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Genetics

Knockout of the abetalipoproteinemia gene in mice: Reduced lipoprotein secretion in heterozygotes and embryonic lethality in homozygotes

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ABSTRACT **Abetalipoproteinemia, an inherited human disease characterized by a near-complete absence of the apolipoprotein (apo) B-containing lipoproteins in the plasma, is caused by mutations in the gene for microsomal triglyceride transfer protein (MTP). We used gene targeting to knock out the mouse MTP gene (*Mttp*). In heterozygous knockout mice (*Mttp*+/−), the MTP mRNA, protein, and activity levels were reduced by 50%, in both liver and intestine. Compared with control mice (*Mttp*+/+), chow-fed *Mttp*+/− mice had reduced plasma levels of low-density lipoprotein cholesterol and had a 28% reduction in plasma apoB100 levels. On a high-fat diet, the *Mttp*+/− mice exhibited a marked reduction in total plasma cholesterol levels, compared with those in *Mttp*+/+ mice. Both the livers of adult *Mttp*+/− mice and the visceral endoderm of the yolk sacs from *Mttp*+/− embryos manifested an accumulation of cytosolic fat. All homozygous embryos (*Mttp*−/−) died during embryonic development. In the visceral endoderm of *Mttp*−/− yolk sacs, lipoprotein synthesis was virtually absent, and there was a marked accumulation of cytosolic fat droplets. In summary, half-normal MTP levels do not support normal levels of lipoprotein synthesis and secretion, and a complete deficiency of MTP causes lethal developmental abnormalities, perhaps because of an impaired capacity of the yolk sac to export lipids to the developing embryo.**

Abetalipoproteinemia is an inherited human disease characterized by extremely low plasma levels of cholesterol and triglycerides and a virtual absence of the apolipoprotein (apo) B-containing lipoproteins [chylomicrons, very-low-density lipoproteins (VLDL), and low-density lipoproteins (LDL)] in the plasma (1, 2). Affected humans manifest intestinal fat malabsorption and frequently develop severe neurological problems as a result of deficient intestinal absorption of vitamin E, a fat-soluble vitamin (3). Abetalipoproteinemia is caused by mutations in the gene for the 97-kDa catalytic subunit of microsomal triglyceride transfer protein (MTP) (4–6). MTP is thought to transfer lipids to the apoB polypeptide chain as it is translated on the ribosome, allowing apoB to translocate into the lumen of the endoplasmic reticulum and assume the proper conformation for lipoprotein assembly (7, 8). In abetalipoproteinemia, apoB is synthesized but cannot form lipoproteins and is degraded (9).

Abetalipoproteinemia is considered to be an autosomal recessive syndrome, requiring two defective MTP alleles for disease expression. Parents of affected patients are said to have plasma lipid levels within the normal range (1). The normal plasma lipid levels in obligate heterozygotes have given rise to the concept that MTP normally is present in great excess within lipoprotein-secreting cells. That is, if MTP normally were present within microsomes in great excess, then half-normal MTP levels in obligate heterozygotes would not be expected to affect lipoprotein secretion rates or plasma lipid levels. Against this concept, however, are recent *in vitro* data on lipoprotein secretion from human hepatoma cells and rat hepatocytes cultured in the presence of MTP inhibitor drugs (refs. 10–13; J. Wetterau, personal communication). These studies have shown that the extent of inhibition of lipoprotein secretion caused by MTP inhibitor drugs is roughly proportional to the extent of inhibition of MTP activity. In other words, inhibiting MTP activity levels by ≈50% results in an ≈50% reduction in lipoprotein secretion. These experiments with MTP inhibitor drugs obviously do not support the concept that MTP is present in great excess within cells and make it difficult to understand why obligate heterozygotes would have normal plasma lipid and lipoprotein levels. There are several potential explanations for this apparent paradox, one being that human "abeta heterozygotes" actually have normal or near-normal MTP levels as a result of a compensatory up-regulation in MTP expression from the normal allele or as a result of a reduction in MTP mRNA or protein turnover. Unfortunately, there are no data to resolve this enigma; no one has measured MTP mRNA, protein, or activity levels in livers or intestines from human heterozygotes. Nor have there been any measurements of lipoprotein secretion from the lipoprotein-secreting tissues of human heterozygotes. Because of logistic and ethical hurdles involved in obtaining intestinal and liver tissue from healthy human subjects, it seems quite unlikely that these issues will ever be satisfactorily addressed in humans.

