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Doubling Expression of the Low Density Lipoprotein Receptor by Truncation of the 3′-Untranslated Region Sequence Ameliorates Type III Hyperlipoproteinemia in Mice Expressing the Human ApoE2 Isoform*

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The primary receptor mediating clearance of apolipoprotein (apo)E- and apoB100-containing lipoproteins from the circulation is the low density lipoprotein (LDL) receptor. Reduced expression of the *LDLR* is believed to be a precipitating factor in the pathogenesis of type III hyperlipoproteinemia (HLP) in some humans homozygous for the apoE2 allele (*APOE*2*). To test the effect of genetic changes in LDL receptor expression on the pathogenesis of type III HLP, we have generated a variant allele at the endogenous mouse *Ldlr* locus that expresses the human LDL receptor transcript. Transcription of the human *LDLR* minigene is regulated by the endogenous mouse promoter sequence, but a truncation of 3′-untranslated region results in increased mRNA stability. Consequently, in liver of heterozygotes, steady state levels of mouse and human *LDLR* transcripts are 50 and 180% the levels of total transcript in wild type mice, respectively. Overall, the 2.3-fold normal level of *LDLR* message in heterozygotes completely ameliorates type III HLP caused by the homozygosity for the human *APOE*2* allele, normalizing their plasma lipoprotein profile. We conclude that a modest increase in expression of the *LDLR* through message stabilization is sufficient to prevent precipitation of type III HLP in mice.

Type III hyperlipoproteinemia (type III HLP)[1] is a disorder of lipoprotein metabolism that leads to elevated plasma cholesterol and triglyceride concentrations due mainly to an increase of apoB-containing remnant lipoproteins. These particles are the product of lipolytic processing of chylomicrons and very low density lipoproteins (VLDL) derived from the intestine and liver, respectively. Like low density lipoproteins (LDL), these are atherogenic lipoproteins, and subjects with type III hyperlipoproteinemia are predisposed to atherosclerosis and premature death from myocardial infarction (1).

Type III HLP is a genetic disease associated with the expression of a metabolically impaired apoE protein (2, 3) or apoE deficiency (4). It is most commonly associated with individuals homozygous for the APOE*2 allele, the product of which has decreased affinity for the LDL receptor compared with other common isoforms. However, homozygosity of APOE*2 is necessary but not sufficient for the common form of type III hyperlipoproteinemia, as only 5–10% of adult homozygotes develop this disorder. In fact, most APOE*2 homozygotes have mild hypocholesterolemia and reduced atherosclerosis risk (5).

It has long been appreciated that other genetic and environmental factors besides possession of two *APOE*2* alleles are required for development of type III HLP. Type III HLP rarely manifests before adulthood, is more prevalent in men than women, and has an earlier age of onset in men than women (6). Women tend to express type III hyperlipoproteinemia only after menopause. Earlier onset is also associated with obesity, excessive alcohol consumption, diabetes mellitus, and hypothyroidism (1). The mechanism by which these conditions induce type III HLP is unclear. One possibility may be that these conditions result in down-regulation of hepatic LDL receptor activity, leading to reduced uptake of atherogenic lipoproteins containing apoE from plasma. For instance, estrogen has been shown to increase hepatic LDL receptor activity (7). The decrease in plasma estrogen levels in postmenopausal women would therefore be thought to lead to decreased hepatic LDL receptor activity and increased circulation time of remnant lipoproteins.

Previously we have generated mice that express human apoE2 in a physiologically regulated manner by replacing the coding sequences of the endogenous mouse *Apoe* gene with the human *APOE*2* allele (*Apoe*$^{2/2}$ mice). These mice exhibit full penetrance of the type III HLP phenotype, regardless of age or gender in the presence of normal murine LDL receptor expression (8). This suggested to us that genetic factors that trigger the type III HLP phenotype in some humans are already present in mice. One possible factor could be a relatively low hepatic level of the LDL receptor in mice. Others have developed transgenic mice expressing human apoE2 that exhibit features of type III HLP when endogenous apoE is absent (9, 10). Unregulated overexpression of *LDLR* gene by adenovirus-mediated gene transfer has been shown to be effective in normalizing the plasma lipid profiles in these mutants (11). Here we

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[1] The abbreviations used are: type III HLP, type III hyperlipoproteinemia; apoE, apolipoprotein E; FPLC, fast performance liquid chromatography; *Gapdh*, glycerophosphate dehydrogenase gene; HDL, high density lipoprotein; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; *Neo*, neomycin phosphotransferase gene; VLDL, very low density lipoprotein; bp, base pair; kb, kilobase pair; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; AREs, AU-rich elements; IDL, intermediate density lipoprotein.

report that moderate and controlled overexpression of the LDL receptor completely ameliorates the type III hyperlipoproteinemia phenotype of the *Apoe*$^{2/2}$ mice. This increase in *LDLR* expression was achieved through enhanced stability of the *LDLR* mRNA in mice engineered to express human LDL receptor in place of the mouse LDL receptor.

EXPERIMENTAL PROCEDURES

Construction of the Human LDLR Replacement Targeting Vector—As a 5′ region of homology, a 7-kb fragment containing intron 1 of the mouse *Ldlr* gene was isolated from plasmid pSIT1. Plasmid pSIT1 was used for a targeted disruption of the murine *Ldlr* gene by Ishibashi *et al*. (12) and was kindly provided by Dr. Shun Ishibashi, University of Tokyo, Japan. A 12-kb *Sal*I/*Not*I fragment containing the human *LDLR* minigene was isolated from plasmid pMY3, which was used for generating *LDLR* transgenic mice by Yokode *et al.* (13) and was kindly provided by Dr. Masahiro Yokode, University of Kyoto, Japan. The human minigene consists of a 7.5-kb fragment of a *Sal*I site in intron 1 through an *Eco*RI site in exon 5 of the human genomic DNA, a 2.2-kb fragment of an *Eco*RI site in exon 5 through a *Sma*I site in exon 18 of the human *LDLR* cDNA (14), and a 0.7-kb fragment of human growth hormone poly(A) addition signal sequence. As a 3′ region of homology, a 1.4-kb fragment from a *Sal*I site in exon 4 through *Sac*I site in intron 4 of the mouse gene was amplified from mouse genomic DNA by PCR using primers designed from published cDNA sequence (15). These fragments were inserted into a TK− Neo vector to yield the targeting construct shown in Fig. 1*A* below. The neomycin phosphotransferase (*Neo*) gene was placed in the same transcriptional orientation as the *LDLR* gene. The human minigene and the *Neo* gene replaces ~8 kb of DNA from an *Xho*I through a *Sal*I site in exon 4 of the mouse *Ldlr* gene.

