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) 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 hypercholesterolemia 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 hyperthyroidism (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 (Apoe2/2 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
report that moderate and controlled overexpression of the LDL receptor completely ameliorates the type III hyperlipoproteinemia phenotype of the Apoe2/2 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 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 pSI1. Plasmid pSI1 was used for a targeted disruption of the murine Ldlr gene by Ishihashi et al. (12) and was kindly provided by Dr. Shun Ishihashi, University of Tokyo. A 12-kb Sall/Not1 fragment containing the human LDLR minigene was isolated from plasmid pMY3, which was used for generating 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 the human genomic DNA, a 2.2-kb fragment of an EcoRI site in exon 5 through a SmaI 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 Sali site in exon 4 through Sali site in exon 4 of the mouse gene was amplified from mouse genomic DNA by PCR using primers derived from published cDNA sequences (15). These fragments were inserted into a TK−Neo vector to yield the targeting construct shown in Fig. 1A 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 Xhol through a Sali 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 TCG TGG 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 Ldlr gene.

Chimera 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 300-6 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 (Ldlrh+/−) were bred with Apoe2/2 mice that were homozygous for targeted replacement of the mouse ApoE gene with the human ApoE2 gene (8). Doubly heterozygous mice were crossed to Apoe2/2 mice again to generate mice homozygous for the human APOE2 gene and heterozygous for the targeted Ldlr locus (Apoe2/2 Ldlrh+/− mice). These mice were further crossed to Apoe2/2 mice to generate littermates homozygous for the human APOE2 gene with wild type mouse Ldlr (Apoe2/2 Ldlr+/− mice) or homozygous for the human APOE2 gene and heterozygous for the human LDLR gene (Apoe2/2 Ldlrh+/− mice) for characterization. All mice used for characterization were a mix of strains 129 and C57BL/6J 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 quantify human and mouse message simultaneously using a primer 5′-GGA GCA CGT CTT GGG GGA ACC 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 25-bp fragments were separated in a denaturing 20% polyacrylamide gel. A primer 5′-GGA GCG AAC TTT ATT ATT GGT 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 a-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 dialedyzed against phosphate-buffered saline, pH 7.4, and subjected to SDS-PAGE analysis as described (20).

**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. 1A) and the targeting construct (Fig. 1B) results in a hybrid gene in which the human LDLR minigene is expressed under the control of the endogenous promoter (Fig. 1C). 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 “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 human 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 (Ldlrh+/−) with no band detected in wild type mice (Ldlr+/−) (Fig. 2A, upper band). The presence of a 4.5-kb mouse Ldlr message was confirmed in both Ldlrh+/− and Ldlrh−/− mice by hybridizing the same blot with a probe specific for the mouse message (Fig. 2A, lower panel). The Ldlrh−/− 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-
Reduction of Plasma Lipids in Mice Expressing hLDLR—

Constitutionally normal mice expressing the stabilized LDL receptor exhibit a plasma lipid profile similar to that described for familial type III hyperlipidemia when fed a high fat diet (21). Thus, the human LDLR was targeted in ES cells to create mice expressing the stabilized human LDL receptor.

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 ARES (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.

To determine if the increased steady state level of the hLDLR mRNA is due to message stabilization, actinomycin D and a-amanitin were administered in vivo to Ldlr<sup>h/+</sup> 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. 3A). The time course analysis (Fig. 3B) 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 ARESs 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<sup>h+</sup>) and homozygous (Ldlr<sup>h+/h+</sup>) 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<sup>h+</sup>, n = 10; 28 ± 8 mg/dl in Ldlr<sup>h+/h+</sup>, n = 8, compared with 85 ± 9 mg/dl in Ldlr<sup>h+/+</sup>, 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, the remaining characteristic was with heterozygous mice and their wild type littermates. There was a trend toward reduction of plasma triglycerides in Ldlr<sup>h+/+</sup> 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. 4A) and FPLC analysis (Fig. 4B) of plasma showed pat-
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body weight) at time 0. Mice were sacrificed at the times post-injection indicated. Each lane represents the RNA from an individual Ldlrh+/m 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 Ldlrh+/m mice, both 
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migrating particles, as well as 
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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. 4C). 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 Ldlrh+/m mice (the protein band corresponding to apoB100 was slightly degraded in this example). Plasma apoE was also reduced in all fractions of plasma from Ldlrh+/m 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 Ldlrh+/m and Ldlrh+/m 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. 4D). This suggests that both wild type and heterozygous mice respond similarly to dietary cholesterol overload.