To gain insights into the relationship between MTP levels and lipoprotein secretion in a mammalian model, we used gene targeting in mouse embryonic stem cells to generate a mouse model for abetalipoproteinemia. Another rationale for these experiments was to assess the importance of MTP in mouse embryonic development. Homozygous apoB-deficient mouse embryos manifest severe neurodevelopmental abnormalities (14–16). It has been hypothesized that these neurodevelopmental abnormalities are a result of the absence of lipoprotein secretion by the yolk sac, causing an impaired delivery of lipid nutrients to the developing embryo (17). If this hypothesis were correct, one would predict that MTP deficiency might

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Abbreviations: MTP, microsomal triglyceride transfer protein; apoB, apolipoprotein B; VLDL, very-low-density lipoproteins; IDL, intermediate density lipoproteins; E, embryonic day; FPLC, fast performance liquid chromatography.

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also cause neurodevelopmental abnormalities. The MTP gene knockout mice described herein test that prediction.

MATERIALS AND METHODS

Generation of MTP Knockout Mice. A PCR from exon 1 of the human *MTP* gene (with oligonucleotides 5′-CTGTAGC-CCACCTGTGTTTAATC-3′ and 5′-AACAGAGGCAGAG-TACGAGGAG-3′) was used to identify a strain 129 P1 clone (Genome Systems, St. Louis) spanning the 5′ portion of the mouse MTP gene (*Mttp*). A sequence-replacement gene-targeting vector was constructed from a 15-kb *Sac*I fragment spanning exons 1–3 of the *Mttp* gene. The coding sequence of exon 1 was interrupted with a *neo* cassette; the long arm contained 6.5 kb of sequences 5′ to exon 1, and the short arm contained 1.5 kb of intron 1 sequences (Fig. 1*A*). The vector was electroporated into RF8 embryonic stem cells (18), and a targeted clone was used to generate heterozygous *Mttp*-deficient mice (19). Nearly all experiments with heterozygous knockout mice were performed with genetically identical F1 mice bred from chimeras (20). Mice were weaned at 21 days of age and fed either a chow diet containing 4.5% fat (Ralston Purina) or a high-fat, high-cholesterol diet (15).

RNase Protection Assays. To measure mRNA levels from liver, intestine, or yolk sac, we used RNase protection assays (21). A 395-bp *Mttp* riboprobe was generated from a plasmid containing a *Cla*I-*Pst*I fragment from the mouse *Mttp* cDNA. A riboprobe for apoB was generated as described previously (22). A mouse β-actin riboprobe spanning β-actin cDNA nucleotides 480–559 was used as a control.

MTP Activity Assays and MTP Western Blots. Microsomal proteins from liver and intestine were isolated and microsomal triglyceride transfer activity was measured as described previously (4, 23). Western blots of microsomal proteins were performed with a rabbit antiserum against bovine MTP (24) and chemiluminescence detection reagents; the intensity of the 97-kDa MTP protein was quantified with a GS300 transmittance/reflectance scanning densitometer (Hoefer).

Measurements of Lipid and ApoB100 Levels. Measurement of cholesterol and triglyceride levels were performed by using enzymatic assays (15), and the distribution of lipids within the plasma lipoprotein fractions was determined by fast performance liquid chromatography (FPLC) (15). Tissue lipids were extracted as described previously (25). The concentration of mouse apoB100 in the plasma was measured with a mAb-based solid-phase sandwich radioimmunoassay by using two mouse apoB100-specific mAbs, LF3 and LF5 (26).

Metabolic Labeling of Primary Hepatocytes. Primary mouse hepatocytes were prepared, and 6×10^5 cells were plated onto collagen-coated 38-mm wells (26). Two hours after hepatocytes were plated, 50 μl of Pro-mix (530 MBq/ml; Amersham) was added to each dish. After a 3-h incubation at 37°C, the medium and cells were collected. The amount of cellular protein in different wells varied by <2%. The amounts of apoB48 and apoB100 in the media were assessed by loading 40 μl of the medium onto SDS-polyacrylamide gels. Dried gels were analyzed by autoradiography and a PhosphorImager (Fuji Bio-Imaging Analyzer, Fuji Medical Systems, Stamford, CT). The density distribution of the ^{35}S-labeled lipoproteins was analyzed by discontinuous sucrose gradient ultracentrifugation (26).

Histology and Electron Microscopy of Tissues. Tissue from adult mice and embryos was examined by routine histologic studies. Electron microscopy of the visceral endoderm of the yolk sacs was performed as described previously (17).

