Molecular bases of low production rates of apolipoprotein B-100 and truncated apoB-82 in a mutant HepG2 cell line generated by targeted modification of the apolipoprotein B gene

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Abstract **In subjects with familial hypobetalipoproteinemia heterozygous for truncated forms of apolipoprotein B, both apoB-100 and the truncated forms are produced at lower than expected rates. We studied the mechanism of low levels of apoB in a cell model produced by targeted modification of the *apob* gene of HepG2 cells. One of the three alleles of *apob* was found to be targeted. The targeted cells expressed apoB-100 and B-82. The media of mutant cells contained 56% of the levels of apoB-100 present in the media of wild-type (WT) HepG2 cells. ApoB-82 was present at 11% of the apoB-100 levels in mutant cell media. An 85-kD protein (apoB-15) representing the N-terminal fragment of apoB was also secreted, but only in the mutant cell media. We examined the mechanism of low levels of apoB-82. Cellular apoB-82 mRNA was 11% of apoB-100 mRNA, lower than the 33% expected, but consistent with relative levels of apoB-82 in the media. ApoB mRNA transcription in WT and the mutant cells did not differ, while the levels of apoB-82 mRNA in nuclei and polysomes were 46% and 12% of the levels of apoB-100 mRNA, respectively, suggesting that the lower levels of apoB-82 mRNA were due to altered message stability. In a pulse/chase experiment with [^{35}S] methionine, at zero time of chase, the amounts of apoB-100 in mutant cells was 66% that of WT levels, consistent with the modification of one allele. The fractions of newly synthesized apoB-100 secreted into the media at 2 h were 10% in the mutant cells and 19% in the WT cells, suggesting greater presecretory degradation of apoB-100 in the mutant cells. Thus, low levels of mutant apoB-82 mRNA gave rise to the low levels of apoB-82, while low levels of apoB-100 were due to low rates of secretion.**—Srivastava, R. A. K., N. Srivastava, M. Averna, A. B. Cefalu, and G. Schonfeld. **Molecular bases of low production rates of apolipoprotein B-100 and truncated apoB-82 in a mutant HepG2 cell line generated by targeted modification of the apolipoprotein B gene.** *J. Lipid Res.* **1999.** 40: **901–912.**

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Familial hypobetalipoproteinemia (FHBL) is an interesting condition defined by <5th percentile apoB and LDL-cholesterol levels segregating in human kindreds (1, 2). Heterozygotes possessing one copy of the mutant allele are usually asymptomatic, but homozygotes may have dietary fat malabsorption and its sequelae for the retina and the nervous system. Some forms of FHBL are caused by a variety of truncation-producing mutations in the *apob* gene (1–4). The plasmas of such heterozygotes contain discrete lipoprotein families possessing apoB-100, apoB-48 (post prandially) and the apoB truncation. Apolipoprotein (apo) B-100 is a 550 kD protein expressed in and secreted by mammalian livers in association with VLDL (5). ApoB-100 also serves as a ligand for the LDL receptor, thus mediating the clearance of apoB-100-containing lipoproteins from plasma (6). A shorter version, apoB-48, representing 48% of the colinear amino terminal sequence of apoB-100, is produced by posttranscriptional editing of the apoB mRNA in enterocytes of all mammals and in the hepatocytes of rodents (7, 8). ApoB-48 is secreted with chylomicrons (8). Plasma levels of apoB are subject to hormonal and dietary regulation (9).

Recent in vivo metabolic studies in humans have shown that the products of both the normal apoB-100 allele and the apoB truncation allele are produced at lower than expected rates in the FHBL heterozygotes (3, 4). We developed a cell model in which the cellular-molecular mechanisms underlying the reduced secretion could be studied. The HepG2 cell line was chosen because it expresses

Abbreviations: apoB, apolipoprotein B; HB, hypobetalipoproteinemia; LDL, low density lipoprotein; apo, apolipoprotein; FHBL, familial hypobetalipoproteinemia; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; WT, wild-type; DTT, dithiothreitol.

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many of the differentiated functions of hepatocytes, including the secretion of most apoproteins and apoB-containing lipoproteins (10). Although the secretion of apoB by HepG2 cells and other hepatomas is regulated to varying extents by posttranscriptional partitioning of apoB-100 between secretion or intracellular degradation (10–12), apoB production should also be altered when one of the alleles directs premature termination of translation, as such mutations affect the abundance of mRNA in most (13–17), but not in all (17–23) cases. To study the effects of a truncation mutation on the secretion of apoB and on cellular apoB mRNA metabolism, we targeted the *apob* gene of HepG2 cells by homologous recombination using the dual selection "in and out" strategy (24). Our results suggest that introduction of a premature translational stop codon into the *apob* gene destabilizes the mutant apoB mRNA, resulting in low levels of apoB-82 production. We also show that the secretion of full-length apoB decreased when one of the alleles synthesized truncated apoB. These mechanisms may account for the low levels of apoB-100 and apoB truncation in heterozygous hypobeta individuals.

MATERIALS AND METHODS

Preparation of targeting vector

An 8.477 kb *Pvu*II fragment of *apob* gene was excised from P1 vector containing the entire human *apob* gene (25). This fragment encompasses part of exon 26, all of exons 27 and 28, part of exon 29, and all of introns 26, 27, and 28 (Fig. 1). The *Pvu*II fragment was cloned into pBSKII vector at *Sma*I site. This vector lacks *Cla*I sites. At 11390 nt position of the apoB cDNA, the *Bgl*II site was restriction digested, filled in, and ligated, resulting in the insertion of 4 additional nucleotides, i.e., a frame-shift of translation and a premature stop codon, UGA, at nucleotide position 11417. Next, a 3.3 kb neo-tk cassette (kindly provided by Dr. Stephen Young, Gladstone Foundation, UCSF) containing its own promoter was cloned at the *Xho*I site in the polylinker region of the vector. This construct on linearization gave rise to 882 bp 5′ arm sufficient for homologous recombination. As a longer 5′ arm has been suggested to increase the targeting efficiency, we prepared another construct which on linearization gave rise to 1.6 kb 5′ arm, and lacked the last two apoB exons.

The linearized constructs (20 μg each) were electroporated into 25 million HepG2 cells, in a cuvette precooled on an ice-bath, at 960 μF and 250 V. After the electroporation, the cells were transferred into 200 ml complete media and distributed in 20 dishes of 100 mm size. The cells were allowed to revive for 24 h prior to putting them in selection media.

Selection and screening for clones

Several electroporations were done. The cells were allowed to grow in the first selection media containing G418 (800 μg/ml) for 2–3 weeks, and cells that survived formed distinct colonies. These colonies were transferred to 24-well plates and grown in the complete media. Genomic DNA was prepared using the PureGene kit (Gentra System, Inc., Research Triangle Park, NC 27709-13159). Clones obtained in the first selection were first screened by PCR using the following primers: 5′ primer (B-1) AAC CCC AAT GGC TAT TCA TTT TCC corresponding to nt 11264–11288, and 3′ primer (B-2) GGT CTG CTC AGG CAC GAT GAT corresponding to nt 11691–11711. The primers flanked the four base insertion site of mutation. The following PCR profile was used: denaturation 94°C for 30 sec, annealing 60°C for 40 sec, and extension 72°C for 40 sec for a total of 30 cycles, yielding products of 445 bp (WT allele) and 449 bp (mutant allele). Because of only 4 nt differences in the PCR products, they show on agarose gel as a single band. We subcloned several preparations of these fragments and sequenced them. All contained the expected sequence plus the four inserted nucleotides (GATC). As discussed above, the insertion of four additional nucleotides (GATC) abolished the *Bgl*II site. Therefore, restriction digestion of the 449 bp PCR product with *Bgl*II for the exogenous "allele" sequences that had been integrated into genome would be expected to yield an undigested product; the endogenous wild-type allele would be digested. PCR-amplified *apob* gene fragment from wild-type cells should be completely digested. As an undigested product may either result from incomplete restriction digestion or from the presence of a mutation, we always restriction digested a PCR product from WT HepG2 cells with *Bgl*II as a control. All HepG2 clones containing the introduced mutation were then screened by Southern blotting for targeting to the *apob* locus (see below).

Second selections for the "out" reaction were performed in the presence of 2 μm ganciclovir (a kind gift from Syntex, Palo Alto, CA). The colonies surviving were screened for the presence of the mutation by PCR. Documentation of the "out step" reaction was achieved by Southern blotting using a 3′ cDNA probe and by PCR analysis using specific primers for apoB (ATTGT AGTTCCCAATTGATC, corresponding to apoB cDNA sequence 7042 to 7023), and for vector (GCTCTAGAACTAGTGGATC, BlueScript sequnce in the polylinker region).

Southern blotting analysis

The strategies for Southern blotting are shown in Figs. 2 and 3. Ten μg of genomic DNA was digested with appropriate restriction endonucleases, and run in a 0.7% agarose gel containing 0.5 μg/ml ethidium bromide at 30 V for 16 h. The DNA from the gel was transferred to GeneScreen nylon membrane (NEN Research Products, Boston) using 0.4 m NaOH as transfer solvent by capillary blotting as described (26). The membrane was baked and hybridization was performed using Rapid-hyb buffer and the protocol provided by the manufacturer (Amersham Life Sciences Inc., Arlington Heights, IL). To document the presence of targeting of the *apob* gene, the cDNA probe was located in the *apob* gene 5′ to the targeted insert (see Fig. 2). To confirm the occurrence of "out step" reaction i.e., the removal of vector and *neo-tk* sequences, we used a cDNA probe 3′ to the targeted insert (Fig. 3). The cDNA probes were labeled with α-^{32}P[dCTP] using random prime kit (United States Biochemicals, Cleveland, OH) as described (26).

