apoB gene (Apob). In a recent study, we reported the development of a mutant Apob allele (the “apoB33-only” allele, or Apob33 allele) in which apoB100 amino acid 3798 was changed to a stop codon (2). The sole product of the Apob33 allele was a truncated apoB, apoB83. The phenotype of heterozygous apoB83-only mice was strikingly similar to that of humans with FHb and an apoB83 mutation. In both human and mouse “apoB83 heterozygotes,” the plasma concentrations of apoB83 were extremely low, and all of the apoB83 in the plasma was confined to the most buoyant very low density lipoprotein (VLDL) particles. An analysis of heterozygous apoB83-only mice revealed dual mechanisms for the “hypobeta” phenotype. First, compared with a wild-type Apob allele, the Apob83 allele was associated with low levels of the apoB mRNA, which led to abnormally low amounts of apoB synthesis and secretion. Second, apoB83 was cleared from the plasma extremely rapidly, far more rapidly than the full-length apoB100.

In the current study, we sought to determine whether these dual “hypobeta” mechanisms (low apoB mRNA levels and rapid clearance of truncated apoB-containing lipoproteins) are specific to the Apob83 mutation or are general features of all Apob nonsense mutations and all truncated apoBs. To investigate that issue, we have used gene-targeting techniques to introduce a nonsense mutation into another site within exon 26 of the mouse Apob gene, generating “apoB39-only” mice. We also sought to further define the impact of Apob gene mutations on mouse development. Homozygous apoB83-only mice had severe neurodevelopmental abnormalities and died during embryonic development or shortly after birth (2). We wanted to determine whether these lethal developmental abnormalities were a general feature of Apob mutations that result in the synthesis of a truncated apoB.

MATERIALS AND METHODS

Generation of Mice with an Apob39 Allele—A gene-targeting vector (Fig. 1) designed to generate apoB39-only mice was constructed from mouse Apob clones 15A5 and 15A6 (strain B10.A) (3). Clone 15A5 begins 55 base pairs (bp) past the beginning of exon 26 and extends to a SacI site located 6540 bp into exon 26. A nonsense mutation was introduced into clone 15A5 by site-directed mutagenesis using oligonucleotide B39stop (see Table I for sequences of all oligonucleotides). The mutagenesis reaction, which involved changing two nucleotides, converted codon 1785 (AAT, specifying Asn) to a stop codon (TAA) and created a new HindIII site. To construct the targeting vector, a 6-kilo-base (kb) insert from the mutant 15A5 clone was excised with SacI and SalI and ligated into the SalI and SacI sites of p15A6. A neomycin resistance gene (neo) driven by the RNA polymerase II promoter was

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The abbreviations used are: FHb, familial hypobetalipoproteinemia; apo, apolipoprotein; LDL, low density lipoprotein(s); VLDL, very low density lipoprotein(s); HDL, high density lipoprotein(s); ES, embryonic stem; bp, base pair(s); kb, kilobase(s); FPLC, fast performance liquid chromatography; PCR, polymerase chain reaction; RT–PCR, reverse transcription–PCR.

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then inserted into the polynucleotide Sal I site. The vector was linearized at a unique Sal I site (608 bp into mouse exon 26) and electroporated into REF embryonic stem cells (strain 129/Sv) (4). Targeted clones were identified by Southern blot analysis of Hind III-digested genomic DNA, using a probe located 5' to the sequences in the gene-targeting vector (2). The gene-targeting vector was designed to insert a subtle mutation into the ApoB gene without disrupting the structure of the ApoB gene or the apoB transcript. The apoB39-only vector is identical to vectors used to insert other subtle mutations into the ApoB gene (2, 3) (e.g., the apoB100-only mutation) and the apoB33-only mutation except for the location of the point mutation.

Targeted ES cells were microinjected into C57BL/6 blastocysts to create male chimeras (5), which were bred with C57BL/6 females to generate heterozygous apoB39-only mice (Apob39/100). The Apob39/100 mice were intercrossed to create homozygotes and also were bred with “apoB100-only” (Apob100/100) and “apoB48-only” (Apob48/48) mice to generate Apob39/100 and Apob39/48 mice. In addition, Apob39/100 mice were mated with Apob39/100 mice to generate Apob39/39 offspring. All mice were weaned at 21 days of age, housed in a barrier facility with a 12-h light/dark cycle, and fed either a chow diet containing 4.5% fat (Ralston Purina, St. Louis, MO) or a high fat diet containing 18.45% butter, 1.25% cholesterol, and 0.5% cholate (ICN Biomedicals, Aurora, OH). All of the mice used in this study were of a mixed genetic background (~50% C57BL/6 and ~50% 129/Sv) (2).