RESULTS

***Mttp* Knockout Mice.** A 15-kb *Sac*I fragment spanning exons 1–3 of the mouse *Mttp* gene was mapped and partially sequenced, revealing an exon/intron structure identical to that of the human and hamster genes (6, 27). A sequence-replacement vector was constructed (Fig. 1*A*) and used to generate heterozygous MTP knockout mice (*Mttp*+/−).‖ *Mttp*+/− mice, which were healthy and fertile, were intercrossed in an attempt to obtain homozygous *Mttp* knockout mice; however, no *Mttp*−/− mice were identified among 123 weaned offspring (Table 1). To investigate this finding, embryos were genotyped at several time points during development (Fig. 1*B* and Table 1). At embryonic day 9.5 (E9.5), *Mttp*−/− embryos were present at the expected Mendelian frequency, but they were retarded in their growth and had open anterior neuropors (Fig. 2*B*). By E10.5, most of the *Mttp*−/− embryos appeared necrotic, and the anterior neuropor remained open (Fig. 2*C*). At this stage, *in situ* hybridization studies revealed *Mttp* expression in the visceral endoderm of the yolk sac, but no signal was detectable within the embryo itself (data not shown). At E14.5, a large fraction of the embryos (32% of the total) was hemorrhagic and in an advanced stage of resorption, and the distribution of the surviving embryos was significantly different from a Mendelian ratio ($P < 0.01$ by χ^2). Only five viable *Mttp*−/− embryos were identified at E14.5, and each had exencephalus (Fig. 2*D*). Thus, most *Mttp*−/− embryos die around E10.5, and the few that survive past this time point have severe neurodevelopmental abnormalities. Why the majority of *Mttp*−/− embryos die around E10.5 is not clear, but it may be relevant that the E9.5 embryos and their yolk sacs were pale. The blood islands in the yolk sac membranes of *Mttp*+/+ and *Mttp*+/− embryos were filled with hematopoietic cells on E10.5 (Fig. 2*E*); in contrast, these cells were rarely observed in the yolk sac blood islands of *Mttp*−/− embryos (Fig. 2*F*). A blood smear from one of the surviving E14.5 *Mttp*−/− embryos revealed very few mature erythrocytes, compared with a blood smear from a *Mttp*+/+ embryo (data not shown).

Disruption of a Single *Mttp* Allele Reduces *MTTP* mRNA Levels and Triglyceride Transfer Activity. As judged by an RNase protection assay, *Mttp* mRNA levels were highest in the intestine, with moderate levels in the liver and lower amounts in the yolk sac, kidney, and heart. As expected, the *Mttp* mRNA was undetectable in the yolk sacs of *Mttp*−/− mice (Fig. 3*A*).

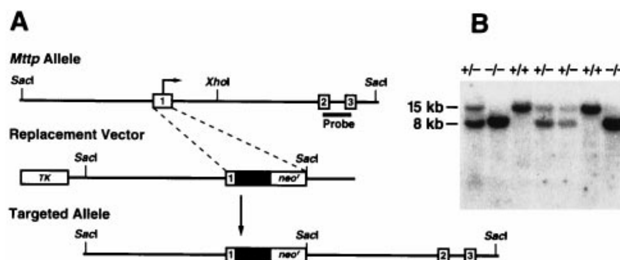

FIG. 1. Targeted disruption of the *Mttp* gene. (*A*) A map of the *Mttp* locus, together with the sequence-replacement gene-targeting vector and the targeted *Mttp* allele. Exons (open boxes), introns (thin line), and start of the MTP ORF (arrow) are indicated. The gene-targeting event replaced exon 1 coding sequences with a cassette containing a neomycin-resistance gene (*neo*) driven by a phosphoglycerate kinase 1 promoter and a green fluorescent protein cDNA fragment (solid box). Analysis of gene-targeting events and mouse genotyping was performed by Southern blot analysis of *Sac*I-digested genomic DNA, using a 1.3-kb probe (thick bar) extending from exon 2 to exon 3. The probe, which was labeled by random priming, was generated by PCR with oligonucleotides 5′-TGAGCGGCTATA-CAAGCTCAC-3′ and 5′-CTGGAAGATGCTCTTCTCGC-3′. (*B*) Southern blot of *Sac*I-digested DNA from E9.5 mouse embryos. The size of the *Sac*I fragment in the targeted allele is 8 kb, versus 15 kb in the wild-type allele.

‖The mutant *Mttp* allele has been designated *Mttp*^{tm1Sgy}. In this paper, we refer to it as *Mttp*−.

Table 1. Genotypes of offspring from intercrosses of *Mttp*+/− mice

Age	*Mttp*+/+	*Mttp*+/−	*Mttp*−/−	Resorbed	Total
E9.5	30	52	26	6	114
E10.5	6	9	9*	2	26
E14.5	16	38	5†	28	87
Postnatal	52	71	0	NA	123

Genotypes were determined by Southern blot analysis as described in the legend to Fig. 1. NA, not applicable.
*Most of these embryos appeared to be necrotic.
†All five embryos had exencephalus.

The *Mttp* mRNA in *Mttp*+/− mice was reduced by one-half in the liver, intestine, and yolk sac (Table 2); thus, there was no evidence that the remaining wild-type allele was up-regulated to compensate for the disrupted *Mttp* allele. We found no evidence that MTP deficiency affected apoB mRNA levels; the apoB mRNA levels were identical in the liver and intestine of adult *Mttp*+/− and *Mttp*+/+ mice, and in the yolk sacs from *Mttp*+/+, *Mttp*+/−, and *Mttp*−/− mice (Table 2).