Detection of apoB-100 and apoB-82 in culture media

WT and the mutant cells were grown in complete media (DMEM plus 10% fetal calf serum) to 80–90% confluency, trypsinized, and washed, and 8×10^6 cells were plated in 75 cm^2 flasks. After 48 h in complete media, the spent media (15 ml) were transferred to sterile tubes and aprotinin (100 U) was added. The media were concentrated in a Centriprep-30 (Amicon, Inc., Beverly, MA). Final volumes were made to 1 ml with EDTA saline and apoprotein was added. Twenty-five μl of the concentrated media was immunoprecipitated using a rabbit anti-human apoB antibody (27, 28). The samples were run in 3–12% gradient SDS-polyacrylamide gels. After electrophoresis, proteins were transferred from the gel to PVDF membranes as described (28), and the membranes were probed with ^{125}I-labeled anti-apoB monoclonal antibody C1.4 (an N-terminal antibody) and also with B1B6 (a C-terminal apoB antibody) as described (29).

Membranes were exposed to X-ray film to visualize and quantitate apoB protein bands (27). Spent media from WT as well as the mutant cells were concentrated and fractionated by ultracentrifugation by adjusting the density to 1.065 g/ml with KBr (26). ApoB proteins were analyzed by Western blotting as described (27).

Pulse–chase experiments

Pulse–chase experiments were carried out to assess the rates of synthesis and secretion of the various forms of apoB into media of WT and the mutant cells. All experiments were carried out in collagen-treated dishes (12). Culture plates (100 mm) were treated with 2 ml of collagen (50 μg/ml) overnight. Next day plates were washed 3 times with PBS. WT and mutant cells (1×10^6/plate) were grown in complete media till the cells became confluent and formed a monolayer. Next day the cells were starved for 75 min in methionine-free media (MEM) followed by incubation in serum-free media with [^{35}S]methionine (35 μCi/ml) procured from ICN Biochemicals (15 mCi/ml). The cells were pulsed for 20 min followed by chase with 1000-fold cold methionine for various time intervals. At indicated time points, the media were removed, aprotinin (100 U) added, and concentrated in Centriprep-30 (Amicon Inc., Beverly, MA). Concentrated media were desalted by the addition of cold saline–EDTA containing protease inhibitors and centrifugation. Cells were washed with cold PBS and scraped off the plate and transferred into a 2-ml Eppendorf tube. Cells were pelleted and 500 μl of ice-cold lysis buffer (150 mm NaCl, 5 mm EDTA, 50 mm Tris, pH 7.4, 0.0625 m sucrose, 0.5% triton X-100, 0.05% sodium dexoycholate) containing protease inhibitors (PMSF, aprotinin, leupeptin, pepstatin A) was added and lysis was allowed to occur overnight in the cold room using a rotary shaker at low speed. Next day tube contents were centrifuged and cell lysates were transferred into another tube and the cell debris was discarded. After determining TCA precipitable counts, equal amounts of counts were used for apoB immunoprecipitation using polyclonal antibody, R197 (27). Newly synthesized apoBs secreted into the media were fractionated by density gradient centrifugation as described (27). In each fraction, apoBs were immunoprecipitated using polyclonal apoB antibody, separated in SDS-PAGE, and apoB bands were visualized by autoradiography. In some experiments cells were fractionated into membrane and lumen as described (12, 30). We also studied the effects of oleic acid on the synthesis and secretion of apoB-100 in WT and the mutant cells. Oleic acid–BSA complex was prepared as described previously (12). Fatty acid-free BSA solution (15%) was adjusted to pH 7.4, and stored frozen in aliquots. Oleic acid (Sigma Chemical Co., St. Louis, MO) was treated with NaOH as described, dried under N_2, and dissolved in serum-free media containing 1.5% BSA so that the oleic acid concentration becomes 0.8 mm. Fresh oleic acid–BSA complex was prepared each time. Cells grown as monolayers were starved for 75 min in methionine-free media (MEM) followed by incubation in serum-free media with [^{35}S]methionine (35 μCi/ml) in the presence of 1.5% BSA or 1.5% BSA/0.8 mm oleate. The processing of cells and media was done as described above.

Preparation of nuclei

Nuclei from WT and the mutant HepG2 cells were prepared as described (31, 32). In brief, 10 million cells were washed with ice-cold wash buffer (20 mm Tris-HCl, pH 7.5, 15 mm NaCl, and 1.1 mm sucrose) and collected by centrifugation at 300 *g* at 4°C. The cells were resuspended in 2.5 ml ice-cold hypotonic buffer (20 mm Tris-HCl, pH 8.0, 4 mm $MgCl_2$, 6 mm $CaCl_2$ and 0.5 mm DTT). The cells were lysed with the addition of 2.5 ml of lysis buffer (0.6 m sucrose, 0.2% Nonidet P-40, and 0.5 mm DTT), and homogenized using a tight-fitting pestle of a homogenizer. Nuclei were pelleted by centrifugation at 1500 *g* for 10 min and resuspended in 0.5 ml of 0.3 m sucrose solution in buffer A (60 mm KCl, 15 mm NaCl, 0.15 mm spermine, 0.5 m spermidine, 14 mm β-mercaptoethanol, 0.5 mm EGTA, 2 mm EDTA, 15 mm HEPES buffer, pH 7.5). The crude nuclear suspension was layered over a 2.5 ml cushion of 1.5 m sucrose in buffer A containing 0.1 mm each of EGTA and EDTA, and centrifuged at 50,000 rpm in a Beckman TL-100 Ultracentrifuge. The clean nuclei obtained as a pellet were either stored in storage buffer (32) or used immediately to perform nuclear run-off assay and to prepare nuclear RNA.

Determination of relative rates of apoB mRNA transcription

Relative rates of apoB mRNA transcription on isolated nuclei were determined as described before (32). In brief, 5 million nuclei each from WT and the mutant cells were taken for transcription assay in a total volume of 200 μl that contained 1 mm each of ribonucleotides CTP, ATP, GTP, and 150 μCi of ^{32}P[UTP], 100 mm tris/HCl, pH 7.9, 50 mm NaCl, 2 mm $MnCl_2$, 4 mm $MgCl_2$, 1 mg/ml heparin sulfate, 0.4 mm EDTA, 0.1 mm PMSF, 1.2 mm DTT, 10 mm creatine phosphate, ribonuclease inhibitor, and 30% glycerol. Transcription was performed at 30°C for 30 min, and reaction was terminated by the addition of 30 U of RNase-free DNase I and incubated at 30°C for an additional 15 min followed by the addition of 2 μl proteinase K (10 mg/ml) and 2 μl of 10% SDS. After incubation at 30°C for 15 min, the contents were extracted with RNAZol™B, and RNA was prepared. The isolated RNA was dissolved in hybridization buffer (20 mm PIPES, pH 6.7, 50% deionized formamide, 2 mm EDTA, 0.8 m NaCl, 0.2% SDS, 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 500 μg/ml salmon sperm DNA). The amounts of radiolabeled UTP incorporated into RNA were determined by TCA precipitation of an aliquot and counting. One million counts were used for each hybridization assay. Recombinant plasmid containing apoB cDNA fragment (10201 nt–10710 nt) was linearized and denatured with NaOH. Three μg linearized recombinant plasmid was immobilized onto nylon membrane by heating at 80°C for 1 h. As an internal control, recombinant plasmid containing γ-actin was also linearized and immobilized onto nylon membrane. Hybridization and washing of the membranes were done as described before (32). The membranes were exposed to X-ray film, and the amounts of the hybridization signal were determined by the intensity of the bands using a SigmaScan Pro4 program.

Preparation of polysomes

Polysomes were prepared as described (32). Ten million HepG2 cells were homogenized in 3 ml of cold 0.25 m sucrose solution containing 50 mm Tris-HCl, pH 7.7, 25 mm NaCl, 5 mm $MgCl_2$, and 500 μg/ml sodium heparin. The homogenate was centrifuged at 15,000 *g* for 10 min at 4°C and the pellet was discarded. The supernatant was treated with 3 ml of solution prepared by mixing equal volumes of 10% of Triton ×100 and 10% sodium deoxycholate. A discontinuous sucrose gradient was prepared as follows: 2 ml of 2.5 m sucrose, 3.0 ml of 1.0 m sucrose, and 0.6 ml of 0.5 m sucrose. Each sucrose solution was made in the buffer used for homogenization that contained 500 μg/ml of sodium heparin. The detergent-treated supernatant (5 ml) was over-layered on the discontinuous gradient, and centrifuged in a Beckman centrifuge at 40,000 rpm for 90 min using SW41 rotor at 4°C. The opalescent band of polysomes at the interface of 2.5 and 1.0 m sucrose gradients was withdrawn with the help of an 18-gauge syringe.

Isolation and analysis of RNA

RNA was isolated from WT and the mutant HepG2 cells using the one-step isolation method (33). Total RNA was also isolated from nuclei and polysomes. In order to avoid any genomic DNA contamination in the nuclear RNA, the nuclear RNA was treated with RQ1 (RNase-free DNase from Promega) for 15 min at 37°C followed by inactivation of RQ1 by heating at 95°C for 5 min and precipitation with isopropanol. The nuclear RNA as well as other RNA preparations did not give any PCR product without reverse transcription, although genomic DNA gave a single specific PCR product under similar conditions. The integrity of apoB mRNA was examined by Northern blotting analysis. Ten micrograms total RNA was electrophoresed in 1.0% agarose gel containing 6% formaldehyde, transferred onto the GeneScreen Nylon membrane (NEN Research Products, Boston), and probed with human-specific cDNA probe corresponding to cDNA sequence 10201 to 10710 as described (34). Absolute levels of apoB-100, apoB-82, and γ-actin mRNA were quantified by RNase protection assay in each RNA preparation as described (28, 30). For total apoB mRNA quantifications, a 510 bp human apoB cDNA (apoB cDNA nucleotides 10201 to 10710) was subcloned into *Pst*I site of the polylinker region of vector pGEM3Zf(+). This vector has T7 and SP6 RNA polymerase promoters flanking the polylinker region. Riboprobes were prepared as described (35). A sense apoB RNA was also synthesized using the same recombinant plasmid, and amounts ranging from 50 to 500 pg were hybridized with the apoB riboprobe. By referring to the calibration curve obtained with sense RNA strand, the counts obtained with RNA were converted into absolute values (pg/μg RNA). As an internal control, a γ-actin riboprobe was used to quantify γ-actin mRNA (30). The apoB riboprobe (510 nt) hybridizes to apoB-100 as well as apoB-82 mRNA. To quantify apoB-82 mRNA, a 449 bp PCR product obtained from the mutated apoB fragment corresponding to nt 11264 to 11711 nt flanking the mutation site (11390 nt) was subcloned in pGEM3Zf(+). The riboprobe prepared using this fragment protects the mutant RNA, but leaves the inserted 4 nt unprotected in the wild-type apoB mRNA giving rise to two fragments of 320 and 125 nt. From the intensity of the 449 nt fragment, apoB-82/B-100 mRNA ratio was determined. This ratio was multiplied with the total apoB mRNA in the mutant cells to obtain absolute levels of apoB-82 and apoB-100 mRNA.