Lipoprotein Fractionation, Western Blot Detection of ApoB, and VLDL Sizing—The distribution of lipoprotein apoB in the lipoprotein fractions was assessed by fractionation on plasma on a fast-performance liquid chromatography (FPLC) column (6). To visualize apoB proteins in mouse plasma or lipoprotein fractions, we performed Western blots of 4% polyacrylamide/SDS gels, using a rabbit anti-serum specific for mouse apoB100 (6). In some experiments, Western blots were performed with a rabbit antiserum against a truncated human apoB protein, apoB37 (7, 8). The diameters of VLDL particles from Apob39/100, Apob48/48, and Apob39/39 mice were determined by electron microscopy of negatively stained fractions (9, 10). For these experiments, VLDL fractions were prepared from mouse plasma by ultracentrifugation (11).

Primer Extension Analysis of the Relative Amounts of ApoB Transcripts Arising from Different ApoB Alleles—The relative amounts of ApoB transcripts from different ApoB alleles were assessed with a primer extension assay similar to the one that we described previously (2). In the current experiments, we assessed the relative proportions of the two different Apob transcripts in the tissues from Apob39/100, Apob39/48, Apob48/48, and Apob39/39 mice. To obtain the template for the primer extension assay, reverse transcription–polymerase chain reaction (RT–PCR) was used to amplify a segment of the apoB gene. Total RNA from the mouse tissues was isolated with the RNA STAT-60 kit (Tel-Test “B”, Inc., Friendswood, TX). Then 5.0 μg of RNA was treated with 2.0 units of DNase I (RNase-free; Boehringer Mannheim) for 30 min at 37 °C. After the DNase was inactivated by heating for 10 min at 95 °C, the first strand of cDNA was synthesized with an RT-PCR kit (Stratagene) and a specific primer (B39cDNA) corresponding to sequences downstream from the apoB39 mutation, within exon 26 of the ApoB gene. After the first strand of cDNA synthesis was completed, we used Taq polymerase (Boehringer Mannheim) and primers B39pe1 and B39pe2 to amplify a 1400-bp segment of the ApoB cDNA. This segment extended from the last 26 bp of exon 26 through the first 1374 bp of exon 2. The corresponding fragment of genomic DNA is ~2 kb in length.

After removal of the unincorporated nucleotides from the enzymatically amplified cDNAs, a 32P-end-labeled oligonucleotide (corresponding to sequences immediately downstream from the apoB39 nonsense mutation (B39pe)) was annealed to the cDNA template and extended with avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) in the presence of dideoxy-CTP (2). Because of the nonsense mutation in the Apob39 allele, the primer extension product from the Apob39 cDNA was 33 bp in length versus 30 bp with the cDNAs from the Apob100, Apob48, Apob39, or Apob83 alleles. The different-sized primer extension products were resolved by electrophoresis on 8% polyacrylamide gels containing 7.5% urea. After electrophoresis, the gels were dried and exposed to autoradiographic film. The radioactivity in each primer extension product was also quantified with the AMBIS radiolabeled imaging system (AMBIS, San Diego, CA). The levels of Apob39 transcripts were expressed as percentages of the transcripts produced by Apob100, Apob48, Apob39, or Apob83 alleles. Data from different animals of the same genotype were expressed as mean ± S.E.

Inhibition of Lipoprotein Clearance in Apob39/100 Mice with Triton WR-1339—Apob39/100 mice (n = 3) were anesthetized with avertin (0.02 mg/kg of body weight) and injected with Triton WR-1339 (500 mg/kg of body weight) (12, 13). A detergent that blocks the processing and uptake of apoB-containing lipoproteins (13). Each mouse was bled from the retroorbital plexus before and after several time points after the injection of Triton WR-1339. The relative levels of apoB39, apoB48, and apoB100 were assessed by Western blots of 4% polyacrylamide/SDS gels, using a rabbit antiserum against mouse apoB (6).

RESULTS

Gene-targeted Mice That Synthesize ApoB39—To generate a mouse model of human FHβ associated with a truncated apoB, we used a gene-targeting vector (Fig. 1) to insert a subtle mutation, N1785Stop, into exon 26 of the mouse ApoB gene. After electroporation of the gene-targeting vector into ES cells, 768 colonies were picked and screened by Southern blots with a 5'-flanking probe. Three targeted clones were identified. The targeting frequency of ~1 in 250 was significantly lower than the ~1 in 50 frequency that we consistently observed with vectors designed to generate apoB48-only, apoB100-only, or apoB83-only mice (2, 3). High percentage male chimeras were

2. From the perspective of the relative levels of apoB proteins in the plasma, mouse strains have been shown to be important. In 1987, Luiss et al. (42) reported that different strains of mice have different “apoB100:apoB48 ratios” in their plasma and that this ratio also varies with different diets. In this study, we analyzed the apoB proteins in mice with a mixed genetic background and were therefore concerned that the mixed genetic background might influence the interpretation of our results. However, in littermates that had been fasted for 4 h, we never observed significant variations in the relative amounts of different apoB proteins in plasma of different animals. In addition, all of the experiments described in this paper were performed on multiple animals of the same genotype. In addition, some of the most important analyses in this paper did not involve comparisons of different animals of the same genotype but instead involved the comparison of two different proteins or two different mRNAs within the same animal.