To assess the possibility that *Mttp*+/− mice might have normal MTP protein or activity levels despite half-normal mRNA levels (e.g., perhaps as a result of decreased turnover of the MTP protein), we compared MTP protein and activity levels in *Mttp*+/− and *Mttp*+/+ mice. Microsomal triglyceride transfer activity in the *Mttp*+/− livers and intestine was one-half of that observed with the *Mttp*+/+ mice (Fig. 3*B*). MTP protein levels in the liver, as assessed by densitometric analysis of Western blots of SDS-polyacrylamide gels, also were half-normal (45 ± 12%; $n = 6$), compared with liver samples from *Mttp*+/+ mice ($n = 6$).

Decreased Levels of Lipids and Apo-B100 in *Mttp*+/− Mice. There were no significant differences in the plasma concentrations of total cholesterol or triglycerides in *Mttp*+/− and *Mttp*+/+ mice (Table 3). However, as judged by FPLC fractionation studies, *Mttp*+/− mice exhibited a small but consistent reduction in intermediate density lipoprotein (IDL)/LDL cholesterol levels (a 28% and a 16% reduction in two independent experiments) (Fig. 4). The reduced concentrations of IDL/LDL cholesterol suggested that the *Mttp*+/− mice might have reduced plasma levels of apoB100. To test that idea, we measured plasma apoB100 levels with a mAb-based

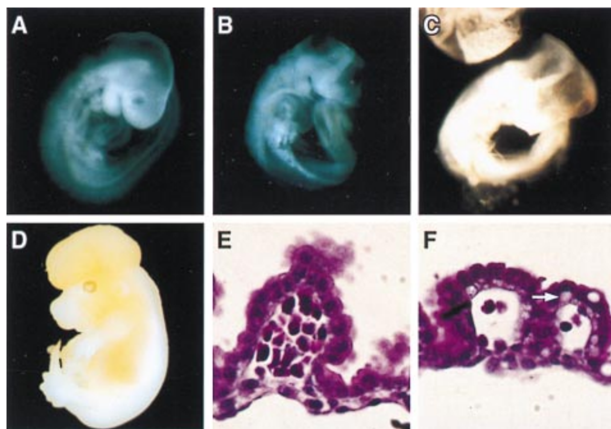

FIG. 2. Phenotype of *Mttp*−/− embryos. (*A*) *Mttp*+/+ embryo at E9.5. (*B*) An E9.5 *Mttp*−/− embryo with incomplete anterior closure of the neural tube. (*C*) An E10.5 *Mttp*−/− embryo that appeared necrotic, and had incomplete anterior closure of the neural tube. (*D*) An E14.5 *Mttp*−/− embryo with exencephalus. (*E* and *F*) Hematoxylin and eosin staining of yolk sac membranes from *Mttp*+/+ (*E*) and *Mttp*−/− (*F*) embryos at E10.5. There were reduced numbers of blood cells in the blood islands of *Mttp*−/− membranes compared with the *Mttp*+/+ membranes. Note the vacuolization at the basolateral surfaces of the *Mttp*−/− visceral endoderm (arrow).

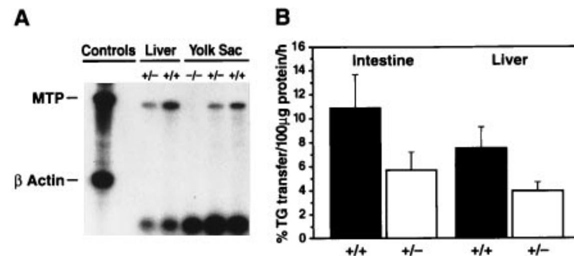

FIG. 3. MTP expression levels in *Mttp* knockout mice. (*A*) An RNase protection assay for the *Mttp* mRNA, using liver and yolk sac RNA samples (5 μg of RNA per lane). The first control lane shows the size of the full-length, undigested riboprobes for *Mttp* and β-actin; the second control lane documents complete digestion of the riboprobes in the presence of RNase but in the absence of cellular RNA. After RNase treatment, the RNA samples were size-fractionated by electrophoresis on 6% denaturing polyacrylamide gels; dried gels were exposed to autoradiographic film and subsequently analyzed with a PhosphorImager. (*B*) Triglyceride transfer assay with cellular extracts from mouse liver and intestine ($n = 10$ for each genotype).

radioimmunoassay. A highly significant ≈28% reduction in apoB100 levels was observed in chow-fed *Mttp*+/− male and female mice, compared with sex-matched *Mttp*+/+ littermate controls (Table 3). Western blots of SDS-polyacrylamide gels (using a polyclonal mouse apoB-specific antiserum) revealed no difference in the ratio of apoB100 to apoB48 in the *Mttp*+/− and *Mttp*+/+ mice (data not shown).

