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Genetics

Apolipoprotein B RNA editing enzyme-deficient mice are viable despite alterations in lipoprotein metabolism

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ABSTRACT **RNA editing in the nucleus of higher eukaryotes results in subtle changes to the RNA sequence, with the ability to effect dramatic changes in biological function. The first example to be described and among the best characterized, is the cytidine-to-uridine editing of apolipoprotein B (apo-B) RNA. The editing of apo-B RNA is mediated by a novel cytidine deaminase, apobec-1, which has acquired the ability to bind RNA. The stop translation codon generated by the editing of apo-B RNA truncates the full-length apo-B100 to form apo-B48. The recent observations of tumor formation in *Apobec-1* transgenic animals, together with the fact that *Apobec-1* is expressed in numerous tissues lacking apo-B, raises the issue of whether this enzyme is essential for a variety of posttranscriptional editing events. To directly test this, mice were created with a null mutation in *Apobec-1* using homologous recombination in embryonic stem cells. Mice, homozygous for this mutation, were viable and made apo-B100 but not apo-B48. The null animals were fertile, and a variety of histological, behavioral, and morphological analyses revealed no phenotype other than abnormalities in lipoprotein metabolism, which included an increased low density lipoprotein fraction and a reduction in high density lipoprotein cholesterol. These studies demonstrate that neither apobec-1 nor apo-B48 is essential for viability and suggest that the major role of apobec-1 may be confined to the modulation of lipid transport.**

The cytidine-to-uridine (C → U) editing of RNA is found in diverse animals and plants. It is most widespread in the mitochondria and chloroplasts of vascular plants, where RNA editing is necessary to produce viable mRNAs (1). In marsupials, it has been reported to affect tRNA in the mitochondria (1). In placental mammals, an example of C → U editing has been described, which edits apolipoprotein (apo)-B RNA in a tissue-specific manner. This editing results in an inframe stop codon (2, 3) generating apo-B48, a 241-kDa protein, required for the assembly of chylomicrons. In humans the full-length molecule, apo-B100 (512 kDa), is only made in the liver for secretion on very low density lipoprotein (VLDL) particles, which are metabolized into low density lipoproteins (LDLs). In contrast to man, the mouse edits apo-B RNA in both the intestine and the liver, which is a likely factor leading to the low levels of LDL in the mouse and a potential factor contributing to the relative resistance of this mammal to atherosclerosis.

Apo-B RNA editing is mediated by a novel cytidine deaminase (4, 5) that binds RNA (6, 7). It is unclear whether apobec-1 arose exclusively to edit apo-B RNA or if it has a multiplicity of RNA targets. Marked overexpression of an *Apobec-1* transgene in rabbits and mice resulted in hepatic dysplasia and hepatocellular carcinoma, respectively (8). Furthermore, much of the editing in these *Apobec-1* transgenic animals represented illegitimate editing, demonstrating the potential of apobec-1 to edit other RNA targets. The observation that apobec-1 is expressed in the rat spleen (9, 10) as well as numerous other tissues that do not secrete apo-B also suggests that there may be other roles for this enzyme. It has thus been argued that the loss of apobec-1-mediated RNA editing might result in a lethal phenotype (1).

No apobec-1 deficiency states have been reported in mammals. To directly investigate the role of *Apobec-1 in vivo*, we have created mice in which this gene was inactivated by homologous recombination in embryonic stem (ES) cells. The consequences of an absence of apobec-1-mediated RNA editing on a variety of biological parameters, including lipoprotein metabolism, were studied in these animals.

MATERIALS AND METHODS

Construction of Targeting Vector. The vector, pPNT (11), which consisted of a neo-resistance cassette (*neo*^{r}), a herpes simplex virus thymidine kinase cassette (*tk*), and a pUC19 vector backbone was used for the construction of the targeting vector. The short arm, a 0.5-kb *Kpn*I fragment containing exon 5, was inserted between the *neo*^{r} and the *tk* cassettes at the *Kpn*I site contained within pPNT. The long arm, a 10.8-kb *Eco*RV fragment, was inserted into the *Xho*I site (using *Sal*I linkers) on the opposing side of *neo*^{r} to generate the final 18.5-kb targeting vector, pPNTEKO.