3. The targeting vectors used to generate the apoB39-only and apoB83-only mice were identical except for the location of the targeted ApoB gene mutations. Before electroporation, both vectors were linearized at the same Sal I site within exon 26 of the ApoB gene. We suspect that the difference in targeting frequency with these two vec-
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bred with C57BL/6 females to generate Apob<sup>39+/+</sup> mice. The Apob<sup>39+/+</sup> mice developed normally, and both males and females were healthy and fertile.

Intercrosses of Apob<sup>39+/+</sup> mice yielded viable homozygous apoB39-only mice (Apob<sup>39/39</sup>). From 170 weaned offspring, there were 43 Apob<sup>+/+</sup>, 102 Apob<sup>39/39</sup>, and 25 Apob<sup>39/39</sup> mice, indicating that about one-half of the Apob<sup>39/39</sup> mice did not survive. The number of homozygous offspring was significantly fewer than predicted (p < 0.05 by x²). Although most of the surviving Apob<sup>39/39</sup> mice were healthy and fertile, three were hydrocephalic. The latter finding strongly suggested that the lethality in the Apob<sup>39/39</sup> mice was a consequence of the same developmental abnormalities previously documented in apoB-deficient mice (2, 14–17) or in mice lacking microsomal triglyceride transfer protein (18).

The Apob<sup>39+/+</sup> mice were mated with Apob<sup>49/48</sup> mice and Apob<sup>100/100</sup> mice to generate Apob<sup>39/48</sup> and Apob<sup>39/100</sup> mice, respectively. ApoB39 was easily detectable in the plasma of fasted Apob<sup>39/48</sup>, Apob<sup>39/100</sup>, and Apob<sup>100/100</sup> mice (Fig. 2). In contrast, apoB39 was virtually undetectable in the plasma of fasted Apob<sup>49/48</sup> mice (Fig. 2A). With the antiserum to mouse apoB100, the apoB39 band in Apob<sup>100/100</sup> mice was only slightly less intense than the apoB100 band (Fig. 2A). However, because that antiserum was developed against the full-length apoB100, we suspected that it contained many more antibodies against apoB100 than against apoB39 and might therefore stain the apoB100 band more intensely than the apoB39 band. To obtain a more accurate view of the relative amounts of apoB39 and apoB100 in the plasma, we performed Western blots with a rabbit antiserum against the amino-terminal 37% of the human apoB molecule, reasoning that that antiserum would be more likely to stain apoB100 and apoB39 equally. With that antiserum, apoB39 was present in low concentrations in the plasma, relative to apoB100 (Fig. 2B). These results were further confirmed by Coomassie Blue-stained SDS-polyacrylamide gels. In the d < 1.21 g/ml lipoprotein fraction, the apoB100 band was 9-fold more intense than the apoB39 band, as judged by densitometry (data not shown).

**Distribution of ApoB39 and ApoB100 within the Plasma Lipoproteins of Chow-fed Mice**—To examine the distribution of apoB39 within the plasma lipoproteins, plasma samples from chow-fed Apob<sup>39/39</sup> (<i>n</i> = 6) mice were pooled and size-fractionated on an FPLC column. Western blot analysis of the FPLC fractions revealed that apoB39 was distributed across a broad range of lipoprotein sizes from the VLDL (fractions 19 and 20) to the high density lipoproteins (HDL) (fractions 29–34); the most intense bands were located in small LDL particles (fractions 25–28) (Fig. 3). In identical experiments performed with Apob<sup>100/100</sup> mice, the apoB100 peak has always eluted slightly earlier (fractions 29–28) (3). That discrepancy led us to suspect that apoB100-containing LDL were larger than apoB39-containing LDL. To directly compare the distribution of apoB100 and apoB39 within the LDL fractions, we size-fractionated pooled plasma from chow-fed Apob<sup>39/100</sup> mice (<i>n</i> = 5) on an FPLC column and performed a Western blot analysis on fractions 23–32. These studies revealed that apoB100-containing particles were larger than apoB39-containing lipoproteins (Fig. 4). Once again, the most intense apoB39 band was located in fractions 27 and 28, whereas the most intense apoB100 band was located in larger particles, fractions 23–26 (Fig. 4). The fact that apoB39 was located in denser lipoproteins, compared with