The livers of *Mttp*+/− mice contained increased amounts of triglycerides (11.8 ± 1.5 mg/g vs. 9.6 ± 1.0 mg/g in *Mttp*+/+ mice, $n = 5$ in each group, $P = 0.04$) and cholesterol (2.9 ± 0.2 mg/g vs. 2.4 ± 0.4 mg/g in *Mttp*+/+ mice, $P = 0.07$). Oil Red O-stained histological sections also revealed increased amounts of neutral lipids in livers of *Mttp*+/− mice compared with *Mttp*+/+ controls (data not shown). The reduction in IDL/LDL cholesterol and apoB100 levels, along with increased liver triglyceride levels, indicated that half-normal levels of MTP activity are insufficient for normal levels of lipoprotein secretion from the liver. Oil Red O staining of intestines from *Mttp*+/+ mice did not reveal any fatty pathology; it is possible that the absence of intestinal fat accumulation could relate to the high *Mttp* mRNA levels in that tissue. Normalized to *Apob* mRNA levels, the levels of the *Mttp* mRNA in the intestine are 5-fold higher than in liver or yolk sac (Table 2).

On a high-fat diet, the total plasma cholesterol levels were ≈20% lower in *Mttp*+/− mice (Table 3) than *Mttp*+/+ mice ($P = 0.007$), as a result of a marked decrease in the amount of VLDL and LDL cholesterol (Fig. 4). ApoB100 plasma levels tended to be lower in the *Mttp*+/− mice than in *Mttp*+/+ mice in two separate experiments, but this difference did not achieve statistical significance (Table 3). However, when a series of dilutions of pooled plasma samples from 6–10 *Mttp*+/− and *Mttp*+/+ mice on a high-fat diet were compared in a radioimmunoassay, we invariably observed a 15–20% reduction in apoB100 levels in the *Mttp*+/− mice (data not shown).

To confirm that the low plasma apoB100 concentrations in *Mttp*+/− mice were caused by a decrease in apoB secretion from cells, we assessed apoB accumulation in the medium of primary hepatocytes prepared from chow-fed *Mttp*+/+ and *Mttp*+/− mice. There was invariably less apoB48 and apoB100 accumulation in the medium of *Mttp*+/− hepatocytes, compared with *Mttp*+/+ primary hepatocytes (Fig. 5*A*). Triglyceride transfer activity levels were half-normal in cultured *Mttp*+/− hepatocytes (Fig. 5*B*); *Mttp*+/− cells had half-normal levels of *Mttp* mRNA (55 ± 8% of *Mttp*+/+ hepatocytes) and normal levels of apoB mRNA (106 ± 8% of *Mttp*+/+ hepatocytes). As judged by PhosphorImager analy-

Table 2. *Mttp* and *Apob* mRNA levels in *Mttp* mutant mice

	Mttp mRNA level			*Apob* mRNA level		
	Liver, $n = 6$	Intestine, $n = 8$	Yolk sac, $n = 2$	Liver, $n = 6$	Intestine, $n = 6$	Yolk sac, $n = 2$
Mttp+/+	0.64 ± 0.05	0.99 ± 0.17	0.18 ± 0.01	3.58 ± 0.31	1.17 ± 0.28	0.94 ± 0.01
Mttp+/−	0.32 ± 0.04	0.57 ± 0.10	0.10 ± 0.01	3.65 ± 0.71	1.01 ± 0.15	0.95 ± 0.02
Mttp−/−	NA	NA	0.01 ± 0.01	NA	NA	0.90 ± 0.03

Mttp and *Apob* mRNA levels were determined by RNase protection assays and were normalized to the signal for β-actin mRNA. Values are reported as means ± SD. NA, not applicable.

sis, there was 19 ± 5% (mean ± SD) less apoB100 and 22 ± 5% less apoB48 in the medium from *Mttp*+/− cells ($P = 0.05$) (Fig. 5). Sucrose density gradient ultracentrifugation of the ^{35}S-labeled lipoproteins revealed no differences in the density profiles of the lipoproteins secreted by *Mttp*+/+ and *Mttp*+/− cells (data not shown).

Reduced *Mttp* Gene Expression Is Associated with Lipid Accumulation in the Visceral Endoderm of the Yolk Sac. Hematoxylin and eosin-stained sections of the yolk sacs of *Mttp*−/− embryos revealed vacuolization of the basolateral surface of the yolk sacs (Fig. 2*F*). Staining of frozen sections with Oil Red O indicated that these vacuoles were cytosolic lipid droplets (Fig. 6*A*). In contrast, only trace amounts of Oil Red O staining were observed in *Mttp*+/+ yolk sacs. The yolk sac membranes of *Mttp*+/− embryos had an intermediate level of fat accumulation, indicating that half-normal levels of MTP activity were insufficient to prevent fat accumulation in that tissue.