Generation of Mice Lacking *Apobec-1*. pPNTEKO was linearized and electroporated into ES cells (12). Drug selection was performed for 10–12 days using both G418 (260 μg/ml) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU; 0.5 μM). Screening for targeting events was achieved by nested PCR using primers specific for *neo*^{r} and a region down stream of the targeting event, as follows. The first primer set consisted of F.1, 5′-ATTGTTTTGCCAAGTTCTATTTCC-3′ and R.1, 5′-AGGATGGGAATAGGAGACTCAGG-3′; the second primer set consisted of Nf.1, 5′-TTTGCCAAGTTCTAATTCCATCAG-3′ and Nr.1, 5′-GGGAATAGGAGACTCAGGAATTC-3′. Genomic Southern blot analysis was used to confirm the correct targeting. The targeted ES cells were used to generate chimeric mice by standard procedures (13). Chimeric males were bred with C57BL/6 × DBA F1 hybrids. Genotypes were determined by multiplex PCR (14) using primer sets specific for *neo*^{r} (neo2.F, 5′-GCAGCCAATATGGGATCG-3′ and neo2.R, 5′-ATCAGAGCAGCCGATTGTCT-3′) and a primer set specific for a region deleted by the targeting of *Apobec-1* (del.F, 5′-TGGGTGGAAGCAAGGATTTC-3′ and del.R, 5′-GTTATGGAAACAGGCTACCAGC-3′).

Gene Expression Analysis of *Apobec-1*-Deficient Mice. *Reverse transcriptase-PCR analysis.* Reverse transcription, using

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Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; ES, embryonic stem.

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RNA isolated from tissue using RNA STAT-60 (Tel-Test, Friendswood, TX), was performed with random primers according to the manufactures instructions (GIBCO/BRL). PCR was consequently performed with primers specific for exon 6 and exon 8 of mouse *Apobec-1*, as follows: m68.F, 5′-TTACCTGGTTCCTGTCCTGG-3′ and m68.R, 5′-TCCTTTGGTAATGGCAGGTT-3′. The control primers spanned four exons of the mouse Huntington disease gene (15), as follows: P11, 5′-CGATGCGGAGTCAGATGTCA-3′ and P14, 5′-GGTCTTTTGCTTGTTCGGGGT-3′.

Primer extension assay: Quantitation of endogenous apo-B RNA editing. Total RNA was extracted from liver samples and subsequently purified by ultracentrifugation through cesium chloride as described (16). The primer extension assay was performed as described (17).

Immunoblot analysis. Total plasma (2 μl) was resolved on precast SDS/4% polyacrylamide gels (NOVEX, San Diego). The proteins were electrotransferred to nitrocellulose and detected with a rabbit anti-rat apo-B antibody. The subsequent detection of the protein–antibody complex was performed with a goat anti-rabbit antibody conjugated to alkaline phosphatase.

Lipid and Lipoprotein Analyses. Lipid measurements were performed on 5- to 8-week-old mice fed either a mouse chow (Purina) or a high-fat diet (1.25% cholesterol/0.5% cholate/16% fat/20% protein). Plasma cholesterol and triglyceride was measured using commercially available enzyme kits (High Performance Cholesterol kit, Boehringer Mannheim; Triglyceride Reagent Set, Seragen; and Triglyceride Blank Blend, Craig Bioproducts). High density lipoprotein (HDL) cholesterol was measured after polyethylene glycol precipitation of apo-B-containing lipoproteins (18).

Calculation of LDL fraction. Equivalent amounts of total plasma, from fasted animals, were analyzed on 2–16% nondenaturing polyacrylamide gels, which was stained for lipid as described (19). The relative contribution of LDL-sized particles was determined by densitometric quantitation of the lipid-staining material in the LDL size range (23–28 nm) and compared with the total lipid-staining material over the LDL to intermediate density lipoprotein/VLDL size range of 23–100 nm.

Vitamin A/fat tolerance test. After an overnight fast, mice were given an intragastric bolus of 3000 units of retinol delivered in 200 μl of corn oil. At times 0, 1, 2, 4, 10, and 24 h after the bolus delivery, 50 μl of blood was drawn from each animal and the plasma lipids were extracted. Analysis was carried out according to Weintraub *et al.* (20).

Chylomicron analysis. Chylomicrons were drawn directly from the intestinal lymph duct with a syringe. Animals were fasted overnight and then given a 200-μl bolus of corn oil by intragastric gavage, 2 h before being sacrificed. The lymph was mixed with an equal volume of 0.2 mg/of BSA per ml, 1 μm μl of the mixture was placed on the grid and stained with sodium phosphotungstate (21).