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**FIG. 1.** Diagram of the “apoB39-only” gene-targeting vector, the mouse apoB gene, and the targeted Apob<sup>39</sup> allele. The gene-targeting vector introduced two nucleotide substitutions within the 5’ portion of exon 26 without changing the structure of the Apob gene or the apoB transcript. The two nucleotide substitutions introduced a new HindIII site and changed codon 1785 (AAT, specifying Asn) to a stop codon (TAA). Targeted Apob alleles were identified by Southern blot analysis of HindIII-cleaved genomic DNA with a flanking probe spanning from intron 24 to exon 25. The wild-type allele yields an 8.0-kb HindIII fragment, whereas the targeted allele yields a 2.5-kb fragment.

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**FIG. 2.** Western blot analysis of the plasma from Apob<sup>39/39</sup>, Apob<sup>39/100</sup>, Apob<sup>100/48</sup>, and Apob<sup>100/100</sup> mice. A, Western blot performed with the antiserum to mouse apoB100. Plasma was obtained from mice after a 4-h fast. As reported previously (2), the concentration of apoB39 in Apob<sup>83/100</sup> mice was so low that it was undetectable. In contrast, apoB39 was easily detectable in the plasma of Apob<sup>39/100</sup> mice. B, Western blot of plasma samples from Apob<sup>39/100</sup> mice (<i>n</i> = 3) with the antiserum to human apoB37 (7, 8).
Apob100, was corroborated by Coomassie Blue-stained SDS-polyacrylamide gels. As judged by densitometry, the apoB100 band was 9-fold more intense in the d < 1.21 g/ml fraction but was 20–30-fold more intense than the apoB39 band in the d < 1.70 g/ml fraction (data not shown).

Response of Apob<sup>39/39</sup> Mice to a High Fat Diet—In Apob<sup>39/39</sup> mice fed a high fat diet, little cholesterol accumulated in the VLDL- and LDL-sized lipoproteins (Fig. 5). In parallel experiments, we documented a striking accumulation of cholesterol in the VLDL/LDL fractions of Apob<sup>48/48</sup> mice when fed a high fat diet (Fig. 5). The size of the VLDL/LDL cholesterol peak in the Apob<sup>48/48</sup> was similar to that of Apob<sup>100/100</sup> and wild-type mice fed a high fat diet (3). We also analyzed the distribution of apoB proteins within the plasma lipoproteins of Apob<sup>39/39</sup> and Apob<sup>39/100</sup> mice fed the high fat diet. In the Apob<sup>39/39</sup> mice, apoB39 was widely distributed within the different lipoprotein fractions, but most of the apoB39 was in small LDL-sized lipoproteins (data not shown). In the Apob<sup>39/100</sup> mice, most of the apoB100 and apoB39 were in the LDL, with the apoB39 peak eluting on slightly smaller particles (Fig. 6).

Sizes of Apob39- and Apob100-containing VLDL—The FPLC fractionation of plasma from Apob<sup>39/39</sup> mice demonstrated that apoB39-containing LDL were smaller than apoB100-containing LDL. To determine whether the apoB39-containing VLDL were also smaller, we isolated VLDL from chow-fed Apob<sup>39/39</sup> and Apob<sup>100/100</sup> mice and measured particle diameters by electron microscopy. The VLDL were significantly smaller in Apob<sup>39/39</sup> mice than in Apob<sup>100/100</sup> mice (mean of 33.9 ± 5.9 versus 44.4 ± 13.4 nm, p < 0.0001). This reduction in diameter corresponds to a ~55% reduction in particle volume. The electron micrographs also revealed that the VLDL from Apob<sup>39/39</sup> mice were homogeneous in size and almost entirely devoid of large lipoprotein particles. In the Apob<sup>39/39</sup> mice, more than 92% of the VLDL particles were between 22 and 39 nm. In contrast, only 50% of the VLDL particles in Apob<sup>100/100</sup> mice were in this size range. Less than 1% of the VLDL particles from Apob<sup>39/39</sup> mice were larger than 50 nm, whereas 24% of the particles in Apob<sup>100/100</sup> mice were larger than 50 nm. We also compared the sizes of VLDL from Apob<sup>39/39</sup> and Apob<sup>48/48</sup> mice. When fed a high fat diet, Apob<sup>39/39</sup> mice had smaller VLDL than Apob<sup>48/48</sup> mice (mean diameter of 30.1 ± 4.8 versus 34.1 ± 8.6 nm, p < 0.0001).