Reverse transcription-polymerase chain reaction (RT-PCR)

Both WT and the mutant apoB mRNA were also quantified by a semiquantitative RT-PCR method. Messenger RNA transcripts from WT and the mutant alleles were distinguished from each other by restriction digestion of the RT-PCR product with the en-

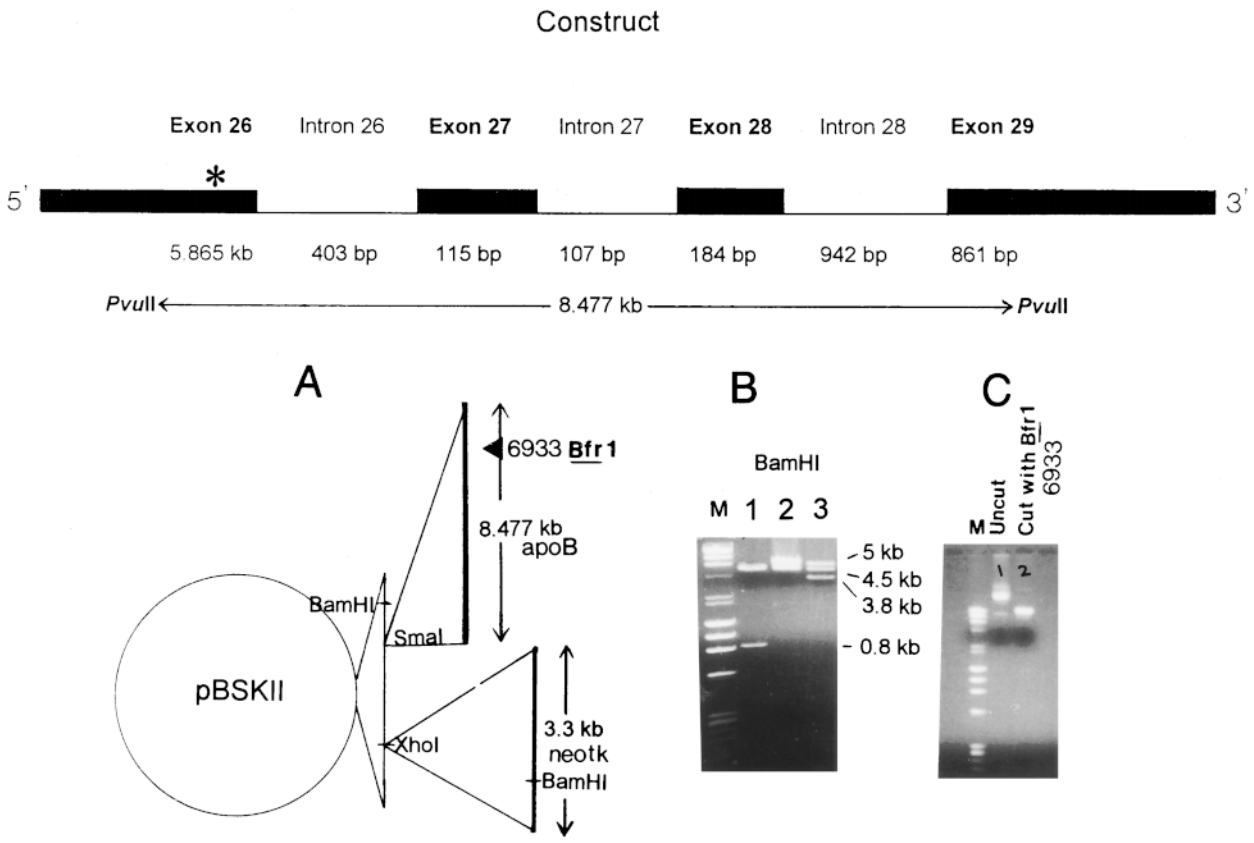

Fig. 1. The *apob* gene fragment used for preparing constructs to target *apob* gene. The *Pvu*II fragment (8.477 kb) was excised from P1 vector containing entire human *apob* gene, and subcloned into *Sma*I site of pBSKII(*Cla*⁻). As shown, this fragment contains most of exon 26, intron 26, exon 27, intron 27, exon 28, intron 28, and part of exon 29 (not to scale). Panel A: The subcloned *apob* gene fragment was bioengineered at *Bgl*II site to generate an inframe stop codon that gives rise to a truncated apoB, B-82. This modification abolishes *Bgl*II site (asterisk). Next a 3.3 kb *neo-tk* cassette containing its own promoter was subcloned at the *Xho*I site of the vector. Panel B: The final construct was examined by digestion with *Bam*HI (lane 3) and by sequencing. As controls, a plasmid containing only neo-tk sequences (lane1), and another plasmid with the *apob* gene fragment without neo-tk (lane 2) were also restriction digested with *Bam*HI. Panel C: Before electroporation the construct was linearized with *Bfr*I, which gives rise to an 882 bp 5′ homologous arm. Lane 1, undigested construct; lane 2, final construct restriction digested with *Bfr*I.

zyme *Bgl*II which was abolished in the mutant allele by targeting. The amount of total RNA needed for RT-PCR was optimized by performing RT-PCR using RNA ranging from 50 ng to 1 μg. We found a linear increase in the PCR product with increasing total RNA up to 500 ng. We, therefore, chose 200 ng total RNA to perform RT-PCR. Total cellular RNA was reverse transcribed using the primer B-2 (GGTCTGCTCAGGCACGATGAT) which is 3′to the mutation site. The reaction mixture for reverse transcription in a total volume of 20 μl contained 4 μl of 25 mm $MgCl_2$, 2 μl of 10× PCR buffer, 2 μl each of 10 mm dNTP, 1 μl of RNase inhibitor, 1 μl RNA sample, 2 μl primer B-2, and 5 μl nuclease-free water. The contents were incubated for 10 min at 70°C and cooled down to 42°C, followed by the addition of 1 μl (2.5 U) of MuLV reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, IN). The reaction was allowed to proceed for 20 min followed by denaturation at 99°C for 5 min and cooling at 4°C for 5 min. For PCR reaction, the following ingredients were added to the above reaction mixture: 4 μl of $MgCl_2$ (25 mm), 8 μl of 10× PCR buffer, 65 μl of sterile water, 1 μl of primer B-1 (AAC CCAATGGCTATTCATTTTCC), and 2.5 U of *Taq* DNA polymerase. Twenty five cycles of PCR were performed using the following profile: 95°C–15 sec, 60°C–30 sec, 72°C–40 sec. An additional profile was performed at 72°C for 7 min.

RESULTS

Targeting of *apob* gene in HepG2 cells

The construct on linearization with *Bfr*I gave rise to a 882 bp 5′ fragment which was sufficient for homologous recombination (**Fig. 1**). As a result of the insertional recombination (“in step”) a portion of *apob* gene becomes duplicated. This region may undergo a second recombination either between the duplicated regions or between the targeted locus and the other *apob* allele. The second recombination, termed as the “out step,” eliminates the