Replacement of the Murine Ldlr Gene with the Truncated Human LDLR Minigene—The targeting vector was linearized and introduced into embryonic stem cells, TC-1, and cultured under selection media as previously described (16). Cells resistant to both G418 and ganciclovir were clonally isolated and screened by PCR using a *Neo*-specific primer 5′-GCT TCC TCG TGC TTT ACG GT-3′ for the targeting construct and a primer 5′-GCA AGA TGG CTC AGC AAG CA-3′ corresponding to intron 4 sequence. Correct targeting was confirmed by Southern blot analysis using a probe derived from exon 4 of the mouse gene.

Chimeras were generated from targeted ES cells and bred with C57BL/6 mice to obtain germ line transmission of the modified chromosome. The genotype of the modified allele (h) in the animals was determined by the presence of a 300-bp PCR fragment produced by using the *Neo*-specific primer above and a 3′ exon 4-specific primer, 5′-GCA GTG CTC CTC ATC TGA C-3′. The wild type mouse *Ldlr* allele (+) was detected as a 380-bp PCR fragment produced by a 5′ exon 4-specific primer 5′-CTC CCA GGA TGA CTT CCG AT-3′ and the 3′ exon 4-specific primer above.

Mice heterozygous for the targeted *Ldlr* gene (*Ldlr*$^{h/+}$) were bred with *Apoe*$^{2/2}$ mice that were homozygous for targeted replacement of the mouse *Apoe* gene with the human *APOE*2* gene (8). Doubly heterozygous mice were crossed to *Apoe*$^{2/2}$ mice again to generate mice homozygous for the human *APOE*2* gene and heterozygous for the targeted *Ldlr* locus (*Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice). These mice were further crossed to *Apoe*$^{2/2}$ mice to generate littermates homozygous for the human *APOE*2* gene with wild type mouse *Ldlr* (*Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice) or homozygous for the human *APOE*2* gene and heterozygous for the human *LDLR* gene (*Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice) for characterization. All mice used for characterization were a mix of strains 129 and C57BL6/J and were fasted for 4 h before plasma lipid analysis. Mice were fed normal chow (Prolab RMH 3000, number 5P76, St. Louis, MO) or western-style diet (0.15% (w/w) cholesterol and 21% (w/w) fat, TK 88137, Teklad Premier, Madison, WI) *ad libitum*.

Hepatic LDLR mRNA Analysis—Mice were sacrificed with an overdose of 2-2-2 tribromoethanol, and livers were harvested, flash frozen in liquid nitrogen, and stored at −70 °C. RNA was prepared from the tissue using Trizol reagent following standard protocols (17). For Northern blot analysis, a 380-bp fragment of mouse exon 4 and a 350-bp fragment of human exon 4 were used as probes specific for the mouse and human genes, respectively. A primer extension assay was designed to quantitate both human and mouse message simultaneously using a primer 5′-GGA GCA CGT CTT GGG GGG ACA GCC T-3′ corresponding to a shared sequence within exon 3 of the two *LDLR* genes. Dideoxy-CTP terminates extension of the primer, resulting in murine message extension by 5 bp and human message extension by 3 bp. The 30- and 28-bp fragments were separated in a denaturing 20% polyacrylamide gel. A primer 5′-GCA GCG AAC TTT ATT GAT GGT ATT 3′ was included in each reaction to determine the expression of the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene as an internal control. Primer extension analysis was performed on 50 μg of total mRNA as previously described (18). Image densitometry was used to determine relative amounts of message (FLA-2000, Fuji Photo Film USA Inc., Elmsford, NY). To determine the decay rate of the *LDLR* mRNA, heterozygous animals were injected via tail vein with actinomycin D (150 μg/100 g body weight) and α-amanitin (50 μg/100 g body weight). Animals were sacrificed at various time points after injection, and liver mRNA was prepared using standard protocols for primer extension analysis.

Plasma Lipid Analysis—Plasma was isolated and total cholesterol, HDL cholesterol, and triglycerides were measured as described previously (19). Equal volumes of plasma from at least five sex- and age-matched animals were pooled, and lipoproteins were separated by ultracentrifugation or by fast protein liquid chromatography (FPLC) as described (20). Lipoprotein fractions were dialyzed against phosphate-buffered saline, pH 7.4, and subjected to SDS-PAGE analysis as described (20).

In Vivo Clearance of VLDL and LDL—VLDL and LDL fractions were isolated from plasma of apoE-deficient mice (21) by ultracentrifugation for use as tracers for the turnover studies. The VLDL and LDL were radioiodinated with ^{125}I and ^{131}I, respectively, using previously published procedures (22, 23). The external jugular vein of recipient mice was exposed and cannulated with a sterile tapered microrenathane catheter, and 3 μCi (specific activity ~10^5 cpm/μg protein) of each radiolabeled ligand were injected. Immediately after dose injection, the catheter was removed, and the skin incision was closed using interrupted sutures. Blood samples were collected by retro-orbital sampling of anesthetized mice at various time points, and radiolabel remaining in the plasma was quantified with a gamma counter.