Although small, this reduction in diameter is predicted to correspond to a 45% reduction in particle volume. Virtually identical differences in VLDL size were observed in chow-fed Apob<sup>39/39</sup> and Apob<sup>39/48</sup> mice. With both diets, there were fewer large particles (>50 nm) in the Apob<sup>39/39</sup> mice than in Apob<sup>48/48</sup> mice.

The Apob<sup>39</sup> Allele Is Associated with Low Levels of ApoB mRNA—In a prior study (2), we demonstrated that the levels of apoB mRNA from an Apob<sup>39</sup> allele were 75–80% lower than those from Apob<sup>100</sup> or Apob<sup>+</sup> alleles (2). To examine if the Apob<sup>39</sup> allele also was associated with low mRNA levels, we used primer extension assays to assess the relative amounts of Apob<sup>39</sup> and Apob<sup>100</sup> transcripts in the liver, intestine, and heart<sup>4</sup> of Apob<sup>39/100</sup> mice (n = 3). Enzymatic amplification of the RNA yielded the expected “cDNA-sized” band of 1400 bp (and none of the ~2-kb “genomic DNA-sized” band) (Fig. 7A). Primer extension experiments revealed that the steady-state levels of Apob<sup>39</sup> mRNA were 60 ± 1, 66 ± 3, and 71 ± 6% lower in the liver, intestine, and heart, respectively, than the levels of the Apob<sup>100</sup> mRNA (Fig. 7B). In the livers of Apob<sup>39/48</sup> mice, there was an identical reduction in Apob<sup>39</sup> transcripts, relative to Apob<sup>48</sup> transcripts (Fig. 7C).

Synthesis and Secretion of ApoB39—The decreased Apob<sup>39</sup> mRNA levels in Apob<sup>39/48</sup> mice suggested that apoB39 synthesis and secretion rates might also be lower, relative to those for Apob48. To test this idea, we prepared primary hepatocytes from Apob<sup>48/48</sup> mice, incubated them with [35S]methionine and

<sup>4</sup> We recently demonstrated that the human and mouse apoB genes are expressed in cardiac muscle (44, 45).
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Fig. 7. Primer extension analysis of the relative proportions of Apob<sup>39</sup> and Apob<sup>100</sup> transcripts in the tissues of Apob<sup>39/100</sup> mice. A, RT-PCR reactions demonstrating the enzymatic amplification of a 1400-bp apoB cDNA fragment from the liver, intestine, and heart from three Apob<sup>39/100</sup> mice. The cDNA was amplified using Taq polymerase and oligonucleotides corresponding to sequences in exon 25 and exon 26 of the Apob gene. Amplification of genomic DNA with the same oligonucleotides yielded a ~2-kb DNA fragment. B, a primer extension assay was used to assess the relative proportions of Apob<sup>39</sup> and Apob<sup>100</sup> transcripts in the tissues from three Apob<sup>39/100</sup> mice, using enzymatically amplified cDNA fragments as templates (see “Materials and Methods”). With the Apob<sup>100</sup> allele (or with the Apob<sup>48</sup> or Apob<sup>1</sup> alleles), the product of the primer extension assay was 33 bp; because of the targeted mutation, the product of the primer extension assay in the Apob<sup>39</sup> allele was 30 bp. C, primer extension assay performed with template cDNA amplified from hepatic RNA of an Apob<sup>39/100</sup> mouse. Identical results were obtained with samples from two other Apob<sup>39/100</sup> mice. Also shown are primer extension experiments with template cDNAs amplified from the hepatic RNA of Apob<sup>100/100</sup>, Apob<sup>39/39</sup>, and Apob<sup>39/39</sup> mice.

<sup>[35]</sup>S)cysteine, and analyzed apoB39 and apoB48 secretion in metabolic labeling/immunoprecipitation experiments. As judged by phosphor imager analysis, the amount of apoB39 in Apob<sup>39/48</sup> hepatocyte lysates was reduced by 60%, and apoB39 secretion into the cell culture medium was reduced by 71 ± 2%, compared with apoB48 (Fig. 8).