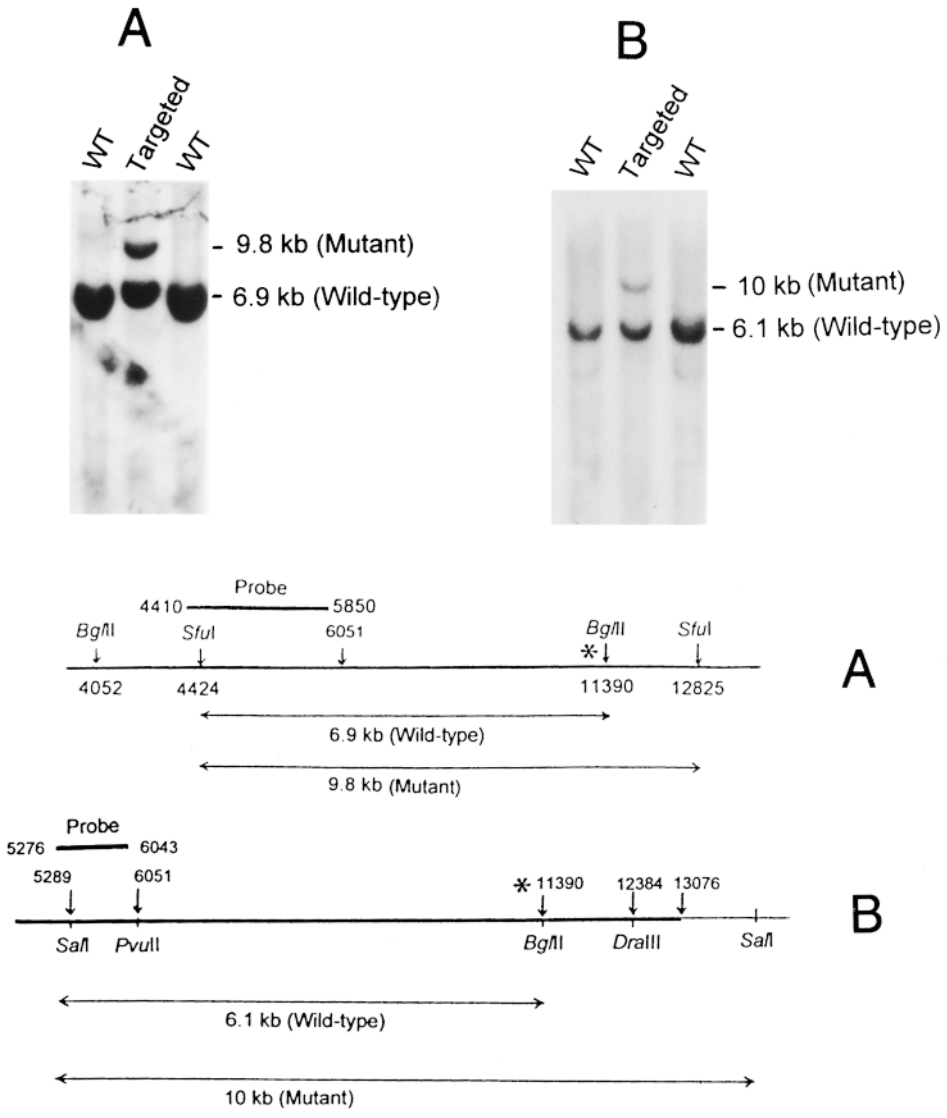

Fig. 2. Southern blotting analysis for the targeting of the *apob* gene. Two different restriction enzymes were used to confirm the targeting of *apob* gene (panels A and B). Ten μg genomic DNA was digested with *Bgl*II and *Sfu*I (panel A), or with *Bgl*II and *Sal*I (panel B), run in an 0.7% agarose gel, transferred onto a nylon membrane, and probed with an apoB cDNA probe outside the construct as indicated. The targeted clone gives rise to a 9.8 kb band (panel A) or 10 kb band (panel B) which correspond to the mutant allele.

vector sequences with a 50% chance of the introduced mutation being retained or removed from the *apob* allele. Of the three electroporations, the second electroporation yielded 400 clones showing the presence of the introduced mutation as determined by restriction digestion with *Bgl*II. Southern blotting analysis showed one clone as having been targeted to the *apob* gene locus. Two different restriction endonucleases and two different apoB probes were used to confirm that the *apob* gene has been targeted (**Fig. 2**).

HepG2 cells contain three alleles of the *apob* gene as chromosome 2 on which *apob* gene maps is trisomic (36). Based on the intensities of the bands on the Southern blot, it appeared that one of the three alleles had been modified. The mutant HepG2 cell was then selected for the "out reaction" in ganciclovir. The cells surviving in ganciclovir were screened for the mutation and removal of the vector sequences. Southern blotting using an apoB probe 3′ to the targeting construct confirmed the removal of the vector sequences (**Fig. 3A**); PCR of the *apob* gene fragment flanking the mutation site followed by restriction digestion with *Bgl*II confirmed the presence of the mutation (Fig. 3B), and PCR using primers in the vector and *apob* gene confirmed the absence of neo-tk sequences (Fig. 3C). Thus, the second selection yielded clones lacking the vector sequences, but retaining the introduced mutation. A portion of the cells obtained after the second selection was transferred into a media-containing G418 to examine the absence of neo-tk and vector sequences. G418 killed WT as well as cells obtained after the second selection, suggesting that the neo-tk sequences have been removed. This experiment also demonstrated the homogeneity of the mutant cells after the second selection.

ApoB forms in the media

Accumulation of apoB in growth media from WT and mutant HepG2 cells was quantified by immunoblotting using culture dishes containing the same numbers of HepG2 cells (**Fig. 4**). Mutant cells synthesized both apoB-100 and the truncated apoB-82, whereas the WT cells synthesized only apoB-100. Media of the mutant cells contained 56% of the amounts of apoB-100 present in the media of WT cells (**Table 1**). ApoB-82 in media of mutant cells accumulated at 11% of the amounts of apoB-100 present in mutant cells (Table 1). The spent media were subjected to ultracentrifugation at the density of 1.065 g/

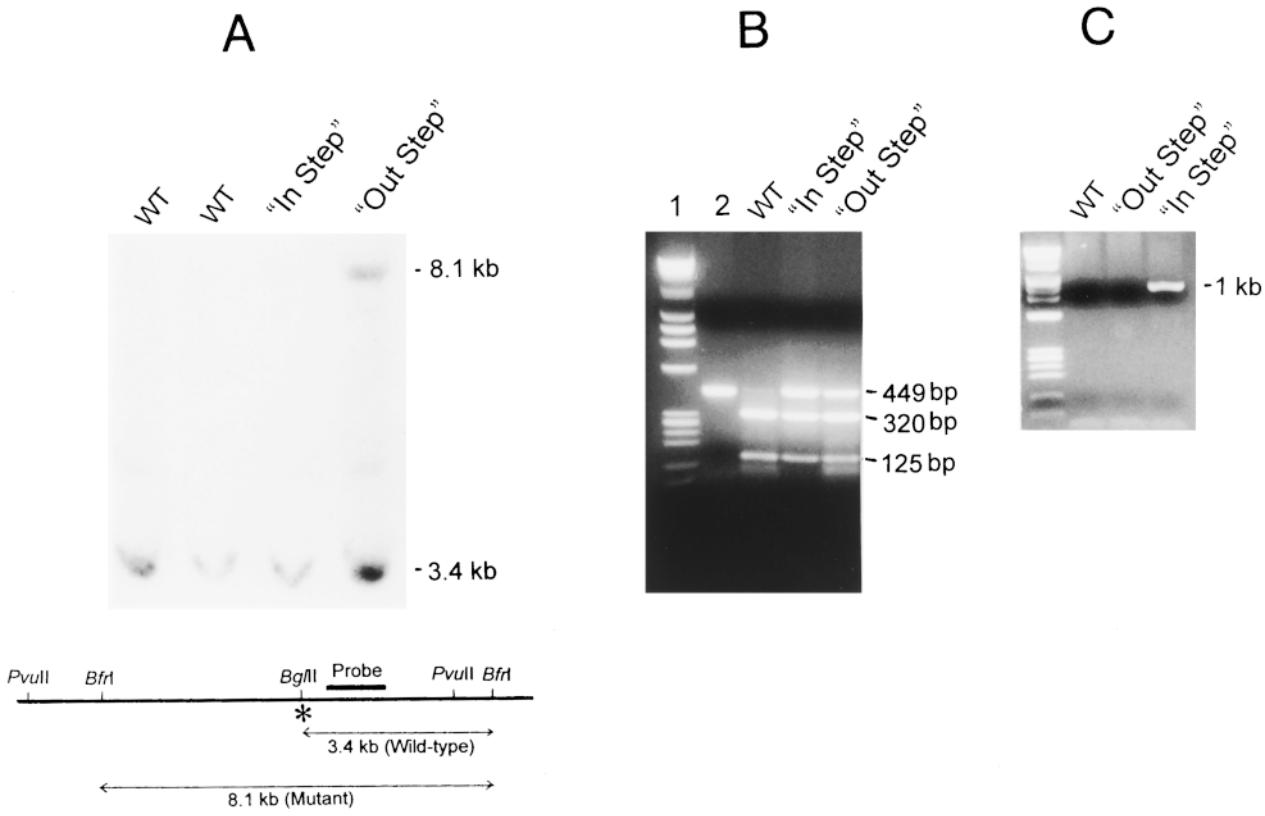

Fig. 3. Confirmation of "out step" reaction. Panel A shows confirmation of the "out step" reaction by Southern blotting. The cells surviving in ganciclovir were examined for retention of the *apob* gene mutation introduced. Genomic DNA was digested with restriction endonucleases *Bfr*I and *Bgl*II. At the 3′ end, *Bfr*I site is 305 nt downstream of the *Pvu*II site (see Fig. 1). The vector and neo-tk sequences are between *Pvu*II and *Bfr*I (3′ end). In the event of "out step" reaction, these sequences will be removed and will light-up an 8.1 kb fragment with the apoB probe indicated. This probe is in intron 26. The 8.1 kb band will not light-up in clones in which "out step" reaction has not occurred. Panel B shows amplification of the genomic DNA from WT and targeted cells ("in step" and "out step") by PCR reaction using primers B1 and B2 (described in Materials and Methods), and digestion with *Bgl*II. Lane 1, DNA size marker; lane 2, PCR product without restriction digestion. Panel C shows amplification of part of neo-tk. Panel C shows that neo-tk sequences are absent in the mutant clone after the "out step" reaction.

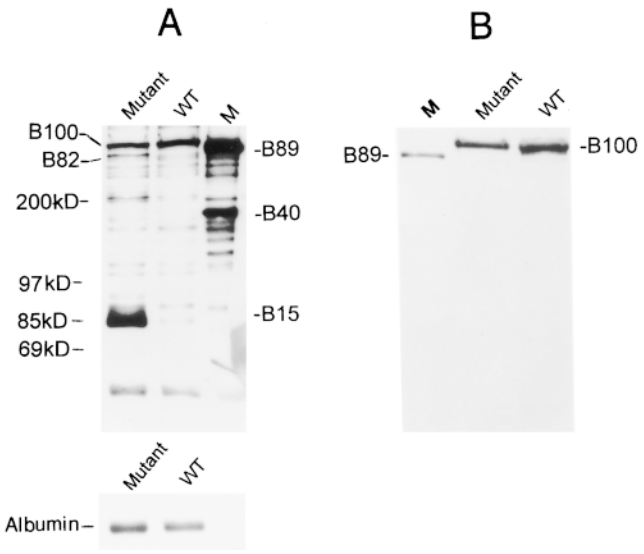

Fig. 4. Identification of apoB-82 in cell culture media of the targeted HepG2 clone. Equal numbers of cells were cultured in each case. After 24 h of culture, aliquots of culture media were run in duplicate in 3–12% SDS-PAGE gels. Panel A shows detection of apoB using an N-terminal apoB monoclonal antibody. Panel B shows detection of apoB using C-terminal apoB monoclonal antibody. Albumin was used as an internal control (the bottom of panel A).The 85 kD fragment of apoB was detected with N-terminal apoB antibody (panel A), but not with C-terminal apoB antibody (panel B). Media from WT and mutant cells have been marked. An apoB-82 was present in mutant cells but not in the WT cells. This experiment was repeated several times, and we consistently observed low levels of apoB-82, and the presence of an 85 kD apoB fragment in the mutant cells. Lane M shows markers for human apoB-89 and B-40.