RESULTS

Replacement of the Mouse Ldlr Gene with the Truncated Human LDLR Minigene—The targeting strategy used to disrupt the mouse *Ldlr* gene and replace it with exons 2–18 of the truncated human *LDLR* minigene is illustrated in Fig. 1. Homologous recombination between the endogenous locus (Fig. 1*A*) and the targeting construct (Fig. 1*B*) results in a hybrid gene in which the human *LDLR* minigene is expressed under the control of the endogenous promoter (Fig. 1*C*). All 5′-regulatory sequences and exon 1 of the endogenous mouse locus are intact, but ~8 kb of mouse DNA spanning 3′ of intron 1 through exon 4 is replaced with the human *LDLR* minigene. The 3′-UTR of the minigene is shortened by a deletion of two of the three 3′ "AU-rich elements" that destabilize the mRNA transcript (24, 25). In addition, the human *LDLR* minigene contains the poly(A) addition signal sequences of the human growth hormone gene. Because exon 1 codes only for the signal peptide sequence, the mature protein transcribed from the chimeric gene is entirely human protein. The modified locus was transmitted to the F1 generation from chimeras that were made from one of the targeted ES cell lines. All F1 heterozygote matings produced normal litter sizes with a normal Mendelian segregation pattern of the modified locus. We designate the modified allele as *hLDLR* (or h) to distinguish it from the wild type mouse *Ldlr* allele (or +).

Increased Steady State LDLR mRNA Levels by Enhanced Message Stability in Heterozygous Mice—The expression of the *LDLR* was confirmed by Northern blot analysis of mRNA with a probe specific for the human *LDLR* mRNA. The human message of ~3 kb was present only in animals heterozygous for the *hLDLR* (*Ldlr*$^{h/+}$) with no band detected in wild type mice (*Ldlr*$^{+/+}$) (Fig. 2*A, upper band*). The presence of a 4.5-kb mouse *Ldlr* message was confirmed in both *Ldlr*$^{+/+}$ and *Ldlr*$^{h/+}$ mice by hybridizing the same blot with a probe specific for the mouse message (Fig. 2*A, lower panel*). The *Ldlr*$^{h/+}$ mice had ~50% of wild type mouse *Ldlr* message levels, in accordance with the loss of one copy of the mouse *Ldlr* gene.

To estimate simultaneously the amount of both human and mouse messages in homozygotes, we devised a primer exten-

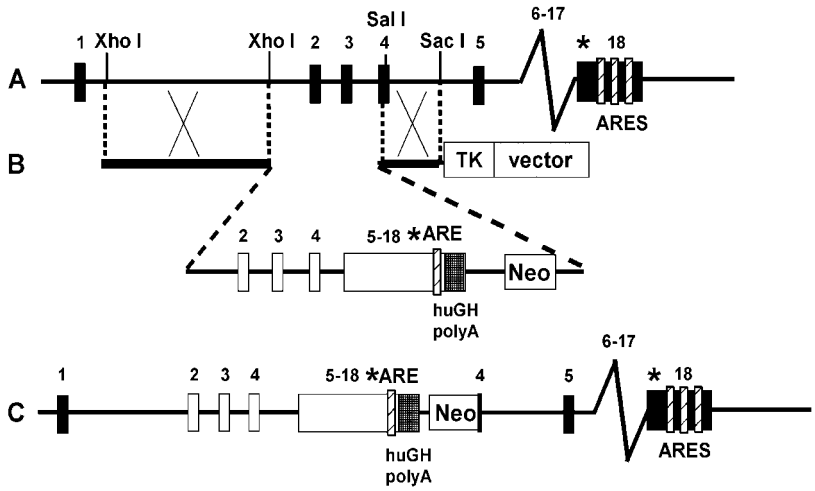

FIG. 1. **Replacement of the murine *Ldlr* gene with the human *LDLR* minigene.** *A,* genomic organization of the mouse *Ldlr* gene containing exons 1–18 (*black boxes*). The three AU-rich elements (*ARES*) in exon 18 are depicted as *white rectangles.* An *asterisk* indicates the position of the stop codon. *B,* the targeting construct containing the 5′ and 3′ arms of mouse homology (*thick black lines*) interrupted by exons 2–4 of the human *LDLR* gene followed by the *LDLR* cDNA starting with exon 5. *White boxes* indicate exons and *thin lines* indicate introns of the human gene. The exon 18 is truncated after the first ARE (*white rectangle*) and followed by the human growth hormone poly(A) addition signal sequence (*black box*). A neomycin-phosphotransferase (*Neo*) and thymidine kinase gene (*TK*) was inserted for selection of targeted ES cell colonies. *C,* the correctly targeted locus results in a chimeric gene encoding the human *LDLR*.

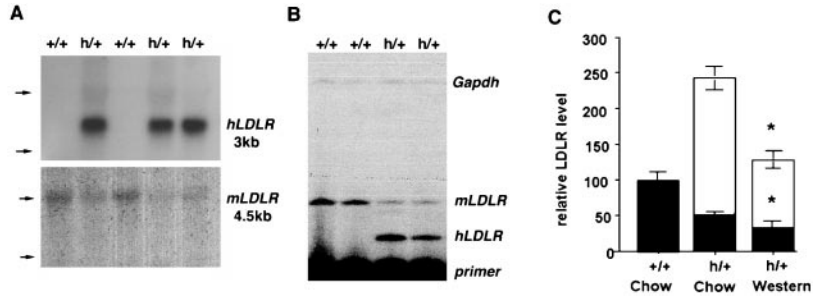

FIG. 2. **Expression of the *LDLR* genes.** *A, upper panel,* Northern blot analysis using a probe specific for the human *LDLR* gene. RNA was isolated from wild type (+/+) or $Ldlr^{h/+}$ (*h*/+) mice as genotyped by PCR analysis. *Arrows* indicate positions for 28 S and 18 S RNA. *Lower panel*, the blot above was stripped and re-hybridized to a probe specific for the mouse *Ldlr* gene. *B,* primer extension analysis of hepatic RNA isolated from wild type mice (+/+) and $Ldlr^{h/+}$ heterozygotes (*h*/+). The bands representing the mouse LDLR, the human *LDLR*, and *Gapdh* transcripts are indicated. *C,* liver RNA was isolated from six female +/+ mice, six female h/+ mice fed normal chow, and nine h/+ mice fed western style diet. Primer extension was performed on individual samples distinguishing human and murine transcripts, and each was quantified by densitometry and values are shown as mean ± S.E. Mouse message, *black box*; human message, *white box*. Both mouse and human LDLR messages are significantly reduced (* $p < 0.001$) in mice fed western-style diet compared with those in mice fed normal chow.