Generation and Analysis of Apob<sup>39/83</sup> Mice—To compare the steady-state levels of the Apob<sup>39</sup> and Apob<sup>83</sup> mRNAs and to gain insights into differences in the metabolism of apoB39- and apoB83-containing lipoproteins, we bred and analyzed Apob<sup>39/83</sup> mice. Since we detected an ~4:1 ratio of Apob<sup>100</sup> and Apob<sup>83</sup> transcripts in the livers of Apob<sup>83/100</sup> mice (2) and an ~2:1 ratio of Apob<sup>100</sup> and Apob<sup>83</sup> transcripts in Apob<sup>39/100</sup> mice (Fig. 7), we suspected that we would observe an ~2:1 ratio of the Apob<sup>39</sup> and Apob<sup>83</sup> transcripts in Apob<sup>39/83</sup> mice. This suspicion was confirmed; primer extension analysis of the liver RNA from an Apob<sup>39/83</sup> mouse revealed that the ratio of apoB39 mRNA to apoB83 mRNA ratio was ~1.8:1 (Fig. 9).

Although the ratio of Apob<sup>39</sup> transcripts to Apob<sup>83</sup> transcripts was ~1.8:1, the ratio of Apob<sup>39</sup> and Apob<sup>83</sup> transcripts in the plasma was quite different. As judged by Western blot analysis, the ratio of apoB39 to apoB83 in the plasma of Apob<sup>39/83</sup> mice was 11:1 (Fig. 10). Given the relatively modest difference in the amounts of Apob<sup>39</sup> and Apob<sup>83</sup> transcripts in

the liver, we suspected that the marked discrepancy in the plasma levels of apoB39 and apoB100 was probably due, in large part, to differences in the rates at which these two proteins were cleared from the plasma. In our previous studies of Apob<sup>83/100</sup> mice, we demonstrated that apoB83-containing lipoproteins were cleared extremely rapidly from the plasma, accounting in large part for the high ratio of apoB100 to apoB83 in the plasma of Apob<sup>83/100</sup> mice (2). Accordingly, we suspected that the high ratio of apoB39 to apoB83 in Apob<sup>39/83</sup> mice was due to the fact that apoB83-containing lipoproteins are cleared from the plasma much more rapidly than apoB39-containing lipoproteins.

Analyzing ApoB Metabolism in Apob<sup>39/100</sup> Mice after an Injection of Triton WR-1339—To gain further insights into the
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The vast majority of the FHβ mutations described to date result in the synthesis and secretion of a truncated form of apoB. While it is clear that the properties of the apoB gene mutations and the truncated apoB proteins must underlie the low plasma cholesterol levels in humans with FHβ, only limited information exists regarding the cellular and molecular mechanisms underlying this disorder (1). Gaining insights into basic mechanisms of FHβ from human studies poses logistic and ethical difficulties because those experiments would require liver and intestinal biopsies from healthy and asymptomatic human subjects. In the current study, to further define the basic mechanisms of FHβ, we used gene targeting in mouse ES cells to introduce a nonsense mutation into exon 26 gene of the mouse Apob gene, generating an authentic FHβ allele that yielded exclusively apoB39. The apoB39-only mice manifested several phenotypes characteristic of hypobetalipoproteinemia (1, 16). First, the plasma concentration of apoB39 in Apob39/100 mice was low, compared with that of apoB100, and second, the plasma of Apob39/39 mice fed a high fat diet contained extremely low levels of VLDL/LDL cholesterol. There are several likely causes for the low plasma apoB39 levels and low plasma cholesterol levels in apoB39-only mice. One is that the apoB39 nonsense mutation causes a 60–70% reduction in apoB mRNA levels, leading to a proportionate decrease in the synthesis and secretion of apoB-containing lipoproteins. Another factor, particularly in the setting of the high fat diet, might be a diminished capacity of apoB39 to assemble a lipoprotein with a large core of neutral lipids. Electron microscopy revealed the VLDL of Apob39/39 mice were small (~34 nm). That finding, along with the absence of fasting chylomicronemia, suggests that the apoB39 molecule may simply be too short to assemble very large, lipid-rich VLDL particles.

Low levels of RNA in association with nonsense mutations have been documented in several human genetic diseases (21–25). The extent to which nonsense mutations reduce mRNA levels can vary substantially, depending on the location of the mutation within the gene. For example, in the case of the β-globin gene, some nonsense mutations markedly reduce mRNA levels, whereas others have no effect on mRNA levels (22). We suspect that the same situation applies to the apoB gene. We previously introduced a nonsense mutation into a “physiologically normal” site in the mouse apoB gene, codon 2153, the same codon that is converted to a stop codon by the intestinal mRNA editing machinery (26, 27). Of note, that mutation had no effect on apoB mRNA levels. More recently, we generated apoB38-only mice by introducing a nonsense mutation into exon 37 and found that that mutation caused a 75–80% reduction in apoB mRNA levels. In the setting of the apoB38-only allele, the reduced mRNA levels likely reflected abnormal mRNA processing or stability, since the levels of apoB38 pre-mRNA were not decreased (2). In the current study, we documented a 60–70% decrease in apoB mRNA levels, leading to a proportionate decrease in the synthesis and secretion of apoB-containing lipoproteins. Another factor, particularly in the setting of the high fat diet, might be a diminished capacity of apoB39 to assemble a lipoprotein with a large core of neutral lipids. Electron microscopy revealed the VLDL of Apob39/39 mice were small (~34 nm). That finding, along with the absence of fasting chylomicronemia, suggests that the apoB39 molecule may simply be too short to assemble very large, lipid-rich VLDL particles.