Nonlipid Phenotypic Analyses. *Morris water maze.* Mice (n = 5) underwent three phases of testing as described (22). First they were trained to find a visible platform, then an invisible platform, and finally, no platform (the probe test). Mice were allowed to search for the platform for a maximum of 60 s and

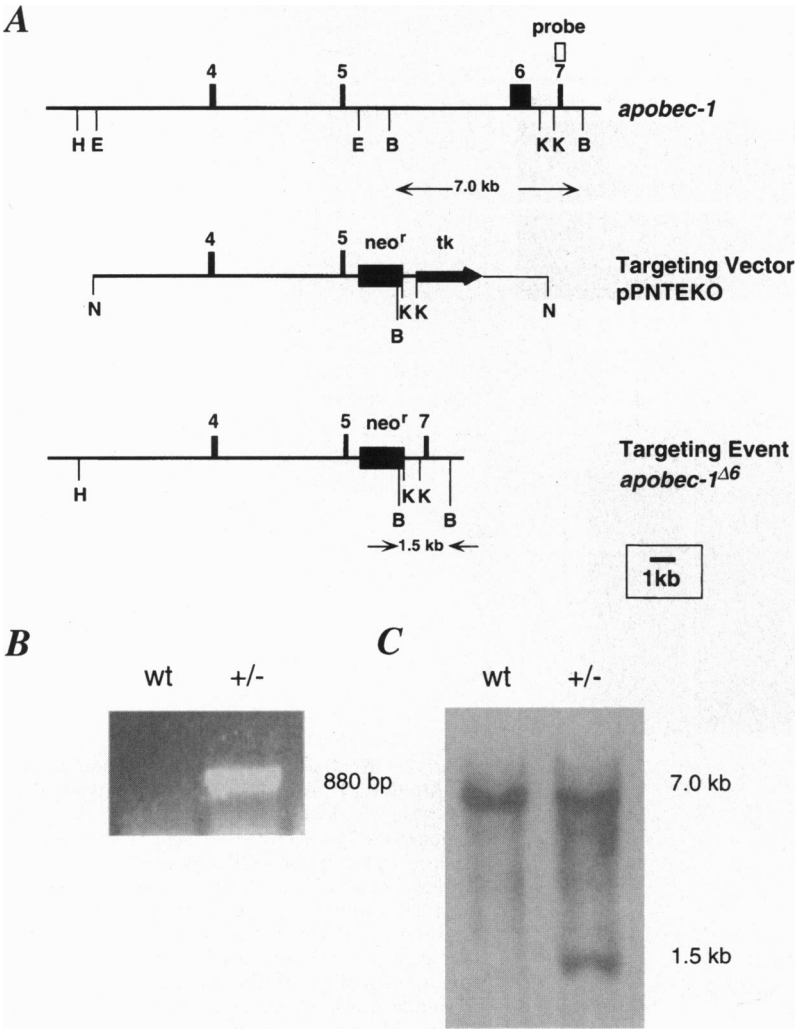

FIG. 1. Targeted disruption of *Apobec-1* by homologous recombination. (*A*) Schematic representation of the *Apobec-1*-targeting strategy. Exons (not drawn to scale) are indicated by raised boxes and are numbered; the gray boxes represent the long and short arms used in the targeting vector, pPNTEKO. The unique *Not*I site in pPNTEKO was used to linearize the vector. E, *Eco*RV; K, *Kpn*I; N, *Not*I; B, *Bgl*II; and H, *Hin*dIII. (*B*) Homologous recombination was detected by nested PCR using primers specific for the *neo*^{r} minigene and a site downstream of the short arm. (*C*) Genomic Southern analysis. DNA was digested by *Bgl*II and hybridized with the radiolabeled probe. The two fragments, corresponding to wild-type (7.0 kb) and the mutant (1.5 kb) alleles, are indicated.

to remain on the platform for 30 s for the first two phases. Data were analyzed by mixed-model ANOVA.

Histological examination. Organs were fixed by perfusion of 4% Bouin's solution. Sections were stained with hematoxylin and eosin. Sections were made of all major organs, including the liver, kidney, spleen testes, thymus, adrenals, bone, spinal cord, and brain. For the brain, six cross sections were made, including analysis of the forebrain, midbrain, and hindbrain.

Electron microscopy of the jejunum. The intestine was first flushed with 37°C saline, and then the jejunum was irrigated and fixed with 2.5% glutaraldehyde in 0.15 M cacodylate buffer, as described (23).