sion strategy utilizing a primer that hybridizes to a sequence in exon 3 shared in both the human and mouse *LDLR* genes (Fig. 2*B*). The murine message is extended 5 base pairs, and the human message is extended 3 base pairs before termination with dideoxy-CTP. In heterozygotes, the steady state level of the human *LDLR* transcript is 3.8 ± 0.3 ($n = 18$) times the level of the mouse transcript ($p < 0.001$). Since the levels of mouse *Ldlr* message in the $Ldlr^{h/+}$ mice are one-half that in wild type mice, total message (mouse + human) is increased 2.3-fold (Fig. 2*C*). There is no down-regulation of the mouse message in response to the higher levels of human message, nor adjustment of total mRNA levels in the $Ldlr^{h/+}$ mice, despite the fact that transcriptional regulatory elements in the *LDLR* gene are intact. The steady state human message is reduced to 68% of the normal chow level in response to dietary cholesterol loading (Fig. 2*C*) indicating that the sterol-responsive transcriptional regulation of the receptor is intact and functional (26).

To determine if the increased steady state level of the *hLDLR* mRNA is due to message stabilization, actinomycin D and α-amanitin were administered *in vivo* to $Ldlr^{h/+}$ mice. These drugs inhibit transcription, allowing for the measurement of mRNA decay rates (27). Differentiation between the mouse and human *LDLR* messages by primer extension was then used to determine relative decay rates of these transcripts in the liver (Fig. 3*A*). The time course analysis (Fig. 3*B*) shows that the human message decayed with a half-life of about 160 min while the mouse message decayed with a half-life of 60 min. This confirms that loss of two AREs in the human *LDLR* transcript results in a large increase in message stability *in vivo* in the liver of mice.

Reduction of Plasma Lipids in Mice Expressing hLDLR—Both heterozygous ($Ldlr^{h/+}$) and homozygous ($Ldlr^{h/h}$) female mice expressing the *hLDLR* exhibited approximately a 4-fold reduction in steady state plasma cholesterol (mainly HDL cholesterol) when fed normal chow (21 ± 3 mg/dl in $Ldlr^{h/+}$, $n = 10$; 28 ± 8 mg/dl in $Ldlr^{h/h}$, $n = 8$, compared with 85 ± 9 mg/dl in $Ldlr^{+/+}$, $n = 12$ $p < 0.00001$). The reduction in plasma cholesterol due to expression of the stabilized *hLDLR* transcript can be viewed as a dominant trait, as there is little difference in plasma cholesterol between heterozygotes and homozygotes for the *hLDLR*. Consequently, all the remaining characterization was with heterozygous mice and their wild type littermates. There was a trend toward reduction of plasma triglycerides in $Ldlr^{h/+}$ mice (29 ± 6 mg/dl, $n = 10$) compared with wild type mice (35 ± 7 mg/dl, $n = 12$), but this reduction was not significant ($p = 0.23$). Both agarose gel electrophoresis (Fig. 4*A*) and FPLC analysis (Fig. 4*B*) of plasma showed pat-

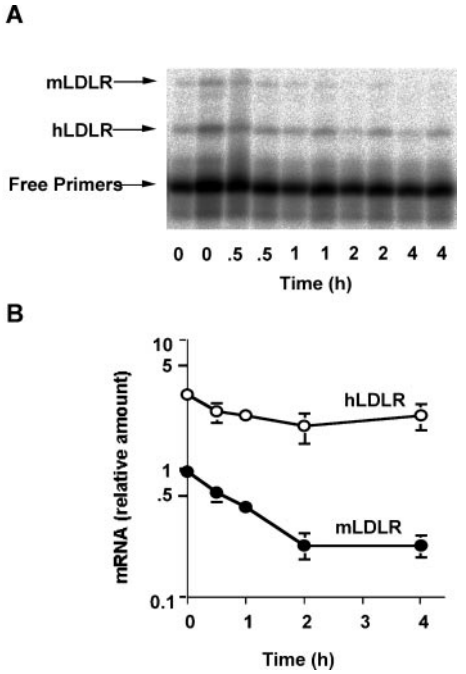

FIG. 3. **Increased human *LDLR* mRNA stability.** *A,* a radiograph of primer extension analysis of liver RNA isolated from individual mice given a bolus tail vein injection of actinomycin D (150 μg/100 g body weight) and α-amanitin (50 μg/100 g body weight) at time 0. Mice were sacrificed at the times post-injection indicated. Each lane represents the RNA from an individual $Ldlr^{h/+}$ mouse. *B,* decay of human and mouse LDLR mRNA in heterozygotes. mRNA levels for hLDLR (*open circles*) and mLDLR (*closed circles*) are expressed relative to the average levels of mLDLR at time 0. Mean ± S.E. from 3 to 6 animals at each time point except for time 0 (10 animals). Half-life for hLDLR (160 min) and for mLDLR (60 min) was calculated from the first 2 h of the data.

terns consistent with a reduction of plasma lipid and HDL cholesterol. In the $Ldlr^{h/+}$ mice, both β- and pre-β-migrating particles, as well as α-migrating particles, were dramatically reduced. SDS-PAGE analysis of plasma lipoproteins isolated by sequential density ultracentrifugation showed that the reduction in plasma lipid was accompanied by reductions in plasma apoproteins B, E, and AI (Fig. 4*C*). Although apoB100 is present in IDL-LDL fractions (p = 1.04–1.06 g/ml) of the wild type mice, it was virtually absent in the IDL-LDL fractions of the $Ldlr^{h/+}$ mice (the protein band corresponding to apoB100 was slightly degraded in this example). Plasma apoE was also reduced in all fractions of plasma from $Ldlr^{h/+}$ mice, with only a small amount of apoE remaining in the largest, lowest density particles (VLDL in fraction p = 1.006 g/ml).