Examination of imidazole-stained yolk sacs by electron microscopy confirmed the presence of massive amounts of cytosolic lipid droplets at the basolateral surfaces of the visceral endoderm of *Mttp*−/− yolk sacs (not shown). Moreover, these studies revealed that defective lipoprotein assembly is the likely cause of this finding. In yolk sacs from E9.5 *Mttp*+/+ and *Mttp*+/− embryos, there were numerous VLDL-sized lipoproteins within the endoplasmic reticulum and Golgi apparatus (Fig. 6*B*). In contrast, lipoprotein production was almost always absent in the visceral endoderm cells from E9.5 *Mttp*−/− embryos (Fig. 6*C*). Only very rare lipid-staining particles were observed in the *Mttp*−/− cells.

DISCUSSION

In this study, we demonstrate that half-normal levels of the MTP mRNA, protein, and triglyceride transfer activity in tissues of *Mttp*+/− mice are insufficient for normal levels of lipoprotein secretion. The hepatocytes of the *Mttp*+/− mice had reduced MTP levels and they exhibited fewer apoB-containing lipoproteins in the incubation medium, compared with hepatocytes from wild-type mice. On a chow diet, plasma levels of IDL/LDL cholesterol and apoB100 were reduced significantly in *Mttp*+/− mice, and on a high-fat diet, total cholesterol as well as VLDL and LDL cholesterol levels were reduced markedly. The lower plasma lipoprotein levels and the reduction in lipoprotein secretion from hepatocytes were accompanied by increased amounts of triglycerides within the liver and visceral yolk sac endoderm of *Mttp*+/− mice. These data indicate that there is no physiological compensation for reduced levels of MTP gene expression in *Mttp*+/− mice and that half-normal levels of MTP gene expression are accompanied by an unequivocal phenotype: decreased secretion of apoB and reduced plasma apoB levels. Thus, at least in the mouse, abetalipoproteinemia is a codominant rather than a recessive syndrome and, in this respect, is similar to familial hypobetalipoproteinemia, a codominant syndrome caused by apoB gene defects (14, 15, 28).

Textbooks (1) and reviews (7) have invariably stated that abetalipoproteinemia in humans is a classic recessive syndrome, with parents having normal plasma lipid levels. Indeed, several parents of abetalipoproteinemia patients have been reported to have total plasma cholesterol levels greater than 250 mg/dl (2, 29, 30), so at least some human "abeta heterozygotes" may be truly unaffected by half-normal MTP levels. On the other hand, it seems possible that heterozygosity for MTP deficiency could reduce LDL cholesterol and apoB levels by an average of ≈20%—similar to what we observed in mice—and that this effect might be overlooked within the extremely broad range of "normal" cholesterol levels. It is noteworthy that several cases of abetalipoproteinemia have

Table 3. Plasma lipid and apoB100 levels

Diet	*n*	Plasma concentration, mg/dl TC	TG	ApoB100
Chow diet				
Mttp+/+ female	14	62 ± 17	38 ± 15	5.3 ± 1.8
Mttp+/− female	13	60 ± 14	36 ± 12	3.8 ± 1.6*
Mttp+/+ male	15	71 ± 22	54 ± 14	4.2 ± 1.4
Mttp+/− male	16	63 ± 21	54 ± 19	3.0 ± 0.8†
High-fat diet (experiment 1, 30 days of diet)				
Mttp+/+ male	6	191 ± 13	23 ± 4	2.8 ± 0.7
Mttp+/− male	6	154 ± 23†	23 ± 10	2.5 ± 0.8
High-fat diet (experiment 2, 35 days of diet)				
Mttp+/+ male	7	211 ± 37	24 ± 4	2.1 ± 0.6‡
Mttp+/− male	10	166 ± 22†	31 ± 11	1.9 ± 0.4‡

Data represent means ± SD. All experiments were in mice after a 4-h fast. TC, total cholesterol; TG, triglycerides.
*$P = 0.02$ vs. *Mttp* +/+ mice.
†$P < 0.001$ vs. *Mttp*+/+ mice.
‡$P < 0.001$ vs. chow diet. Statistical significance determined by two-tailed *t* test.