TABLE 1. Levels of apoB-100 and B-82 protein, and apoB and γ-actin mRNA in WT and mutant cells

	Wild-Type	Mutant	% Change
ApoB mRNA			
Total apoB mRNA[a]	211 ± 14	172 ± 16	−18
ApoB-100 mRNA	206 ± 17	140 ± 11	−32
ApoB-82 mRNA[b]		24 ± 4	
γ-actin mRNA	27 ± 4	25 ± 3	
ApoB protein[c]			
ApoB-100 protein[d]	100	56 ± 8	−44
ApoB-82 protein[e]		11 ± 4	

[a] Total apoB mRNA was quantified by RNase protection assay (pg/μg RNA). Ten μg total RNA were hybridized with the apoB riboprobe (32), and RNase-resistant hybrids were precipitated with TCA, and counted on glass fiber filters. Absolute levels of apoB mRNA were determined by referring to a calibration curve with in vitro synthesized sense RNA. Results are means ± SD.

[b] Mutant apoB mRNA was determined from the RNase protection assay as well as from RT-PCR quantification method.

[c] ApoB protein level was determined by Western blotting as shown in Fig. 4.

[d] The value of apoB-100 in the mutant cells was obtained from 4 independent experiments, and is presented as % of apoB-100 in the wild-type cells.

[e] The value of apoB-82 represents percent of apoB-100 in the mutant cells obtained from four independent experiments.

ml. ApoB-100 and B-82 floated at d < 1.065 g/ml. Mutant cells, but not WT cells, also secreted an 85 kD N-terminal fragment of apoB (apoB-15) (Fig. 4A). The 85 kD protein is an N-terminal fragment of apoB (apoB-15) as it reacted with the N-terminal anti-apoB antibody (Fig. 4A), but not with the C-terminal apoB antibody (Fig. 4B). To determine the flotation characteristics of apoB-15, we pulsed cells with [^{35}S]methionine, and collected media after 120 min of chase. The media were concentrated and density gradient ultracentrifugation was performed (**Fig. 5**). ApoB-15 was present in fractions denser than HDL. ApoB-15 was not detected in the WT media.

ApoB mRNA levels

Based on the low rates of secretion of truncated apoB, B-82, we predicted that mutant cells would contain low levels of apoB-82 mRNA. In RNase protection assays, total apoB mRNA was reduced by 18% in the mutant cells while γ-actin mRNA showed similar concentrations in WT and the mutant cells (Table 1). Northern blotting analysis showed an intact single 14 kb apoB mRNA band with no apparent degradation (not shown). To quantify apoB-100 and apoB-82 mRNAs, we developed another RNase protection assay using the mutant apoB mRNA riboprobe (**Fig. 6**), that exploited the presence of four inserted nucleotides in the mutant allele. The mutant riboprobe completely protected the mutant apoB mRNA from RNase digestion. Indeed, the protected 449 bp fragment corresponding to the mutant message was seen in the mutant cells, but not in WT cells. The region of the riboprobe bearing the 4 nucleotide insertion comprising the mutation remains single stranded and susceptible to RNase digestion, yielding two fragments of 320 and 125 nt (Fig. 6). Mutant cell apoB-100 mRNA was reduced to 68% of WT levels, apoB-82 mRNA level was 11% of that of apoB-100 mRNA in the mutant cells (Table 1).

To ascertain the mechanism for the low levels of mutant apoB mRNA, we measured relative rates of apoB mRNA transcription in wild-type and the mutant cell nuclei (**Fig. 7**). The relative rates of apoB mRNA transcription in the mutant cells were comparable with wild-type cells, suggesting that WT and the mutant *apob* alleles are transcribed at the same rates. We also quantified apoB mRNA levels in RNA isolated from nuclei and polysomes (**Fig. 8**). Total cellular and polysomal apoB-82 mRNA levels (panels A and B) were found to be similar to each other at 10–12% of apoB-100 mRNA, but the levels of apoB-82 mRNA in the nuclei were 46% of apoB-100 mRNA in the same nuclei (panel B). The data suggest that the cytoplasmic stability of the mutant message has been altered.

Synthesis and secretion of apoB forms

The differing levels of apoB in the media suggested that rates of secretion may have differed for apoB-100 and apoB-82 in mutant and WT cell lines. To test this, cells were pulsed with [^{35}S]methionine for 20 min and chased for 120 min. The amounts of apoB-100 in mutant cell media were ~50% of those in WT media, and the amounts of apo-B82 were about 10% of the amounts of apoB-100 in the same mutant cell media. The similar relative accumu-

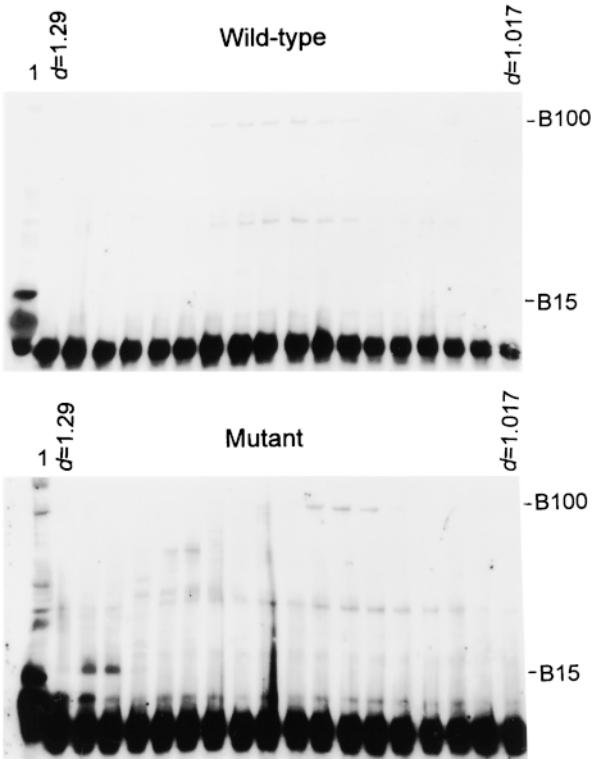

Fig. 5. Density distributions of apoBs. WT and mutant cells were pulsed with [^{35}S]methionine and chased for 120 min in the presence of 1000-fold excess of cold methionine. The media, 15 ml, were concentrated in Centricon-30, desalted in the presence of protease inhibitors, and subjected to ultracentrifugation. After the centrifugation, aliquots of the tube contents were collected from the bottom of the tube, dialyzed, and an aliquot was used for immunoprecipitation using polyclonal anti-apoB antibody, R197, and separated in SDS-PAGE. Lane 1, an aliquot of mutant cell media immunoprecipitated after [^{35}S]methionine pulse and 120 min chase to mark the position of B-15.

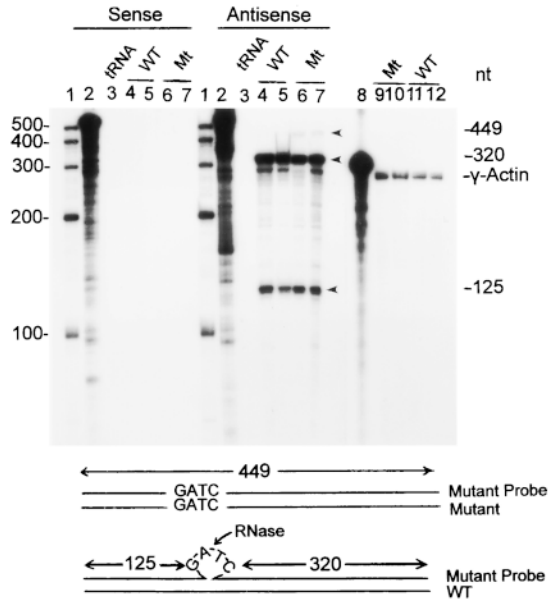

Fig. 6. ApoB mRNA quantification by RNase protection assay. The strategy of the protection assay is illustrated below the figure. Five micrograms of total RNA was used for each assay. After hybridization with riboprobe, RNAs were digested with Rnase A + T1, precipitated, and the protected fragment was separated on a sequencing gel. Lane 1 shows RNA size markers synthesized by in vitro transcription; lane 2, riboprobe; lane 3, riboprobe hybridized with yeast tRNA. Lanes 4 and 5 show mRNA from WT cells, and lanes 6 and 7 show mRNA from apoB-82 mutant cells. The protected fragments are indicated by arrows. The labeled RNA probe synthesized on the sense strand of the recombinant plasmid did not hybridize to the total RNA. Lane 8 shows γ-actin riboprobe; lanes 9 and 10, mutant mRNA; lanes 11 and 12, WT mRNA.

lated quantities and rates of secretion suggest that altered secretory rates were responsible for the differences in the quantities accumulated in the media.