When fed a diet containing 0.15% (w/w) cholesterol and 21% (w/w) fat, plasma cholesterol levels in $Ldlr^{+/+}$ and $Ldlr^{h/+}$ mice increased to 128 ± 10 and 81 ± 6 mg/dl, respectively. This increase was due mainly to the increase in HDL cholesterol, as shown by FPLC analysis of plasma from both groups of mice (Fig. 4*D*). This suggests that both wild type and heterozygous mice respond similarly to dietary cholesterol overload.

Increased LDLR Level Ameliorates Type III Hyperlipoproteinemia in $Apoe^{2/2}$ $Ldlr^{h/+}$ *Mice*—When a copy of the *hLDLR* allele was introduced into the mice expressing human *APOE*2* in place of mouse *Apoe*, these mice ($Apoe^{2/2}$ $Ldlr^{h/+}$) exhibited a 2.5-fold increase in steady state total LDL receptor mRNA levels when compared with $Apoe^{2/2}$ mice (data not shown). The ratios between the mouse *Ldlr* and *hLDLR* mRNA levels were similar in $Apoe^{+/+}$ $Ldlr^{h/+}$ and $Apoe^{2/2}$ $Ldlr^{h/+}$ mice.

The $Apoe^{2/2}$ $Ldlr^{+/+}$ mice maintained on normal chow had type III HLP, with plasma cholesterol levels of 268 ± 12 mg/dl and triglycerides of 157 ± 22 mg/dl. In contrast, their $Apoe^{2/2}$ $Ldlr^{h/+}$ littermates had normal plasma cholesterol and triglyc-

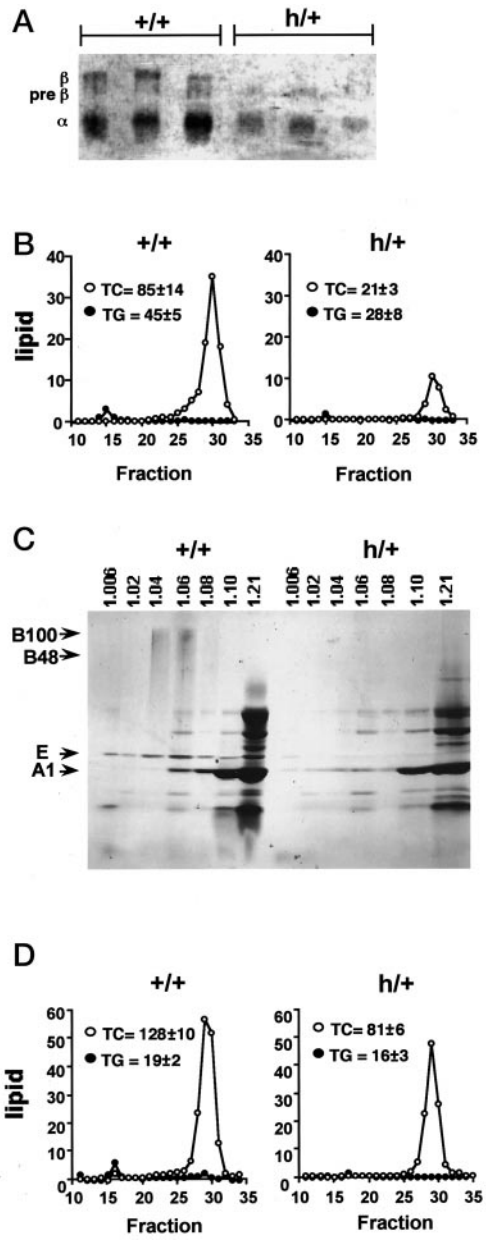

FIG. 4. **Plasma lipid and apolipoprotein distribution in wild type (+/+) or heterozygote (h/+) mice.** *A,* fasted plasma (1 μl) from males fed normal chow was electrophoresed in a 1% agarose gel and stained with Fat Red 7B. The positions of α-, β-, and pre-β-lipoproteins are indicated. *B,* pooled plasma (100 μl) from 6 male mice fed normal chow was analyzed by FPLC on a Superose 6B column. 0.5-ml fractions were collected, and lipid was measured as μg/fraction. *TC*, total cholesterol (*open circles*); *TG*, triglycerides (*closed circles*). The *numerical values* represent the total plasma levels in mg/dl ± S.D. from six animals. *C,* pooled plasma (1 ml) from $Apoe^{+/+}$ $Ldlr^{+/+}$ (+/+). and $Apoe^{+/+}$ $Ldlr^{h/+}$ (h/+) mice fed normal chow was fractionated by sequential density ultracentrifugation, and each fraction was electrophoresed in a denaturing 3–20% SDS-PAGE gradient gel and stained with Coomassie Brilliant Blue. Density fractions are as follows: *1.006*, <1.006 g/ml; *1.02*, 1.006–1.02 g/ml; *1.04,* 1.02–1.04 g/ml; *1.06*, 1.04–1.06 g/ml; *1.08*, 1.06–1.08 g/ml; *1.10*, 1.08–1.10 g/ml; and *1.21*, 1.10–1.21 g/ml. *D,* 100 μl of plasma pooled from at least five male mice fed western-style diet was analyzed by FPLC on a Superose 6B column. 0.5-ml fractions were collected, and lipid was measured as μg/fraction. *TC*, total cholesterol (*open circles*); *TG,* triglycerides (*closed circles*). The numerical values represent the total plasma levels in mg/dl ± S.D.

eride levels of 83 ± 4 and 40 ± 5 mg/dl, respectively. These values in the $Apoe^{2/2}$ $Ldlr^{h/+}$ mice are similar to those of wild type mice, suggesting that the 2.5-fold increase in *LDLR* expression is sufficient to normalize the plasma lipids of the

Apoe$^{2/2}$ mice. The marked reductions in plasma cholesterol and triglycerides observed in the *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice can be accounted for by the reduction in β-VLDL, as shown by agarose gel electrophoresis of whole plasma (Fig. 5*A*). The lipid content of α-migrating HDL particles in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice was not reduced in the presence of the *hLDLR* allele, which is in distinct contrast to the marked reduction of HDL in *Apoe*$^{+/+}$ *Ldlr*$^{h/+}$ mice, which have wild type mouse apoE protein.