RESULTS

Targeted Disruption of *Apobec-1*. To create a targeting vector suitable for the disruption of *Apobec-1*, the replacement vector, pPNTEKO, was designed to delete exon 6, which encodes the catalytic domain of the protein (Fig. 1*A*). This mutant allele will allow for the translation of only 7% of apobec-1. Analysis of 600 ES cells targeted with pPNTEKO by both PCR (Fig. 1*B*) and Southern blotting (Fig. 1*C*) identified a single clone with the predicted disruption of the murine *Apobec-1* gene, designated *Apobec-1*$^{\Delta 6}$. The targeted clone was micro-injected into C57BL/6 blastocysts, resulting in chimeric animals, one of which gave germ-line transmission of the *Apobec-1*$^{\Delta 6}$ mutation. Heterozygous mice derived from these targeted cells produced *Apobec-1*$^{\Delta 6}$ homozygotes (Fig. 2*A*) in approximately the Mendelian-predicted ratio: wild type, 32 mice; heterozygous *Apobec-1*$^{\Delta 6}$, 83 mice; and homozygous *Apobec-1*$^{\Delta 6}$, 39 mice. Both male and female homozygous knock-out mice were viable and reproduced normally.

Expression of Apobec-1. Analysis of *Apobec-1* expression by reverse transcriptase-PCR (Fig. 2*B*) revealed an absence of *Apobec-1* mRNA in the homozygous knock-out mice, while it was present in the liver, intestine, spleen, brain, and kidney of control animals. This confirmed the predicted deficiency of *Apobec-1* transcripts following the targeting of this gene. To investigate whether editing of apo-B RNA can occur in the absence of *Apobec-1*, we examined liver and intestinal extracts for the presence of edited apo-B transcripts. The primer extension assay demonstrated a complete lack of apo-B RNA editing in both the liver and intestine in the *Apobec-1* null mice, while approximately 70% and 98%, respectively, of apo-B