Increased LDLR Level Ameliorates Type III Hyperlipoproteinemia in Apoe+/– Ldlrh+/m 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+/– Ldlrh+/m) exhibited a 2.5-fold increase in steady state total LDL receptor mRNA levels when compared with Apoe+/– mice (data not shown). The ratios between the mouse Ldrl and hLDLR mRNA levels were similar in Apoe+/– Ldlrh+/m and Apoe+/– Ldlrh+/m mice.

The Apoe+/– Ldlrh+/m 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+/– Ldlrh+/m littermates had normal plasma cholesterol and triglyceride levels of 83 ± 4 and 40 ± 5 mg/dl, respectively. These values in the Apoe+/– Ldlrh+/m 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<sup>2/2</sup> mice. The marked reductions in plasma cholesterol and triglycerides observed in the Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice can be accounted for by the reduction in β-VLDL, as shown by agarose gel electrophoresis of whole plasma (Fig. 5A). The lipid content of α-migrating HDL particles in Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice was not reduced in the presence of the hLDLR allele, which is in distinct contrast to the marked reduction of HDL in Apoe<sup>+</sup>/+ Ldl<sup>h<sup>+</sup></sup> mice, which have wild type mouse apoE protein.

FPLC analysis of plasma from the Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice (Fig. 5B) 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<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice. In addition, VLDL triglycerides are dramatically reduced in Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> 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<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice is 0.67, the ratio in Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> 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. 5C). As previously reported, Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice fed normal chow have large amounts of plasma apoB, especially apoB48, as well as apoE in the VLDL fractions (8). In contrast, Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> 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<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice do not show any reduction in apoAI (or cholesterol) in the HDL fractions, whereas Apoe<sup>+</sup>/+ Ldl<sup>h<sup>+</sup></sup> mice have significant reductions in both.

When challenged by western style diet for 3 weeks, Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice were still protected from type III HLP. Cholesterol levels increased in Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice (172 ± 43 mg/dl n = 5) but were significantly lower than in Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice (546 ± 30 mg/dl n = 3, p < 0.0001). The triglycerides in Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice did not increase compared with levels on normal chow (35 ± 3 mg/dl n = 5), whereas they doubled in the Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice (310 ± 84 mg/dl n = 3). FPLC analysis of plasma from Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice (Fig. 5D) 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<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice had markedly increased VLDL triglyceride as well as VLDL-LDL cholesterol with little change in HDL cholesterol (Fig. 5D). 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<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> Mice**—To ascertain whether the normalized plasma lipid and lipoprotein levels in Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> 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<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice compared with Apoe<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice. This is consistent with our previous observations that Apoe<sup>2/2</sup> 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<sup>2/2</sup> Ldl<sup>h<sup>+</sup></sup> mice result 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.

**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
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 Aponeo2/2 Ldlr<sup>+/-</sup> 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 Aponeo2/2 Ldlr<sup>-/-</sup> mice are more resistant to diet-induced hyperlipidemia than Aponeo<sup>2/2</sup> Ldlr<sup>-/-</sup> 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 nonmammalian sequence UUAUUAUUAU. 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<sup>h/-</sup> 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<sup>h/-</sup> 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<sup>h/-</sup> 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<sup>h/-</sup> mice in response to increased dietary cholesterol, as in Ldlr<sup>-/-</sup> mice. This suggests the regulation of transcription of the LDLR gene by increased cellular sterol levels in Ldlr<sup>h/-</sup> mice is intact.

Whereas many factors besides decreased LDL receptor activity may precipitate type III HLP in human APOE<sup>2</sup> 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<sup>2</sup> homozygotes are slightly hypolipidemic, but all Aposeo2/2 Ldlr<sup>-/-</sup> 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 (3). 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 Aposeo2/2 Ldlr<sup>h/-</sup> 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. The results were similar when human cells were used. 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<sup>−/−</sup> Ldlrh<sup>−/+</sup> 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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