Histology of Liver and Intestine from Apob39/39 Mice—The reduced apoB secretion in the setting of the Apob39 allele, along with the smaller size of apoB39-VLDL, led us to consider the possibility that the Apob39/39 mouse might develop fatty pathology in the intestine and liver (as a result of an abnormally low capacity to export neutral lipids). Oil Red O and hematoxylin and eosin staining showed no fatty pathology in the intestines or livers of chow-fed Apob39/39 mice (data not shown). On the high fat diet, the Apob39/39 mice developed fatty pathology in the liver, but that pathology is characteristic of the high fat diet in wild-type mice (20) and was also observed in Apob48/48 and Apob100/100 mice. There was no fatty pathology in the intestines of Apob39/39 mice fed the high fat diet (data not shown).

**DISCUSSION**

Metabolism of apoB39, relative to that of apoB48 and apoB100, we assessed the relative levels of apoB39, apoB48, and apoB100 in the plasma of Apob39/39 mice, both at base line and at several time points after the injection of Triton WR-1339, a detergent that inhibits the lipolytic processing and clearance of apoB-containing lipoproteins. After injection of the detergent, apoB48 accumulated in the plasma, relative to apoB100 (Fig. 11). This finding is consistent with the data of Li and co-workers (13). The accumulation of apoB48 in the setting of normal amounts of neutral lipid synthesis) (1). Similarly, the plasma concentration of apoB39 in Apob39/100 mice was low, compared with that of apoB100, and second, the plasma of Apob39/39 mice fed a high fat diet contained extremely low levels of VLDL/LDL cholesterol. There are several likely causes for the low plasma apoB39 levels and low plasma cholesterol levels in apoB39-only mice. One is that the apoB39 nonsense mutation causes a 60–70% reduction in apoB mRNA levels, leading to a proportionate decrease in the synthesis and secretion of apoB-containing lipoproteins. Another factor, particularly in the setting of the high fat diet, might be a diminished capacity of apoB39 to assemble a lipoprotein with a large core of neutral lipids. Electron microscopy revealed the VLDL of Apob39/39 mice were small (~34 nm). That finding, along with the absence of fasting chylomicronemia, suggests that the apoB39 molecule may simply be too short to assemble very large, lipid-rich VLDL particles.
During the final phases of our investigation of the Apob<sup>39</sup> allele in mice, we fortuitously obtained a duodenal biopsy from a human “apoB37 heterozygote” (7, 28) (subject 18 from Ref. 28) during an elective cholecystectomy. Interestingly, primer extension studies of the duodenal RNA from that subject revealed that the apoB37 mRNA was reduced by 60%, relative to the apoB mRNA from the wild-type allele. Those results are quite consistent with the results that we obtained with the Apob<sup>39</sup> allele in mice.

In Apob<sup>83/100</sup> mice, lipoproteins containing apoB83 were cleared far more rapidly from the plasma than apoB100 (2). The rapid clearance of apoB83-containing lipoproteins was demonstrated in experiments in which the uptake of lipoproteins in Apob<sup>83/100</sup> mice was blocked by an intravenous injection of the detergent Triton WR-1339. After the Triton injection, the amount of apoB83 in the plasma increased sharply, relative to apoB100. That result indicated that apoB83 is cleared much more rapidly than apoB100 under normal circumstances (i.e. in the absence of detergent). Other experiments indicated that the enhanced clearance of apoB83 was dependent both on the LDL receptor and on apoE (2). In generating the apoB39-only mice, one of our principal goals was to determine whether rapid clearance was a general property of all truncated apoB83 or whether there were intrinsic differences in the metabolic properties of apoB39- and apoB83-containing lipoproteins. Several lines of evidence strongly favor the latter scenario. First, in the plasma of Apob<sup>83/83</sup> mice, the concentration of apoB39 was at least 11-fold greater than that of apoB83, although the amount of the apoB39 mRNA was less than 2-fold greater. Second, when we injected Triton WR-1339 into an Apob<sup>83/83</sup> mouse, apoB39 did not accumulate relative to apoB100. However, apoB48 did accumulate, relative to both apoB100 and apoB39, indicating that the apoB48-containing lipoproteins are normally cleared more rapidly than those containing either apoB39 or apoB100.