FPLC analysis of plasma from the *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice (Fig. 5*B*) confirmed that reductions in plasma cholesterol were due to the reduction of cholesterol in large lipoproteins (in the VLDL and IDL range). There was no change in HDL cholesterol compared with *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice. In addition, VLDL triglycerides are dramatically reduced in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice, whereas triglycerides in remnant particles (fractions 19–25) are virtually eliminated. One diagnostic criterion for type III HLP is a ratio of cholesterol/triglyceride in VLDL larger than 0.3. Although the ratio in *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice is 0.67, the ratio in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice is 0.2, less than the value required for the diagnosis of type III HLP in humans.

The amelioration of type III HLP in the mice with modestly increased LDL receptor levels is further shown by SDS-PAGE analysis of apoproteins in lipoproteins isolated by ultracentrifugation (Fig. 5*C*). As previously reported, *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice fed normal chow have large amounts of plasma apoB, especially apoB48, as well as apoE in the VLDL fractions (8). In contrast, *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice have markedly reduced apoB and apoE levels in all the non-HDL lipoprotein classes. One important difference between mice expressing mouse apoE and mice expressing human *APOE*2* is that the *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice do not show any reduction in apoAI (or cholesterol) in the HDL fractions, whereas *Apoe*$^{+/+}$ *Ldlr*$^{h/+}$ mice have significant reductions in both.

When challenged by western style diet for 3 weeks, *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice were still protected from type III HLP. Cholesterol levels increased in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice (172 ± 43 mg/dl $n = 5$) but were significantly lower than in *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice (546 ± 30 mg/dl, $n = 3, p < 0.0001$). The triglycerides in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice did not increase compared with levels on normal chow (35 ± 3 mg/dl $n = 5$), whereas they doubled in the *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice (310 ± 84 mg/dl $n = 3$). FPLC analysis of plasma from *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice (Fig. 5*D*) fed a high cholesterol diet demonstrated the continued protection against type III HLP, with increased cholesterol predominantly in the HDL fractions. In contrast, the *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice had markedly increased VLDL triglyceride as well as VLDL-LDL cholesterol with little change in HDL cholesterol (Fig. 5*D*). Taken together, these results demonstrate that a modest increase in the expression of LDL receptor mRNA can ameliorate the type III HLP phenotype in mice, making them more resistant to diet-induced hyperlipidemia.

Increased Clearance of Non-HDL Lipoproteins from Plasma of Apoe$^{2/2}$ *Ldlr*$^{h/+}$ *Mice*—To ascertain whether the normalized plasma lipid and lipoprotein levels in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice are due to an increase in functional LDL receptor activity, the clearance of radiolabeled VLDL and LDL obtained from mice deficient in apoE was measured. Fig. 6 shows that at 3 h after injection, significantly more VLDL and LDL remain in the plasma of *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice compared with *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice. This is consistent with our previous observations that *Apoe*$^{2/2}$ mice are unable to clear VLDL from the plasma completely 4 h post-injection (8). We conclude that the increased steady state *LDLR* mRNA levels in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice results in an increase in functional LDL receptor activity, with an increased fractional catabolic rate of VLDL, as well as LDL, and normalization of plasma lipid levels.