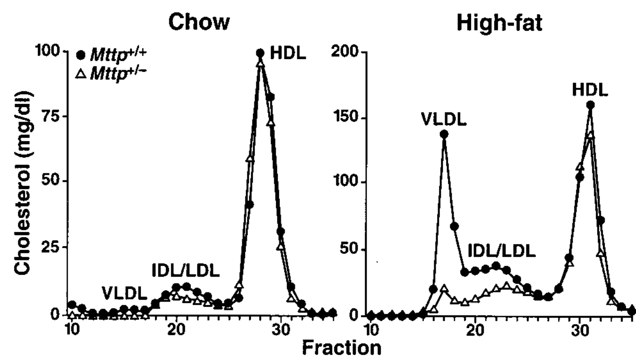

FIG. 4. Distribution of cholesterol within the plasma lipoproteins of *Mttp*+/+ and *Mttp*+/− mice. The plasma lipoproteins were size-fractionated on an FPLC column, and the cholesterol concentration in each fraction was measured enzymatically (15). (*Left*) Distribution of cholesterol in the plasma of mice fed a chow diet. The study was performed with plasma pooled from three female mice in each group. The FPLC profiles did not suggest that the size of *Mttp*+/+ and *Mttp*+/− lipoproteins differed. This conclusion was supported by electron microscopy, which showed the VLDL size distribution to be identical in chow-fed *Mttp*+/− and *Mttp*+/+ mice. (*Right*) Distribution of plasma cholesterol after 5 weeks on a high-fat diet. The study was performed with plasma pooled from four male mice in each group. Once again, electron microscopy revealed no differences in the VLDL size distribution in *Mttp*+/− and *Mttp*+/+ mice.

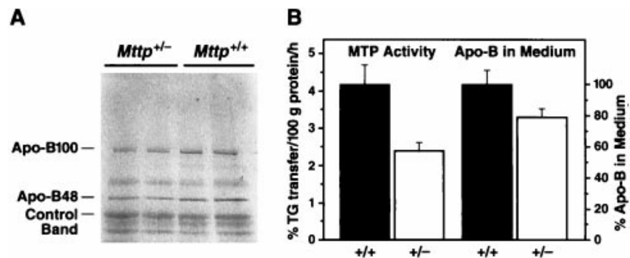

FIG. 5. ApoB accumulation in the medium of primary hepatocytes prepared from *Mttp*+/+ and *Mttp*+/− mice. (*A*) Media samples from primary hepatocytes were size-fractionated on SDS-polyacrylamide gels, and the ^{35}S-labeled proteins were visualized by autoradiography. (*B*) Triglyceride transfer activity and quantification of total apoB secretion in *Mttp*+/+ and *Mttp*+/− primary hepatocytes. Triglyceride transfer assays were performed as described (4, 23); the amounts of ^{35}S-labeled apoB48 and apoB100 in the media were quantified with a PhosphorImager and normalized to the amount of a non-apoB protein in the media ("control band"). The amount of total apoB (apoB100 and apoB48) in the medium was 21 ± 5% less in *Mttp*+/− primary hepatocytes, compared with *Mttp*+/+ cells ($P = 0.05$ by *t* test, $n = 4$ mice). This experiment was repeated twice, with the same results.

been described in which the parents had fairly low plasma lipid levels. Prior to the era of molecular genetics, Salt *et al.* (31) reported a case of "a-β-lipoproteinemia" (the first case given this designation) in which both parents had very low total plasma cholesterol levels (127 and 107 mg/dl). Although it might be argued that both parents actually may have had an *APOB* null allele, our results in *Mttp*+/− mice make it plausible that the parents of Salt's patient might have had hypocholesterolemia on the basis of reduced MTP activity levels. In the era of molecular genetics, there have been only a handful of genotypically proven human "MTP heterozygotes" for which lipid and apoB levels have been reported (29). From 15 heterozygotes, 7 had fairly low total cholesterol levels (133, 137, 148, 151, 161, 164, and 164 mg/dl), and 6 had distinctly low apoB levels (15, 28, 25, 38, 42, and 45 mg/dl) (29). These observations suggest that heterozygous MTP deficiency might result in hypocholesterolemia, at least in some humans. However, defining the effect of heterozygous MTP deficiency in humans in a definitive fashion obviously would require analyzing many large human kindreds with large numbers of genotypically proven heterozygotes.

The current studies provide new information regarding apoB mRNA levels in the setting of MTP deficiency. *APOB* mRNA levels have been reported to be significantly elevated in human patients with abetalipoproteinemia (32–34), suggesting the possibility of a feedback up-regulation of apoB gene transcription in the setting of deficient lipoprotein assembly. Using controlled RNase protection assays, we found *Apob* mRNA levels to be identical in *Mttp*+/+ and *Mttp*+/− adult mice and in *Mttp*+/+, *Mttp*+/−, and *Mttp*−/− yolk sacs. Our results are consistent with prior studies showing very small or absent changes in hepatic apoB mRNA levels in response to profound dietary interventions (35, 36) or genetic interventions that cause massive fat accumulation in the liver (37); however, we recognize that studies with livers from adult mice with complete MTP deficiency (i.e., liver-specific knockout mice) would be helpful in further analyzing this issue.