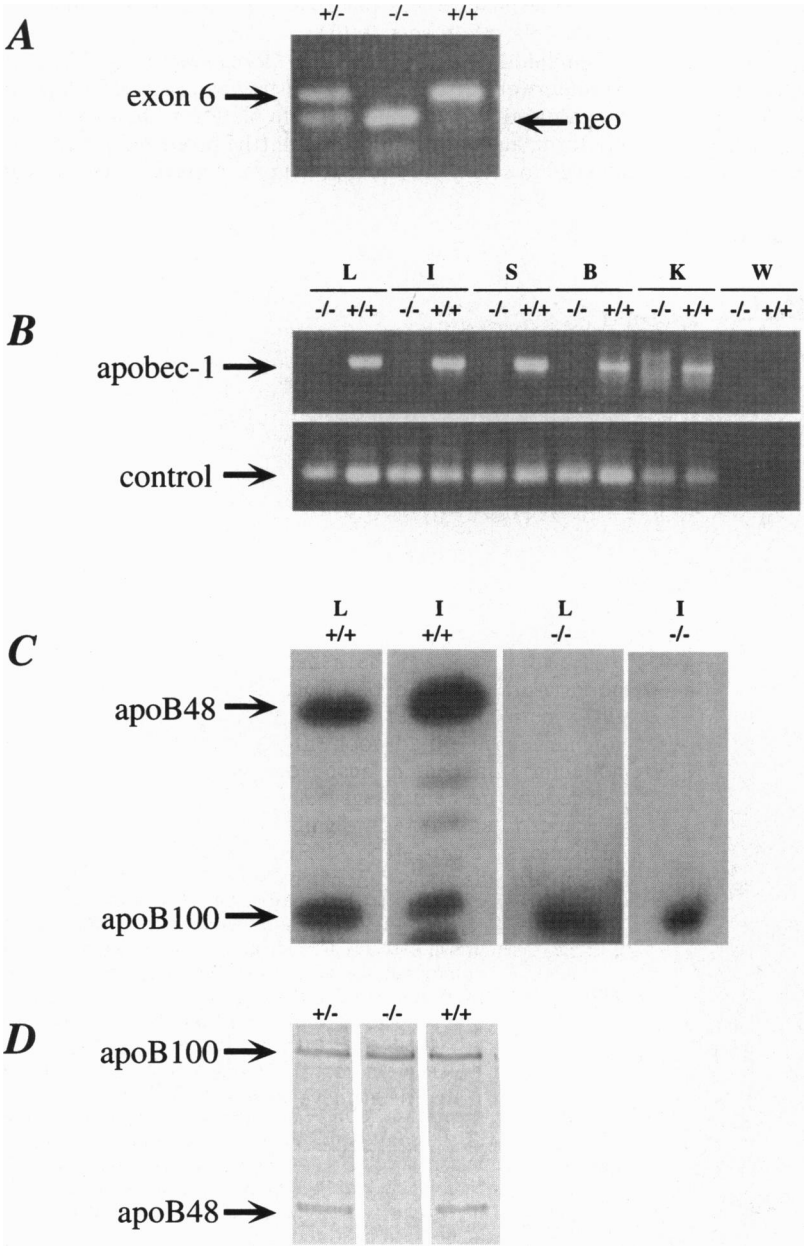

FIG. 2. Analysis of *Apobec-1* expression in mice. (*A*) Multiplex PCR-identified mice genotypes. Control (wt, +/+), heterozygous *Apobec-1*$^{\Delta 6}$(+/−), and homozygous *Apobec-1*$^{\Delta 6}$ (−/−) mice are shown. (*B*) Reverse transcriptase-PCR was used to detect the presence of *Apobec-1* RNA in various tissues. L, liver; I, intestine; S, spleen; B, brain; K, kidney; and W, water (no RNA control). (*C*) The primer extension assay was performed to detect edited and unedited forms of apo-B RNA obtained from intestine (I) and liver (L) biopsies. (*D*) Western analysis was performed on total plasma using apo-B anti-sera.

transcripts were edited in the liver and intestine of control mice (Fig. 2*C*). The percentage of RNA editing (apo-B48/apo-B48 + apo-B100) was assessed by quantitation of the apo-B100 and apo-B48 primer extension signals using a PhosphorImager (Molecular Dynamics). This indicated that there was no redundancy for the role of apobec-1 in the editing of apo-B RNA. Immunoblot analysis of the plasma of apobec-1-deficient mice revealed a complete absence of apo-B48 (Fig. 2*D*). In contrast to *in vitro* studies using apo-B mini-genes (24), these results indicate an absolute requirement for *Apobec-1* and apo-B editing for the production of apo-B48 *in vivo*.

Behavior and Histological Analyses. Due to expression of *Apobec-1* in the brain, quantitative behavioral assays, which assess learning and memory (Morris water maze) (22), were performed. This analysis revealed no significant differences between the apobec-1-deficient and control mice (data not shown). Histological examination by light microscopy of the brain, liver, spleen, kidney, and testes also revealed no abnormalities in apobec-1-deficient animals (R. Bronson, personal communication). Mice homozygous for *Apobec-1*$^{\Delta 6}$ were also indistinguishable from control animals as judged by fertility, weight, appearance, and gross behavior.

Electron Microscopy of Intestinal Enterocytes and Chylomicrons. The one human condition noted for absence of apo-B48 in the plasma is chylomicron-retention disease, which is characterized by a lack of circulating chylomicrons and an accumulation of lipid in the intestinal absorptive cells (25). Electron microscopy of the lipoproteins isolated from intestinal lymph ((Fig. 3 *A* and *D*) of animals on both chow and high-fat diets revealed no differences in the size of chylomicrons: chow diet (controls), 106 ± 51 nm; *Apobec-1*$^{\Delta 6}$ homozygotes, 123 ± 59 nm (Fig. 3 *B* and *E*); high-fat diet (controls), 142 ± 82 nm; and *Apobec-1*$^{\Delta 6}$ homozygotes, 117 ± 82 nm. There were also no apparent abnormalities in the ultrastructure of the intestinal absorptive cells (Fig. 3 *C* and *F*) of the apobec-1-null mice. Together these observations imply no intrinsic requirement for apobec-1 nor apo-B48 in the assembly of chylomicrons or the transport of intestinally absorbed lipid.

Vitamin A/Fat Tolerance Test. To examine post-prandial chylomicron metabolism in apo-B100 only (apobec-1-deficient) mice, we undertook vitamin A/fat tolerance studies. The orally administered vitamin A (retinol) was used to observe chylomicron metabolism in the plasma (Fig. 4). Retinol delivered to the gut is esterified, secreted into plasma packaged into chylomicrons, and finally removed from the plasma by the liver. There is little recirculation of the retinyl esters (26). The results reveal a similar level of retinol in the