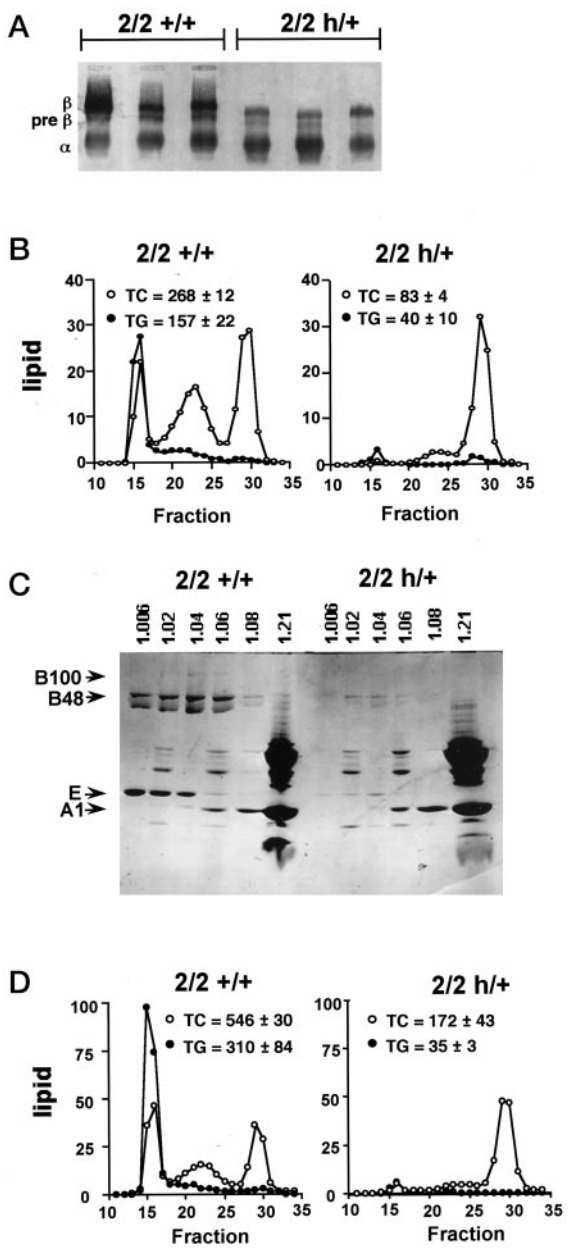

FIG. 5. **Plasma lipid and apolipoprotein distribution in mice expressing human apoE2 without (2/2 +/+) and with a human *LDLR* allele (2/2 h/+).** *A,* fasted plasma (1 μl) from males fed normal chow was electrophoresed in a 1% agarose gel and stained with Fat Red 7B. *B,* pooled plasma (100 μl) from 6 male mice fed normal chow was analyzed by FPLC on a Superose 6B column. 0.5-ml fractions were collected, and lipid was measured as μg/fraction. *TC,* total cholesterol (*open circles*); *TG,* triglycerides (*closed circles*). The numerical values represent the total plasma levels in mg/dl ± S.D. *C,* pooled plasma (1 ml) from *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ (*2/2 +/+*) and *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ (*2/2 h/+*) mice fed normal chow was fractionated by sequential density ultracentrifugation. Each fraction was electrophoresed in a denaturing 3–20% SDS-PAGE gradient gel and stained with Coomassie Brilliant Blue. Density fractions are as follows: *1.006*, <1.006 g/ml; *1.02*, 1.006–1.02 g/ml; *1.04,* 1.02–1.04 g/ml; *1.06*, 1.04–1.06 g/ml; *1.08*, 1.06–1.08 g/ml; *1.10*, 1.08–1.10 g/ml; and *1.21*, 1.10–1.21 g/ml. *D,* 100 μl of plasma pooled from at least five male mice fed western-style diet was analyzed by FPLC on a Superose 6B column. 0.5-ml fractions were collected, and lipid was measured as μg/fraction. *TC*, total cholesterol (*open circles*); *TG,* triglycerides (*closed circles*). The *numerical values* represent the total plasma levels in mg/dl ± S.D.

DISCUSSION

In marked contrast to humans, in which type III HLP affects 5–10% of *APOE*2* homozygotes, all mice homozygous for targeted replacement of human *APOE*2* exhibit features of type

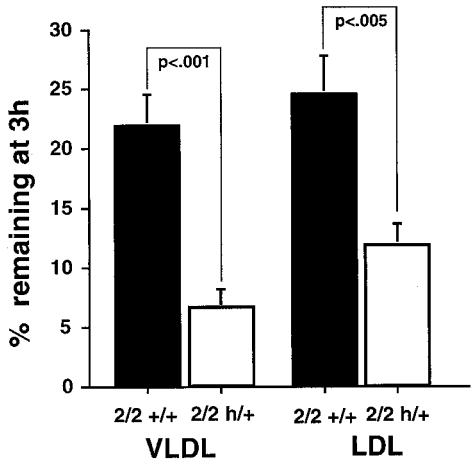

FIG. 6. **Plasma VLDL and LDL clearance rates in mice fed normal chow.** VLDL and LDL were isolated from apoE-deficient mice by ultracentrifugation. VLDL labeled with ^{125}I and LDL labeled with ^{131}I were injected via jugular vein into *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ (*2/2 +/+*) or *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ (*2/2 h/+*) mice. At 3 h post-injection, mice were bled retro-orbitally, and the remaining radiolabel in plasma was measured. Values shown are percentage of injected radiolabel amount remaining at 3 h in mean ± S.D.

III hyperlipoproteinemia regardless of age or gender (8). In the current study, we have modestly increased steady state hepatic *LDLR* mRNA levels by replacing the endogenous mouse *Ldlr* gene with an *hLDLR* minigene with increased mRNA stability. The *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice carrying this allele exhibit a 2.5-fold increase in total hepatic *LDLR* message, increased clearance of VLDL and LDL particles from the plasma, and a normal plasma lipid phenotype. Not only is this modest increase in hepatic *LDLR* mRNA sufficient to ameliorate the type III hyperlipoproteinemia, the *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ mice are more resistant to diet-induced hyperlipidemia than *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice.

Brown and Goldstein (26) have demonstrated a regulatory pathway that results in the down-regulation of *LDLR* gene transcription in response to increased intracellular sterol levels. However, regulation of transcription is only one method by which steady state levels of mRNA are altered. While the 5′ regulatory elements of the human *LDLR* have been studied in detail, the significance of 3′ regulatory elements in the regulation of cellular LDL receptor activity is only beginning to be appreciated. A few studies of the *LDLR* 3′-UTR indicate that it may play a significant role in regulation of *LDLR* mRNA levels. Wilson *et al*. (28) have identified three AU-rich elements (AREs) at positions 2690, 3257, and 3438 of the human transcript (14) based on sequence homology with the nonameric sequence UUAUUUAUU. This sequence is the minimal element contributing to a rapid turnover of several mRNAs such as those encoding immediate early genes and cytokines (24, 25). By fusing the 3′-UTR sequence of the human *LDLR* to the coding region of human β-globin gene, Wilson *et al*. (28) demonstrated that the 3′-UTR sequence of the *LDLR* gene confers a short constitutive half-life to the otherwise stable β-globin transcript in cultured cells. They showed that fusion constructs containing all three AREs have a 10-fold higher mRNA turnover rate compared with constructs lacking all three AREs. The three AREs contributed in an additive fashion to the mRNA destabilization rate, with constructs containing only the 5′-most ARE having just a 3-fold increase in the mRNA degradation rate. Since the chimeric transcript produced by *Ldlr*$^{h/+}$ mice contains only human sequence up to nucleotide 2804, it lacks two of the AREs. The lack of these two AREs results in a 3-fold increase in the stability of the *LDLR* message *in vitro* (28), an increase in stability similar to what is seen in the mice *in vivo* in our current study. Thus in *Ldlr*$^{h/+}$ mice there was a 3.8-fold higher steady state level of chimeric human message compared with wild type mouse message. Since both *mLDLR* and *hLDLR* alleles have identical transcriptional regulatory sequences, the increase in steady state levels of *hLDLR* message over the wild type *mLDLR* message must be the direct result of this increase in message stability.