To examine the intracellular proportions of synthesized apoB-100 that are secreted into the media, WT and mutant HepG2 cells were pulsed with [^{35}S]methionine and chased for 120 min in the presence of 1000-fold excess cold methionine. We found that secretion of apoB-100 into the media was linear for 180 min. The amounts of apoB-100 in the cells and in the media were determined by immunoprecipitation and SDS-PAGE analysis. In the WT cells, ~20% newly synthesized apoB-100 is secreted, whereas in the mutant cells about 10% of newly synthesized apoB-100 is secreted (**Table 2**). After 120 min of chase 15% of newly synthsized apoB-100 remains within the WT cells, and for the mutant cells it is ~19%. In comparison to WT cells, mutant cells synthesized 66.5% the amounts of apoB-100 at zero time of chase; respective secretion rates were 15% and 7%. As presecretory degradation of apoB-100 in mutant cells was more compared to WT cells, we examined the effects of oleic acid on the restoration of apoB-100 degradation. As shown in **Table 3**,

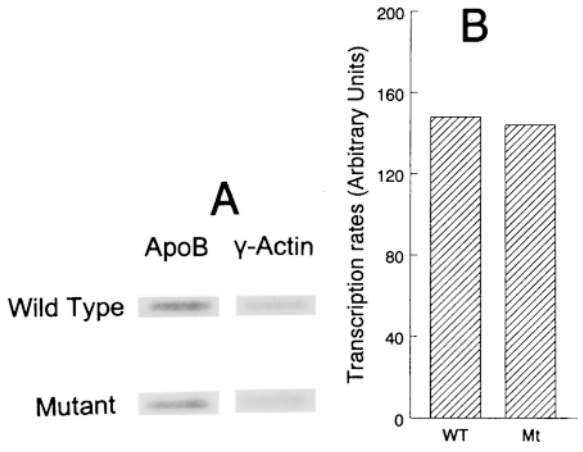

Fig. 7. Relative rates of apoB mRNA transcription in isolated nuclei. Nuclei were isolated from WT and the mutant cells, and 5 million cells were used for the nuclear run-on assay. After the transcription total RNA were extracted and one million counts were used for hybridization to the membrane bound apoB and γ-actin probes. Panel A shows the results of transcription assay and panel B shows the relative rates of apoB mRNA transcription after correction with internal control, γ-actin.

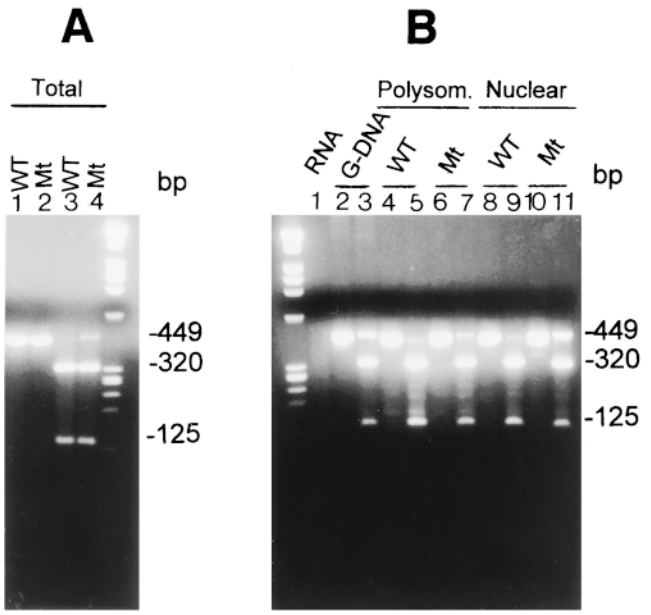

Fig. 8. Quantification of WT and apoB-82 mutant apoB mRNA in various cellular fractions by RT-PCR. Two hundred nanograms of total RNA from various cellular fractions was reverse transcribed and amplified by polymerase chain reaction as indicated. The PCR product was digested with *Bgl*II and run in a 1.5% agarose gel. Panel A shows RT-PCR, and digestion of RT-PCR product with *Bgl*II; WT, wild-type; Mt, mutant. Panel B shows RT-PCR of RNA from polysomal and nuclear fractions of WT and the mutant cells. Lane 1, PCR of nuclear RNA sample without RT to check for genomic DNA contamination; lanes 2 and 3, PCR of genomic DNA from mutant cells and digestion with *Bgl*II; lanes 4, 5, polysomal RNA from WT cells; lanes 6, 7, polysomal RNA from mutant cells; lanes 8, 9, nuclear RNA from WT cells; lanes 10, 11, nuclear RNA from mutant cells.

providing oleic acid reduced apoB-100 degradation in both types of cells. The fraction of newly synthesized apoB-100 secreted into the media in the presence of oleic acid was 34% for WT and 35% for the mutant cells, but as less apoB-100 was synthesized by the mutant cells, less was secreted. We also explored whether the increased degradation of apoB-100 occurs before translocation of apoB-100 into the ER lumen or after translocation has occurred. To examine this, we pulsed cells with [^{35}S] methionine, and fractionated the cells into ER membrane and lumen. Lumenal contents of apoB-100 in the mutant cells were found to be 52% compared to WT cells, suggesting that the enhanced presecretory degradation of apoB-100 may have occurred prior to their translocation into the lumen.

TABLE 2. Synthesis and secretion of newly synthesized apoB-100 in wild-type and mutant cells

Cell	Cellular Synthesis[a]	Retained in Cells[b]	Secreted into Media[c]
Wild-type	65,000	10,121 (15.6%)[d]	12,850 (19.8%)[e]
Mutant	43,250	8,120 (18.8%)[d]	4,519 (10.4%)[e]

Cells were pulsed and chased as described in the text. ApoB was immunoprecipitated and run in SDS-PAGE gel, and the band corresponding to apoB-100 was cut out and counted. The data shown are means of duplicate experiments.

[a] Cellular synthesis represents counts in apoB-100 at zero chase time.
[b] ApoB-100 retained in the cells after 120 min of chase.
[c] Secretion of apoB-100 into media was determined 120 min after chase.
[d] Percent of total cellular cpms retained in cells.
[e] Percent of total cellular cpms secreted into media.

TABLE 3. Effects of oleate on synthesis and secretion of apoB-100

Cells	Cellular Synthesis[a]	Retained in Cells[b]	Secretion into Media[c]
Wild-type	114,450	22,890 (20%)[d]	39,290 (34.3%)[e]
Mutant	78,525	17,295 (22%)[d]	27,653 (35.2)[e]

Pulse/chase experiment was carried out in the presence of oleate as described in the text. After immunoprecipitation and SDS-PAGE apoB-100 band was cut out and counted.

[a] Cellular synthesis represents counts in apoB-100 at zero chase time.
[b] ApoB-100 retained in the cell after 120 min of chase.
[c] Secretion of apoB-100 into media was determined 120 min after chase.
[d] Percent of total cpms retained in the cells.
[e] Percent of total cellular cpms secreted into media.

DISCUSSION

The targeted HepG2 cell line expressing apoB-82 truncation

The aim of the present study was to develop an in vitro model in which to study the cellular and molecular mechanisms that may cause the low in vivo production rates of apoB in FHBL heterozygotes bearing long truncated forms of apoB (e.g., B-89, B-87, B-75). An insertion-type construct was used to introduce the mutation into the endogenous *apob* gene and to remove the vector sequences in the "out-step" selection, so as to obtain genomic DNA and mRNA that resemble natural mutations driven by the endogenous promoter. Various lengths of human apoB have been expressed in rat hepatocytes and COS cells, and valuable data have been obtained on the relationship between lengths of truncations and the sizes and densities of secreted particles (37). However, these cells differ from the naturally occurring mutations in several respects: *a*) the expression of cloned apoB cDNA is driven by a heterologous promoter that could result in the overloading the cells with varying lengths of apoB mRNA and apoB proteins; *b*) the insertion of apoB cDNA in the HepG2 chromosome is random and not targeted to the *apob* gene; and *c*) more than one copy of the recombinant plasmid containing apoB cDNA becomes inserted making it difficult to study control. The HepG2 cell has its own limitations. It is trisomic with respect to chromosome 2 (36), and unlike normal hepatocytes, it secretes primarily LDL and not VLDL particles (11). This limits the usefulness of HepG2 cells for studies of VLDL assembly. Nevertheless, the HepG2 cell is suitable to study apoB mRNA metabolism and apoB synthesis and secretion. We, therefore, chose HepG2 cells to target *apob* gene and introduce a premature translational stop codon. Successful targeting of one

of three chromosomes was documented by PCR-based and Southern blot analyses (Figs. 2 and 3).

Secretion of apoB

As HepG2 cells have three *apob* alleles, and only one allele was targeted in our experiments (Figs. 2 and 3), we expected levels of mutant apoB to be 33% and levels of apoB-100 to be 67% when compared to WT cells. Instead, the respective levels were 5.5 and 56%. The low relative levels of both apoB-82 and apoB-100 resembled plasma levels of truncated apoBs and apoB-100 in FHBL human and mouse heterozygotes. For truncations, these range from 5–25% of apoB-100 (38–42). For apoB-100, levels range from 30–50% of normal (3, 4). Another truncation, apoB-15, was also found in mutant cell media exclusively in fractions denser than HDL (Fig. 5). This is compatible with previous reports on the density distributions of short apoB truncations (37). The mutant cell line exhibited characterisctics similar to the WT cells with regard to triglyceride synthesis, suggesting that the mutant cells retained this characteristic. As triglyceride synthesis was found to be similar in WT and the mutant cell line (data not shown), lower than expected levels of apoB-100 in the mutant cell resulted from the introduction of premature translational stop codon in one of the *apob* alleles, and not because of the low availability of triglycerides.