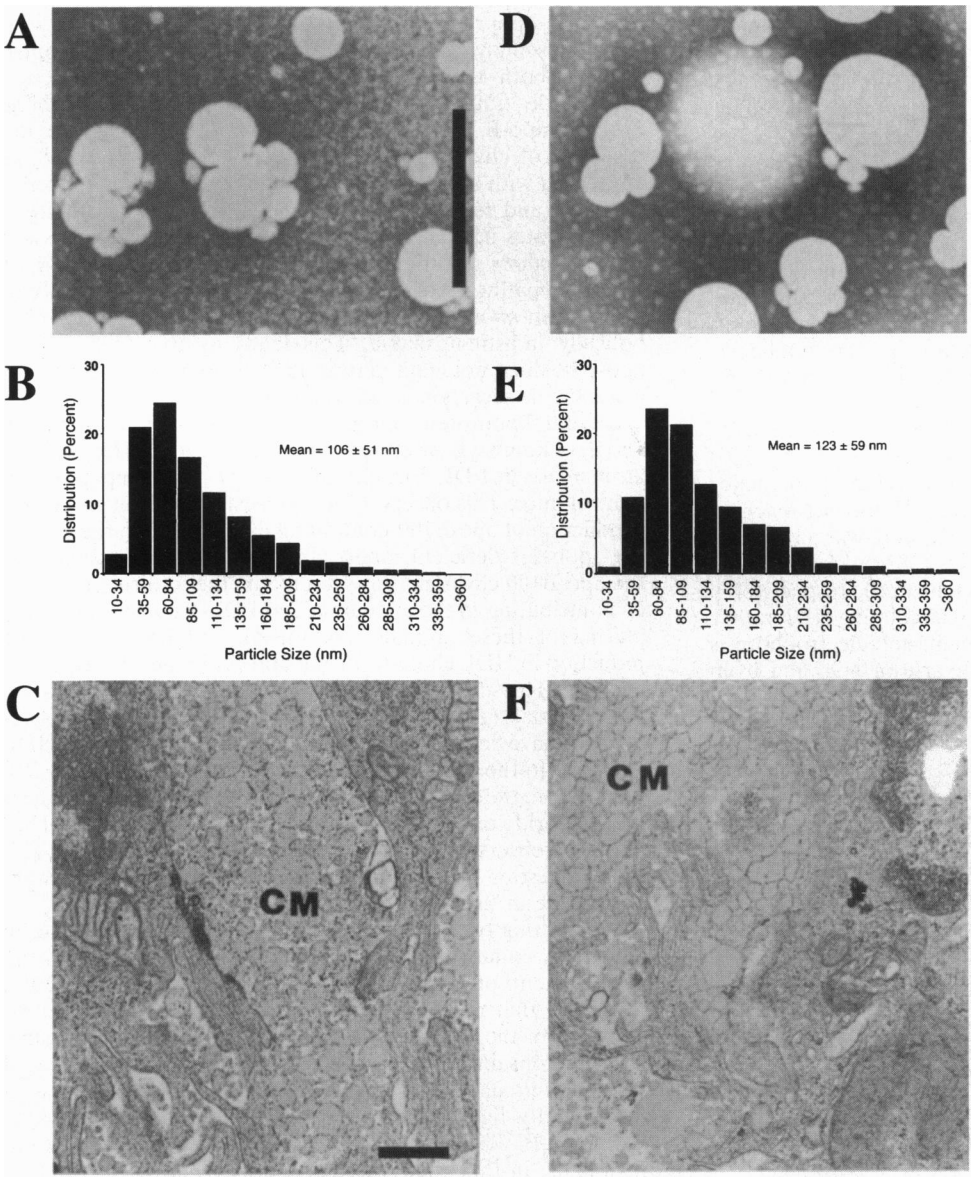

FIG. 3. Electron microscopy of intestinal lymph lipoproteins and intestinal absorptive cells. Negatively stained images of lipoproteins present in the intestinal lymph, together with a histogram of the size distribution of the particles, for control (*A* and *B*) and apobec-1-deficient mice (*D* and *E*); the results represent the mean of values obtained for each genotype (n = 3). The lower micrographs shows presence of chylomicrons (CM) in the basolateral intracellular space of the intestinal cells, for control (C) and apobec-1-deficient mice (F). Bar = 500 nm, in the respective pairs of micrographs.

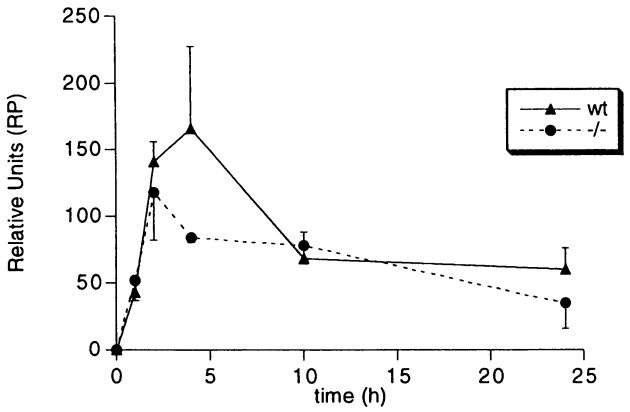

FIG. 4. Vitamin A/fat tolerance test in mice lacking apobec-1. Plasma was taken from mice (n = 3) at the indicated times after administration of corn oil and retinyl palmitate (RP) by intragastric gavage. Plasma lipids were extracted and resuspended in ethanol in preparation for reverse-phase chromatography. The area under the peak corresponding to RP was normalized to an internal control, retinyl acetate (RA). The values represent the mean ± SE.