It is important to note that we found neither down-regulation of the mouse gene accompanying the stabilized human gene nor normalization of the total *LDLR* mRNA levels in heterozygotes. Furthermore, the messages for both *mLDLR* and *hLDLR* alleles are reduced in response to increased dietary cholesterol to similar degrees, maintaining the same steady state ratio observed in mice fed normal chow. This suggests that there is no feedback mechanism to adjust the *LDLR* mRNA levels through transcriptional regulation when message stability is increased *in vivo*.

Transgenic mice overproducing the human LDL receptor were reported previously by Yokode *et al.* (13). These authors used the mouse metallothionein-I promoter sequence to drive expression of the human minigene in the transgenic mice. Similar to our *Ldlr*$^{h/+}$ mice, the transgenic mice on low fat diet had markedly reduced levels of plasma cholesterol and HDL cholesterol as well as apolipoprotein B and E. The most likely explanation for the low levels of HDL cholesterol in mice with increased LDL receptor activity is increased receptor-mediated clearance of HDL particles with apoE. However, whether other mechanisms such as inhibition of lipoprotein secretion by increased hepatic LDL receptor activity (29) contribute to lower HDL cholesterol in these mice require further studies. The transgenic mice generated by Yokode *et al.* (13) are completely protected against diet-induced hypercholesterolemia because of their unregulated overexpression of the *LDLR*. In contrast, HDL cholesterol increased in *Ldlr*$^{h/+}$ mice in response to increased dietary cholesterol, as in *Ldlr*$^{+/+}$ mice. This suggests the regulation of transcription of the *LDLR* gene by increased cellular sterol levels in *Ldlr*$^{h/+}$ mice is intact.

Whereas many factors besides decreased LDL receptor activity may precipitate type III HLP in human *APOE***2* homozygotes (30), it is clear that high hepatic expression of the LDL receptor is sufficient to overcome this phenotype in mice. Previous studies that test the effect of LDL receptor overexpression on type III HLP in mice have used very high levels of uncontrolled overproduction of the LDL receptor (9, 31). Our study is the first to show that as little as a 2.5-fold increase in LDL receptor mRNA results in increased clearance of VLDL and a 3–4-fold reduction of plasma cholesterol and triglycerides in mice expressing apoE2 fed normal chow. The fact that most human *APOE***2* homozygotes are slightly hypolipidemic, but all *Apoe*$^{2/2}$ *Ldlr*$^{+/+}$ mice are hyperlipidemic, suggests a possibility that the set point of expression of the *LDLR* may be higher in humans than in mice. Murine apoE can efficiently mediate clearance of plasma lipoproteins by LDL receptor-independent mechanisms[2] Thus, a relatively low set point of *LDLR* expression may not influence the overall metabolism of apoE-containing lipoproteins in mice except when mouse apoE is replaced with human apoE2, which is far less effective in mediating LDL receptor-dependent uptake of apoE-containing lipoproteins.

Three-quarters of LDL receptor proteins in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ heterozygotes are of human sequence. Our unpublished studies using mouse fibroblast cells show that VLDL containing mouse apoE or human apoE3 or apoE4 isoforms bind to the mouse LDL receptor equally well, whereas VLDL with apoE2 binds

[2] C. Knouff, V. Clavey, and N. Maeda, unpublished observations.

with similar affinity but with 50% B_{max} compared with VLDL containing the other apoE isoforms.[2] The results were similar when human cells were used.[2] Thus, species differences in the affinity of apoE for the LDL receptor do not appear to be playing a significant role. In contrast, Corsini *et al*. (32) have shown that human LDL has much lower affinity to mouse receptor than to human receptor, suggesting significant species effects are present in the interaction between apoB100 and the LDL receptor. Although we did not observe any increase of LDL in either heterozygotes (Fig. 4) or homozygotes (data not shown), our present study was carried out in *Apoe*$^{2/2}$ *Ldlr*$^{h/+}$ heterozygotes to reduce the potential complexities induced by interactions between mouse LDL and the human LDL receptor.

Several studies have shown that cellular LDL receptor activity can be regulated by post-transcriptional mechanisms, including changes in mRNA stability as well as altered protein stability. For example, treatment of HepG2 cells with phorbol-12-myristate-13-acetate has been shown to increase the stability of *LDLR* mRNA 2–2.5-fold by an indirect effect of destabilization of the actin cytoskeleton by this agent (33). Sequences in the extreme 3′-UTR of the *LDLR* have been shown to confer association of this RNA with the actin cytoskeleton. Lack of this region inhibits the PMA-induced stabilization of this RNA (28). Gemfibrozil, a fibrate drug used in the treatment of type III hyperlipoproteinemia in humans, also has been shown to enhance *LDLR* mRNA stability in human hepatoma cells (34). In this study pravastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, significantly increased sterol-responsive element-dependent transcription (with no effect on *LDLR* mRNA stability), whereas gemfibrozil increased *LDLR* mRNA stability 4–6-fold with no change in transcription rate. Whereas both drugs have efficacy in treating type III hyperlipoproteinemia, pravastatin lowers LDL cholesterol preferentially, and gemfibrozil treatment results in larger reductions in VLDL cholesterol and triglycerides (35). Whether these differences are the result of different mechanisms leading to increased LDL receptor activity or are due to other effects of these drugs on peripheral lipolysis or hepatic VLDL secretion has not been resolved.

That the regulation of *LDLR* mRNA stability may have a physiological role is suggested by the fact that depletion of hepatic sterol by the inhibition of squalene synthase results in increased *LDLR* mRNA transcription, stability, and translation in rats (36). Whether alterations of *LDLR* mRNA levels in humans due to regulated alterations in mRNA stability can be a "precipitating factor" for type III hyperlipoproteinemia is unclear. Nevertheless, our present study makes clear that changes in hepatic *LDLR* levels due solely to differences on *LDLR* mRNA stability can have a profound effect on plasma lipid levels in a mouse model of type III HLP.

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