Mutant cells produce an N-terminal apoB fragment of 85 kD size

A novel finding in the present study was the secretion of apoB-15 by the mutant HepG2 cells (Fig. 5). Production of a short truncated apoB, B-21, has been reported in human aortic endothelial (43) and bovine endothelial cells (44). Bovine endothelial cells synthesize apoB-100 intracellularly, but secrete only a 116 kD N-terminal fragment. In addition to the 116 kDa fragment of apoB, human aortic endothelial cells (HAEC) also produce an 85 kD protein that binds with lipoprotein lipase (43). A similar sized (85 kD) N-terminal fragment of apoB has also been reported to be secreted by CHO cells transfected with apoB cDNA that synthesizes apoB-53 (45). These CHO cells expressed apoB-53 intracellularly, but secreted mostly apoB-15 (85 kD fragment) into the media. Both CHO as well as endothelial cells are unable to translocate and secrete apolipoprotein B which leads to its complete degradation in the endoplasmic reticulum. Thus, these cells incapable of secreting lipoproteins secrete partially degraded apoBs. The limitation may be due to the absence of microsomal triglyceride transfer protein (46) and the resulting inability to assemble apoB with lipids, or an imbalance between the availability of apoB and the availability of lipids that protect apoB from degradation, for hepatic export. Thus, in the mutant HepG2 cells, apoB-15 appears to be produced as a result of the inability of apoB to translocate across the ER membrane. In the mutant cell line the production of apoB-15 did not result from the low availability of triglycerides, as triglyceride synthesis in the mutant cells was comparable to that in WT cells (not shown). Further studies will be needed to determine the exact cellular mechanism that results in production of apoB-15.

Low levels of apoB-82 are caused by low levels of mutant apoB mRNA

Three processes could have led to low levels of apoB-82 in the media: *a*) low levels of mutant apoB mRNA resulting in low rates of translation; *b*) higher than normal presecretory degradation of truncated apoB; or a combination of *a*) and *b*). The levels of apoB-82 mRNA were 11% of the apoB-100 in mutant cells and 5.5% of mRNA in WT cells (Table 1). ApoB-100 mRNA in the mutant cells was 68% of the total apoB mRNA in the WT cells (Table 1). As HepG2 cells have three alleles of *apob*, the levels of mutant apoB-82 and apoB-100 mRNA should have been 33% and 67% of the apoB mRNA in the WT cells. The 68% for apoB-100 mRNA is consistent with one of three alleles having been targeted. That apoB-82 was present at 5.5% of normal instead of 33% is compatible with low translation rates of apoB-82 and with the similarly low secretory rates discussed below.

The premature stop codon could have affected either the transcription or the stability of the apoB-82 mRNA. To distinguish between those possibilities, we fractionated the mutant cells and quantified apoB-100 and B-82 mRNA in their nuclei and polysomes. ApoB-82 mRNA in nuclei was present at 46% of apoB-100 mRNA in the same nuclei as expected from the targeting of one of three alleles. As the presence of a nonsense codon may affect nuclear transport of mutant mRNA (47), normal levels of mutant apoB mRNA in the present study could reflect either altered transport and/or altered transcription of mutant mRNA transcript. Measurements of relative rates of apoB mRNA transcription ruled out changes in the transcription rates. As the levels of apoB-82 mRNA in polysomes was ~11%, similar to that found in total cellular mRNA, it suggests that the low levels of mutant apoB mRNA were caused by the increased rates of cytoplasmic degradation. Premature stop codons decrease cytoplasmic stability in some genes (13–17), but not in others (18–21). Our data are consistent with a recent report on the mouse model of hypobetalipoproteinemia (48). In the apoB-100/B-83 heterozygotes (48), nuclear levels of precursor apoB mRNA did not change, but the mutant apoB mRNA after splicing showed 76% reduction. We found ~70% reduction in the mutant apoB mRNA levels. Thus, our data as well as those of others (48) support the "translational/translocation" model (22) which proposes that polysomal translation starts as soon as mature mRNA emerges from the nucleus, and the presence of a premature stop codon interrupts this process, thereby providing signals and/or access to nucleases, leading to the degradation of the mRNA. Our results are not compatible with the "nuclear scanning" model, which postulates that mRNAs are scanned within nuclei for the presence of premature stop codons and the presence of such codons leads to slowed processing of the mRNAs, leading to low RNA phenotypes (47).

Low levels of apoB-100 in the targeted cell line are caused by greater presecretory degradation

To test whether low levels of apoB-100 and apoB-82 were due to changes in rates of secretion, [^{35}S]methionine pulse experiments were performed that demonstrated decreased rates of secretion of both apoB forms into mutant cell basal media. The media levels of newly synthesized apoB-100 in the mutant cells were about 50% when compared to WT cells. As one of the three *apob* alleles was targeted in the mutant cells, we expected the levels of apoB-100 in the media of mutant cells to be 67% of the WT media levels. Why are the levels of apoB-100 in the mutant cell media lower than the expected levels of 67%? To examine the mechanism, we determined the total amounts of apoB-100 synthesized in WT and mutant cells at zero time of chase, and the amounts of apoB-100 secreted into media after 120 min of chase (Table 2). The presecretory degradation of apoB-100 in the mutant cells was higher compared to WT cells. These results are compatible with in vivo data reported in FHBL humans heterozygous for truncations for apoB (3) and support the suitability of this cell line as a model for cellular studies of FHBL mechanisms. Oleate increases the secretion of apoB-100 by rescuing apoB-100 from intracellular degradation. If the presecretory degradation of apoB-100 was more in the mutant cells, then providing oleate should restore apoB-100 secretion in mutant cells to expected levels. We examined this possibility. In the presence of oleate the fraction of newly synthesized apoB-100 secreted into the mutant cell media was similar to WT cell media (Table 3). This suggests that the intracellular apoB-100 degradation occurred more in the mutant cells than in WT cells. At this point we do not have experimental evidence to provide a definitive mechanism for the greater degradation and lower secretion of apoB-100 in the mutant cells. It is possible that the presence of a premature translational stop codon in the apoB coding region interferes with co-translational translocation of apoB-82, which in turn may block the efficient translational translocation of apoB-100 leading to the co-translational degradation of apoB-100 (49, 50). Indeed, we found that the amounts of apoB-100 secreted into the ER lumen of mutant cells were lower, suggesting that the degradation of apoB occurred prior to their translocation into the ER lumen.

In summary, we have introduced stop codon in one of the alleles of *apob* gene in HepG2 cells by targeted modification, and demonstrated that nonsense mutation affected the metabolism of apoB mRNA and the various apoB proteins secreted.

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REFERENCES

1. Schonfeld, G. 1995. The hypobetalipoproteinemias. *Annu. Rev. Nutr.* **15:** 23–34.
2. Linton, M. F., R. V. Farese, Jr., and S. G. Young. 1993. Familial hypobetalipoproteinemia. *J. Lipid Res.* **34:** 521–541.
3. Aguilar-Salinas, C. A., P. H. R. Barrett, K. G. Parhofer, S. G. Young, D. Tessereau, J. Bateman, C. Quinn, and G. Schonfeld. 1995. Apolipoprotein B100 production is decreased in subjects heterozygous for truncations of apolipoprotein B. *Arterioscler. Thromb.* **15:** 71–80.
4. Parhofer, K. G., P. H. R. Barrett, D. M. Bier, and G. Schonfeld. 1996. Positive linear correlations between the length of truncated apolipoprotein B and its secretion rate: in vivo studies in apoB-89, apoB-75, apoB-54.8, and apoB-31 heterozygotes. *J. Lipid Res.* **37:** 844–852.
5. Schumaker, V. N., M. L. Phillips, and J. E. Chatterton. 1994. Apolipoprotein B and low density lipoprotein structure: implications for biosynthesis of triglyceride-rich lipoproteins. *Adv. Protein Chem.* **45:** 205–248.
6. Spady, D. K., and J. M. Dietschy. 1988. Interaction of dietary cholesterol and triglycerides in the regulation of hepatic low density lipoprotein transport in the hamster. *J. Clin. Invest.* **81:** 300–309.
7. Lau, P. P., W. J. Xiong, H. J. Zhu, S. H. Chen, and L. Chan. 1991. Apolipoprotein B mRNA editing is an intracellular event that occurs posttranscriptionally coincident with splicing and polyadenylation. *J. Biol. Chem.* **266:** 20550–20554.
8. Greeve, J., I. Altkemper, J. H. Dieterich, H. Greten, and E. Windler. 1993. Apolipoprotein B mRNA editing in 12 different mammalian species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins. *J. Lipid Res.* **34:** 1367–1383.
9. Goldberg, A., and G. Schonfeld. 1985. Effects of diet on lipoprotein metabolism. *Annu. Rev. Nutr.* **5:** 195–212.
10. Pullinger, C. R., J. D. North, B-B. Teng, V. A. Rifici, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constituently expressed in HepG2 cells: regulation by oleic acid, albumin, and insulin, and measurement of the mRNA half life. *J. Lipid Res.* **30:** 1065–1077.
11. Moberley, J. B., T. G. Cole, D. H. Alpers, and G. Schonfeld. 1990. Oleic acid stimulation of apolipoprotein secretion from HepG2 and Caco2 cells occurs posttranscriptionally. *Biochim. Biophys. Acta.* **1042:** 70–80.
12. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleic acid stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266:** 5080–5086.
13. Hamosh, A., B. C. Trapnell, P. L. Zeitlin, C. Montrose-Rafizadeh, B. J. Rosenstein, R. G. Crystal, and G. R. Cutting. 1991. Severe deficiency of cystic fibrosis transmembrane conductance regulator messenger RNA carrying nonsense mutation R553X and W1316X in respiratory epithelial cells and patients with cystic fibrosis. *J. Clin Invest.* **88:** 1880–1885.
14. Chang, J. C., and Y. W. Kan. 1979. β° thalassemia: a nonsense mutation in man. *Proc. Natl. Acad. Sci. USA.* **76:** 2886–2889.
15. Longo, N., S. D. Langley, D. Griffin, and L. J. Elaas. 1992. Reduced mRNA and a nonsense mutation in the insulin receptor gene produce heritable severe insulin resistance. *Am. J. Hum. Genet.* **50:** 998–1007.
16. Mashima, Y., A. Murakami, R. G. Weleber, N. G. Kennaway, L. Clarke, T. Shiono, and G. Inana. 1992. Nonsense codon mutations of the ornithine aminotransferase gene with decreased levels of mutant mRNA in gyrate atrophy. *Am. J. Hum. Genet.* **51:** 81–91.
17. Frangi, D., M. Cicardi, A. Sica, F. Colotta, A. Agostoni, and A. E. Davus. 1991. Nonsense mutations affect C1 inhibitor messenger RNA levels in patients with type I hereditary angioneurotic edema. *J. Clin. Invest.* **88:** 755–759.
18. Liebhaber, S. A., M. B. Coleman, J. G. Adams, F. E. Cash, and M. H. Steinberg. 1987. Molecular basis for nondeletion alpha-thalassemia in American blacks. Alpha 2<116GAG . . . . . UAG>. *J. Clin. Invest.* **80:** 154–159.
19. Winslow, R. M., M-L. Swenberg, E. Gross, P. A. Chervenick, R. R. Buchman, and W. F. Anderson. 1976. Hemoglobin Mc Kees Rocks <alpha 2 beta 2145Tyr leads to term>. A human "nonsense" mutation leading to a shortened beta-chain. *J. Clin. Invest.* **57:** 772–781.
20. Lehrman, M. A., W. J. Schneider, M. S. Brown, C. G. Davis, A. Elhammer, D. W. Russell, and J. L. Goldstein. 1987. The Lebanese al-