plasma for both the control and apobec-1-deficient mice over the time course of the experiment. Hence the metabolism (production and clearance) of chylomicrons, whether they contained only apo-B48 or apo-B100, was indistinguishable in these studies.

Analysis of Plasma Lipoproteins. A notable result of apobec-1 deficiency on mice fed a chow diet was the appearance in the plasma of a distinct LDL-sized fraction with a major particle size of 26 nm in diameter. Gradient gel electrophoresis of apo-B-containing lipoproteins from *Apobec-1*-null mice revealed a prominent LDL-sized particle (Fig. 5), which was only a minor component of the LDL/VLDL-sized particles in the control mice (63 ± 5% and 23 ± 5%, respectively; n = 3, $P < 0.001$). Separation of plasma lipoproteins by gel permeation chromatography, followed by compositional analysis, indicated that this LDL-sized fraction in the apobec-1-deficient mice was cholesterol-rich and triglyceride-poor, similar to human LDL (data not shown).

The apobec-1-deficient mice displayed a reduced total plasma cholesterol when fed the chow diet (Table 1). This reflected a reduction in HDL cholesterol (Table 1), the major plasma cholesterol fraction in the mouse. While not reaching statistical significance (n = 3, P = 0.06), there was a concomitant reduction in apo-AI protein levels (283 ± 96 and 140 ± 17, for wild-type and *Apobec-1*-deficient mice, respectively), suggesting a reduction in the amount of circulating HDL rather than the HDL particle size. Gradient gel electrophoresis revealed a homogenous HDL-particle size of ≈9.7 nm from

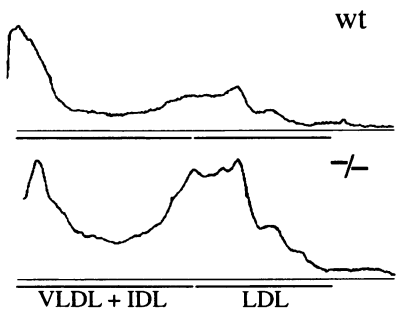

FIG. 5. Gradient gel electrophoresis of apo-B-containing lipoproteins. Equivalent amounts of total were separated on 2–16% nondenaturing polyacrylamide gels, stained for lipid, and scanned densitometrically. The LDL and intermediate density lipoprotein/VLDL size ranges are 23–28 nm and 28–100 nm, respectively. wt, wild-type; −/−, homozygous *Apobec-1*$^{\Delta 6}$.

both wild-type and apobec-1-deficient mice. This data further suggests that the decrease in plasma cholesterol reflects fewer circulating particles.

No effect on HDL cholesterol levels was noted in either the wild-type or the apobec-1-deficient mice fed a high-fat diet (Table 1), while total cholesterol levels doubled in all animals fed this diet. Gel permeation analysis of the plasma from these animals revealed that the elevated cholesterol was confined to the VLDL/intermediate density lipoprotein fraction (data not shown). The high-fat diet also caused a decrease in plasma triglycerides in the control mice ($P < 0.005$), which was not apparent in the apobec-1-deficient animals.

DISCUSSION

This study demonstrates the viability of *Apobec-1* knock-out mice and the absence of a discernible phenotype, other than that associated with lipoprotein metabolism. This is a somewhat unexpected finding, considering the expression of *Apobec-1* in multiple tissues (9, 10), tumor formation in *Apobec-1* transgenic studies (8), and the absence of individuals lacking apobec-1. Detecting subtle phenotypic consequences of gene targeting in mice has proven to be a significant challenge in many studies. Despite this caveat, the results of the present study support the hypothesis that the primary, if not exclusive, function of *Apobec-1*-mediated RNA editing in mammals is to modulate lipoprotein metabolism.