Most of the homozygous *Mttp* knockout embryos died at midgestation, and the few that survived past that time point had exencephalus, a phenotype first observed by Homanics *et al.* (14) in the setting of apoB70 mice and later by others in the setting of *Apob* gene knockouts (15, 16). At the light and electron microscopy level, there was a substantial accumulation of cytosolic fat in the *Mttp*−/− visceral yolk sac endoderm, reflecting deficient lipoprotein secretion—virtually identical to pathology observed in *Apob*−/− yolk sacs

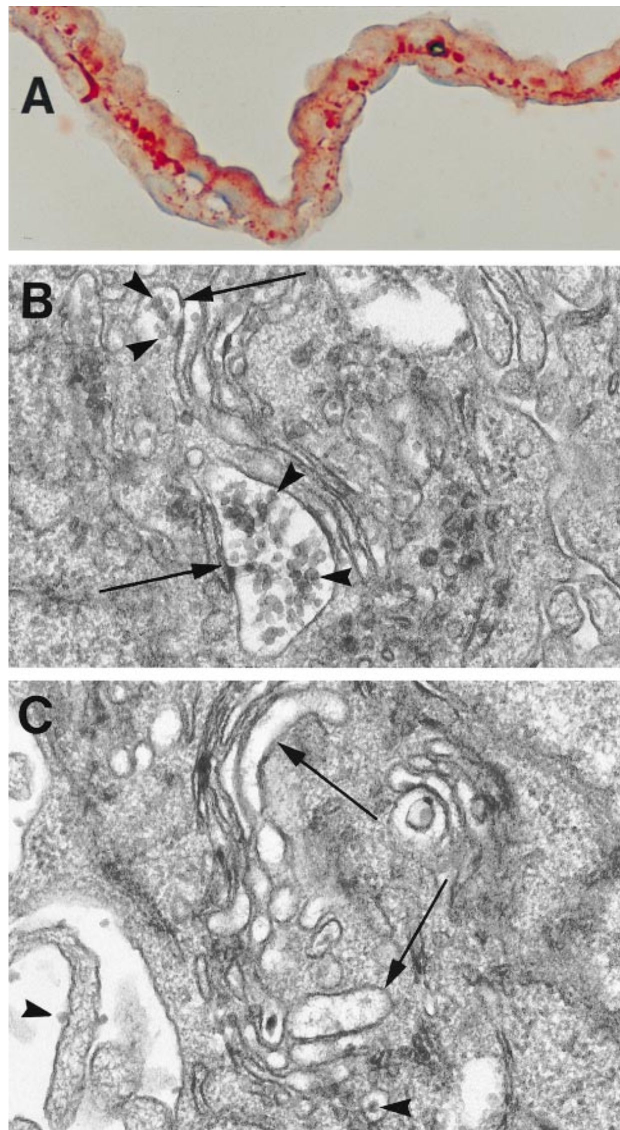

FIG. 6. Examination of the yolk sac membranes of mouse embryos at E9.5. (*A*) Oil red O staining of the yolk sac membrane from an *Mttp*−/− embryo. (*B* and *C*) Electron micrographs of imidazole-stained visceral endoderm cells from the yolk sacs of an *Mttp*+/− embryo (*B*) and an *Mttp*−/− embryo (*C*). In the *Mttp*+/− cells, there were invariably clusters of lipid-staining nascent VLDL particles (arrowheads) filling Golgi secretory vesicles (arrows). The lipoproteins were ≈300–800 Å in diameter. The clusters of lipoproteins were invariably absent from Golgi vesicles (arrows) of *Mttp*−/− yolk sac cells. However, a few lipid-staining particles were identified occasionally in the Golgi compartments and in the adjacent extracellular space (arrowheads) in *Mttp*−/− yolk sacs. (×34,500.)

(17). In their analysis of *Apob*−/− embryos, Farese *et al.* (17) suggested that deficient yolk sac lipoprotein production might reduce the delivery of lipid nutrients to the developing embryo at a critical time point when the chorioallantoic placenta is not yet established. In this study, we noted that the *Mttp*−/− embryos were pale and appeared to be deficient in erythropoiesis. Defects in erythropoiesis are known to be a cause of midgestational embryonic death (38). We speculate that impaired lipid nutrition might interfere with the capacity of the embryo to generate new red blood cells. The cause of the neurodevelopmental abnormalities has not been defined precisely, but we speculate that this finding could be a result of either deficient delivery of lipids for cell membranes in the brain or, more simply, a deficiency of fuel for developing neurons.

At the ultrastructural level, there were subtle differences in the yolk sacs of *Mttp*−/− embryos, compared with *Apob*−/− yolk sacs (17). In the *Apob*−/− yolk sacs, apoB synthesis and secretion appeared to be completely absent, whereas very rare lipid-staining particles were observed within the Golgi compartments of the *Mttp*−/− yolk sacs. These results suggest the possibility that lipoprotein assembly could occur in the absence of MTP, but at an extremely low efficiency. This finding would be consistent with reports of trace levels of apoB-containing lipoproteins in the plasma of patients with abetalipoproteinemia (34, 39–41).

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