The portion of the apoB100 molecule that binds to the LDL receptor is located near amino acids 3300–3500, far downstream from the carboxyl terminus of apoB48 (29–31). Consequently, the clearance of apoB48-containing lipoproteins from the plasma is entirely dependent on the binding of apoE on the surface of the lipoprotein to either the LDL receptor or the LDL receptor-related protein (32–34). In the case of the LDL receptor-related protein, binding of lipoproteins appears to require that the surface of the lipoprotein be enriched with a supplemental “dose” of apoE (35, 36). The fact that apoB48 accumulated relative to apoB39 in the Triton WR-1339 experiments suggests that apoE-mediated clearance may be less efficient for apoB39-containing lipoproteins. In view of the small size of apoB39-containing VLDL, it is tempting to speculate that apoB39-containing particles cannot accommodate as many molecules of apoE on their surface, compared with apoB48-containing lipoproteins. Of course, apoB39 does contain the portion of the apoB molecule that interacts with the LDL receptor. Thus, we suspect that the extremely low levels of apoB83 in the plasma of Apob<sup>83/83</sup> mice relate in large part to the ability of apoB83 to bind to the LDL receptor (2).

ApoB39 was distributed widely within the VLDL, intermediate density lipoproteins, LDL, and HDL fractions of apoB39-only mice, in a manner virtually identical to that described for similarly sized truncated apoB83 in humans with FHβ (1, 37). Within the LDL fractions, apoB39-containing particles were smaller than those containing apoB100, presumably because of a decreased ability of apoB39 to bind lipids. The distribution of apoB39 within the plasma lipoproteins was markedly different from that of apoB83, which was confined to the largest particles within the VLDL fraction, both in humans and in mice (2, 38). The absence of apoB83 from the LDL probably reflects the extraordinarily rapid clearance of apoB83-containing VLDL by lipoprotein receptors in the liver (2).

Approximately one-half of the predicted number of Apob<sup>39/39</sup> mice were viable, and about 10% of those developed hydrocephalus. This phenotype is similar to that reported for homozygous apoB70 mice, which have low apoB mRNA levels as a result of an insertional mutation within the Apob gene (14). The Apob<sup>83/83</sup> mice were more severely affected, all of them dying before birth or shortly after birth and the majority manifesting severe neurodevelopmental abnormalities. Farese et al. (39) reported data suggesting that the developmental abnormalities in apoB knockout mice were due to an absence of lipoprotein production by the visceral endoderm of the yolk sac, causing impaired delivery of lipid nutrients to the developing embryo. With that concept in mind, we believe that the most parsimonious explanation for the more severe developmental abnormalities in the Apob<sup>83/83</sup> mice (than in Apob<sup>39/39</sup> mice) is that the lower level of apoB83 mRNA resulted in a more severe nutritional deficit in the developing mouse embryos. However, other factors could contribute to the more severe phenotype in the Apob<sup>83/83</sup> mice. As noted earlier, apoB83 is taken up very rapidly by cellular lipoprotein receptors, far more rapidly than apoB39 or apoB100 (2). We can imagine that the peculiar properties of apoB39 might cause the yolk sac lipoproteins to be taken up and metabolized by the “wrong” cell types within the developing Apob<sup>83/83</sup> embryo, thereby preventing them from reaching the critical cell types within the developing central nervous system.

In the past, we (7, 28, 40) and others (41) have documented that truncated apoB proteins shorter than apoB48 or apoB100 can assemble large and buoyant lipoproteins. Those observations have occasionally led us to wonder why the extraordinary size of the apoB proteins has been conserved during tens of millions of years of mammalian evolution. In other words, why has evolution not “whittled away” at the length of the apoB molecule? The current studies suggest several likely explanations. First, the embryonic lethality in the homozygous apoB83-only and apoB39-only mice suggests that nonsense mutations within the apoB transcript may not always be well tolerated, both from the standpoint of normal embryonic development and from the standpoint of maintaining normal levels of the apoB mRNA. Second, the apoB39-only mice have shown that whittling away 358 amino acids from apoB48 results in plasma lipoproteins that are small and have distinctly different metabolic properties. Given the central role of the apoB-containing lipoproteins in delivering lipids, antioxidant vitamins, and fuel to cells, we suspect that mutations affecting the intrinsic metabolic properties of lipoproteins might be poorly tolerated by evolution.

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A Gene-targeted Mouse Model for Familial Hypobetalipoproteinemia: LOW LEVELS OF APOLIPOPROTEIN B mRNA IN ASSOCIATION WITH A NONSENSE MUTATION IN EXON 26 OF THE APOLIPOPROTEIN B GENE

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