The assembly and secretion of apo-B-containing lipoproteins in both the liver and intestine requires microsomal triglyceride transfer protein, whereas a second metabolic process, apo-B editing, occurs in the intestine before the assembly of chylomicrons. The extent to which apo-B48, as compared with apo-B100, dictates the process of chylomicron assembly and secretion has been unclear. The present study demonstrates normal lipid absorption from the intestine and no anomalous accumulation of lipid in the cytoplasm or secretory pathways of intestinal cells. Furthermore, chylomicrons from *apobec-1* knock-out and control mice are metabolically indistinguishable. This leads us to conclude that apo-B48 does not have any unique properties essential for assembly or secretion of chylomicrons.

Several lipoprotein anomalies were demonstrated in the *apobec-1* homozygous knock-out mice. A major difference was an increase in LDL particles in *apobec-1*-null compared with control mice. This observation is consistent with the exclusive production of apo-B100-containing lipoproteins in the liver in the apobec-1-deficient mouse. In addition, it is possible that the apo-B100 chylomicrons secreted by the intestine may also be contributing to the appearance of LDL-size particles in the plasma of these animals. An unexpected finding was the reduction in HDL cholesterol and apo-AI in the apobec-1-null animals. This may reflect the mixed genetic background of these animals (129/Sv, C57BL/6, and DBA/2). Genetic backgrounds have been shown to have differing effects on the HDL response to the high-fat diet in these strains of mice (28, 29). In addition, the absence of apo-B48 may result in disturbances in triglyceride metabolism. A decrease in HDL cholesterol has also been observed in apo-B and apo-CIII transgenic mice (27, 30), suggesting that a multiplicity of biochemical pathways participate in the regulation of HDL cholesterol levels.

The editing of apo-B RNA appears to have arrived late in evolution, coinciding with the appearance of apo-E and the development of a lymphatic drainage system in placental mammals that channels chylomicrons from the intestine directly into the systemic circulation. Once in the plasma, chylomicrons undergo lipolysis and are then efficiently cleared from the circulation by the liver via apo-E, which acts as a high-affinity ligand for lipoprotein receptors. Birds lacking both apo-B editing and apo-E, also differ from placental mammals, in that their vascular anatomy allows for dietary

Table 1. Lipoprotein analysis of *Apobec-1*-deficient mice

		TC		TG		HDLc		apoB‡	
mice	diet*	mg/dl	*n*	mg/dl	*n*	mg/dl	*n*	mg/dl	*n*
wt	Chow	137 ± 17	6	41 ± 7	6	104 ± 24†	6	79 ± 33	3
+/−	Chow	126 ± 25†	10	36 ± 11	10	88 ± 19†	10		
−/−	Chow	106 ± 23	13	37 ± 10	12	65 ± 18	13	81 ± 27	3
wt	High-fat	240 ± 35	9	21 ± 13	9	100 ± 36	8	70 ± 14	6
+/−	High-fat	253 ± 80	13	31 ± 42	13	98 ± 25	13		
−/−	High-fat	211 ± 54	11	35 ± 19	11	118 ± 73	10	65 ± 22	3

All analyses were performed on individual littermates after overnight fasting. Blood was drawn by tail bleed into EDTA tubes. The values for the high-fat diet were determined after the animals had been on the diet for 12 days. HDLc was determined by precipitation (27). Data are shown as mean value ± standard deviation. wt, Wild type; (+/−), heterozygous for *Apobec-1*$^{\Delta 6}$; (−/−), homozygous for *Apobec-1*$^{\Delta 6}$; TC, total cholesterol; TG, total triglyceride; and HDLc, HDL cholesterol.

*Diets: refer to *Materials and Methods*.

†$P < 0.05$, compared with −/− mice.

‡apo-Al and apo-B levels were measured directly on total plasma by immunoassay (27).

lipids and vitamins to be delivered directly to the liver via portal circulation (31). Considering these anatomical and biochemical differences between birds and placental mammals, apo-B RNA editing and apo-E may have coevolved in placental mammals to facilitate the targeting of intestinally derived lipids and vitamins from the systemic circulation to the liver. Whether the absence of apobec-1 has a deleterious effect on the aging animal or the free-living rodent is unclear at present. It is possible, however, that altered targeting of triglycerides or lipid-soluble vitamins derived from the diet, might produce subtle disadvantages and provide the basis for the genetic selection of this unusual form of metabolic processing.

Note. During the preparation of this manuscript, we became aware of data generated by another group that demonstrates that *Apobec-1*$^{-/-}$ mice fail to edit endogenous apo-B mRNA but are otherwise healthy and manifest no obvious systemic abnormalities (N. O. Davidson, K. Hirano, L. M. Powell-Braxton, and S. G. Young, personal communication).

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