lele at the low density lipoprotein receptor locus. Nonsense mutation produces truncated receptor that is retained in endoplasmic reticulum. *J. Biol. Chem.* **262:** 401–410.
21. Farese, R. V., Jr., M. M. Veniant, C. M Cham, L. M. Flynn, V. Pierotti, J. F. Loring, M. Traber, S. Ruland, R. S. Stokowski, D. Huszar, and S. G. Young. 1996. Phenotypic analysis of mice expressing exclusively apolipoprotein B100 or apolipoprotein B48. *Proc. Natl. Acad. Sci. USA.* **93:** 6393–6398.
22. Cooper, D. N. 1993. Regulatory mutations and human disease. *Ann. Med.* **25:** 11–17.
23. Selby, S. L., and Z. Yao. 1995. Level of apolipoprotein B mRNA has an important effect on the synthesis and secretion of apolipoprotein B-containing lipoproteins. Studies on transfected hepatoma cell lines expressing recombinant human apolipoprotein B. *Arterioscler. Thromb. Vas. Biol.* **15:** 1900–1910.
24. Valancius, V., and O. Smithies. 1991. Testing "In-Out" targeting procedure for making subtle genomic modifications in mouse embryonic stem cells. *Mol. Cell Biol.* **11:** 1402–1408.
25. Linton, M. F., R .V. Farese, Jr., G. Chiesa, D. S. Grass, P. Chin, R. E. Hammer, H. H. Hobbs, and S. G. Young. 1993. Transgenic mice expressing high plasma concentrations of human apolipoprotein B100 and lipoprotein (a). *J. Clin. Invest.* **92:** 3029–3037.
26. Srivastava, R. A. K., S. Jiao, J. Tang, B. A. Pfleger, R. T. Kitchens, and G. Schonfeld. 1991. In vivo regulation of LDL receptor and apoB gene expressions in inbred strains of mice by dietary fatty acids and cholesterol. *Biochim. Biophys. Acta.* **1086:** 29–43.
27. Srivastava, N., D. Noto, M. Averna, J. Pulai, R. A. K. Srivastava, T. G. Cole, M. A. Latour, B. W. Patterson, and G. Schonfeld. 1996. A new apolipoprotein B truncation (apoB-43.7) in familial hypobetalipoproteinemia: genetic and metabolic studies. *Metabolism.* **45:** 1296–1304.
28. Srivastava, R. A. K., J. Tang, E. S. Krul, B. A. Pfleger, R. T. Kitchens, and G. Schonfeld. 1992. Dietary fatty acids and cholesterol differ in their effects on the in vivo regulation of apoAI and AII gene expressions. *Biochim. Biophys. Acta.* **1125:** 251–261.
29. Krul, E. S., Y. Kleiman, M. Kinoshita, B. Pfleger, K. Oida, A. Law, J. Scott, R. Pease, and G. Schonfeld. 1988. Regional specificities of monoclonal anti-human apolipoprotein B antibodies. *J. Lipid Res.* **29:** 937–948.
30. Bonnardel, J. A., and R. A. Davis. 1995. In HepG2 cells, translocation, not degradation, determines the fate of de novo synthesized apolipoprotein B. *J. Biol. Chem.* **270:** 28892–28896.
31. Srivastava, R. A. K., H. Ito, M. Hess, N. Srivastava, and G. Schonfeld. 1995. Regulation of low density lipoprotein receptor gene expression in HepG2 and Caco2 cells in response to palmitate, oleate, and 25-hydroxycholesterol. *J. Lipid Res.* **36:** 1434–1446.
32. Srivastava, R. A. K., D. Baumann, and G. Schonfeld. 1993. In vivo regulation of low density lipoprotein receptor by estrogen differs at the posttranscriptional level in rat and mouse. *Eur. J. Biochem.* **216:** 527–538.
33. Srivastava, R. A. K., N. Srivastava, and G. Schonfeld. 1992. Expression of low density lipoprotein receptor, apolipoprotein AI, AII, and AIV in various rat organs utilizing an efficient and rapid method for RNA isolation. *Biochem. Int.* **27:** 85–95.
34. Srivastava, R. A. K., and G. Schonfeld. 1991. Use of riboprobes for Northern blotting analysis. *Bio/Techniques.* **11:** 584–587.
35. Srivastava, R. A. K., B. A. Pfleger, and G. Schonfeld. 1991. Expression of LDL receptor, apolipoprotein B, apolipoprotein AI, and apolipoprotein AIV genes in various organs of mouse by a novel solution hybridization assay. *Biochim. Biophys. Acta.* **1090:** 95–101.
36. Farese, R. V., Jr., L. M. Flynn, and S. G. Young. 1992. Modification of the apolipoprotein B gene in HepG2 cells by gene targeting. *J. Clin. Invest.* **90:** 256–261.
37. McLeod, R. S., Y. Zhao, S. L. Selby, J. Westerlund, and Z. Yao. 1994. Carboxy-terminal truncation impairs lipid recruitment by apolipoprotein B100 but does not affect secretion of the truncated apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* **269:** 2852–2862.
38. Farese, R. V., Jr., A. Garg, V. R. Pierotti, G. L. Vega, and S. G. Young. 1992. A truncated species of apolipoprotein B, B-83, associated with hypobetalipoproteinemia. *J. Lipid Res.* **33:** 569–577.
39. Wagner, R. D., E. S. Krul, J. Tang, K. G. Parhofer, K. Garlock, P. Talmud, and G. Schonfeld. 1991. ApoB-54.8, a truncated apolipoprotein found in the apoB gene and hypobetalipoproteinemia. *J. Lipid Res.* **32:** 1001–1011.
40. Groenewegen, W. A., M. R. Averna, J. Pulai, E. S. Krul, and G. Schonfeld. 1994. Apolipoprotein B-38.9 does not associate with apo[a] and forms two distinct HDL density particles populations that are larger than HDL. *J. Lipid Res.* **35:** 1012–1025.
41. Homanics, G. E., T. J. Smith, S. H. Zhang,, D. Lee, S. G. Young, and N. Maeda. 1993. Targeted modification of the apolipoprotein B gene results in hypobetalipoproteinemia and developmental abnormalities in mice. *Proc. Natl. Acad. Sci. USA.* **90:** 2389–2393.
42. Farese, R. V., Jr., S. L. Ruland, L. M. Flynn, R. P. Stokowski, and S. G. Young. 1995. Knockout of the mouse apolipoprotein B gene results in embryonic lethality in homozygotes and protection against diet-induced hypercholesterolemia in heterozygotes. *Proc. Natl. Acad. Sci. USA.* **92:** 1774–1778.
43. Wolle, J., E. Ferguson, L. J. Devall, R. S. Newton, and U. Saxena. 1995. Identification of a novel 85 kD lipoprotein lipase binding protein on human aortic endothelial cell surface. *Biochem. Biophys. Res. Commun.* **216:** 906–912.
44. Sivaram, P., T. Vanni-Reyes, and I. Goldberg. 1996. Endothelial cells synthesize and process apolipoprotein B. *J. Biol. Chem.* **271:** 15261–15266.
45. Du, E. Z., J. Kurth, S. Wang, P. Humiston, and R. A. Davis. 1994. Proteolysis-coupled secretion of the N-terminus of apolipoprotein B. Characterization of a transient, translocation arrested intermediate. *J. Biol. Chem.* **269:** 24169–24176.
46. Wang, S., R. S. McLeod, D. A. Gordon, and Z. Yao. 1996. The microsomal triglyceride transfer protein facilitates assembly of apolipoprotein B-containing lipoproteins and decreases cotranslational degradation of apolipoprotein B in transfected COS-7 cells. *J. Biol. Chem.* **271:** 14124–14133.
47. Urlaub, G., P. J. Mitchell, C. J. Ciudad, and L. A. Chasin. 1989. Nonsense mutation in the dihydrofolate reductase gene affect RNA processing. *Mol. Cell Biol.* **9:** 2868–2880.
48. Kim, E., C. M. Cham, M. M. Veniant, P. Ambroziak, and S. G. Young. 1998. Dual mechanisms for the low plasma levels of truncated apolipoprotein B proteins in familial hypobetalipoproteinemia. Analysis of a new mouse model with a nonsense mutation in the apoB gene. *J. Clin. Invest.* **101:** 1468–1477.
49. Benoist, F., and T. Grand-Perret. 1997. Cotranslational degradation of apolipoprotein B100 by the proteasome is prevented by microsomal triglyceride transfer protein. Synchronized translational studies in HepG2 cells treated with an inhibitor of microsomal triglyceride transfer protein. *J. Biol. Chem.* **272:** 20435–20442.
50. Adeli, K. 1994. Regulated intracellular degradation of apolipoprotein B in semipermeable HepG2 cells. *J. Biol. Chem.* **269:** 9166–9175.