Hammerhead Ribozyme Cleavage of Apolipoprotein B mRNA Generates a Truncated Protein*

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Jian-Ping Wang‡‡, Munechika Enjoji‡, Margret Tiebel‡, Scott Ochsner‡, Lawrence Chan‡, and Ba-Bie Teng‡‡‡‡

From the **Research Center for Human Genetics, Institute of Molecular Medicine, University of Texas, Houston, Texas 77030 and the ‡Departments of Medicine and Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Target substrate-specific hammerhead ribozymes cleave the specific mRNA and results in the inhibition of gene expression. In humans, overproduction of apolipoprotein B (apoB) is positively associated with premature coronary artery diseases. To modulate apoB gene expression, we designed hammerhead ribozymes targeted at AUA⁶⁶⁶⁶ and GUA⁶⁶⁷⁷ of apoB mRNA, designated RB16 and RB15, respectively, and investigated their effects on apoB mRNA in HepG2 cells. The results demonstrated that RB15 and RB16 ribozyme RNAs cleaved apoB RNA efficiently in vitro. Both ribozymes, RB15 and RB16, were used to construct recombinant adeno viral vectors, designated AvRB15 and AvRB16, respectively, for in vivo gene transfer. HepG2 cells were infected with 2 × 10⁵ plaque-forming units of AvRB15 for 5, 10, 15, and 24 h. An RNase protection assay showed that the expression of the RB15 transcript was time-dependent; it increased ~300-fold from 5 to 24 h. Using reverse ligation-mediated polymerase chain reaction, the 3′ cleavage product of apoB mRNA was detected, and the exact cleavage site of apoB mRNA was confirmed by sequencing. Importantly, the levels of apoB mRNA in HepG2 cells decreased ~80% after AvRB15 infection. Pulse/chase experiments on HepG2 cells treated with AvRB15 and AvRB16 demonstrated that ribozyme cleavage produced a truncated protein that was secreted at a density of 1.063–1.210 g/ml. The cleavage activity of RB15 on apoB mRNA was more efficient than that of RB16. Moreover, pulse/chase experiments in HepG2 cells treated with AvRB15 revealed that most of the truncated apoB protein was degraded intracellularly. We conclude that hammerhead ribozyme targeted at GUA⁶⁶⁷⁷ of apoB mRNA cleaves apoB mRNA, results in decreased apoB mRNA levels, and generates a truncated apoB of the expected size in vivo. Thus, the therapeutic application of ribozyme in regulating apoB production holds promise.

Apolipoprotein B (apoB) is a large hydrophobic protein that is synthesized in the liver and small intestine of mammals. ApoB plays an essential, although incompletely understood, role in the assembly and secretion of triglyceride-rich lipoproteins, chylomicrons, and very low density lipoproteins (VLDLs). It also functions in the catabolic clearance of low density lipoprotein (LDL), the major plasma cholesterol transport vehicle in humans (1). Mamalian apoB exists in two forms, apoB100 and apoB48 (2). Both are products of a single gene, which is expressed and processed in a tissue- and species-specific fashion (3, 4). In humans, apoB100 is produced in the liver and assembled as part of VLDL, which is metabolized in the circulation to intermediate density lipoprotein (IDL) and finally to LDL. Unlike other mammalian apolipoproteins, apoB100 does not exchange between lipoprotein particles during this metabolic process and is present in LDL as the sole protein component. Elevated plasma concentrations of apoB100 and LDL are established risk factors for atherosclerotic coronary disease (5). Patients with familial hypercholesterolemia, familial defective apoB100, and familial combined hyperlipidemia or hyperapolipoproteinemia all suffer from either overproduction of apoB100 or decreased clearance of apoB100. The most common of these disorders, familial combined hyperlipidemia, affects 1/50 of the population and is the result of overproduction of apoB100. On the other hand, heterozygous individuals with familial hypobetalipoproteinemia have reduced levels of apoB100, and their plasma LDL cholesterol levels are less than half of normal. Moreover, they appear to be protected from atherosclerotic disease (6). Familial hypobetalipoproteinemia is characterized by the presence of truncated apoB produced by one of the alleles, and the levels of truncated apoB are barely detectable (7). In most cases, the underlying mechanism for the reduced apoB concentration is unclear; it could be the result of an inability of the truncated apoB variants to assemble and be secreted as normal triglyceride-rich lipoprotein. Truncated apoB may be preferentially degraded intracellularly. Alternatively, secreted truncated forms of apoB lipoprotein particles may be rapidly cleared from the circulation, as demonstrated with the apoB50 lipoprotein particle (8). Kinetic turnover studies of apoB89 showed an increased catabolic rate (9), whereas affected individuals with apoB75 had diminished production and increased catabolism (10). Transgenic mice expressing apoB83 had decreased...
apoB mRNA levels, and reduced apoB secretion that was removed rapidly from the plasma compared with apoB100 (11).

We sought to develop a gene therapy strategy to decrease apoB100 production and to alter lipoprotein metabolism for the prevention of coronary artery disease. In this context, the reduction of gene expression using the hammerhead ribozyme is an attractive approach. Ribozymes are small RNA molecules with enzymatic RNA cleaving activity (12). Several ribozyme classes (or catalytic motifs) have been identified, each mediating a naturally occurring biological process. The hammerhead ribozyme self-cleaves at a specific phosphodiester bond to produce 2′,3′-cyclic phosphate and 5′-hydroxyl termini (13, 14). Uhlenbeck (15) and Haseloff and Gerlach (16) engineered substrate-specific hammerhead ribozymes that efficiently cleave target substrate RNAs in trans. A ribozyme designed for cleavage of specific RNA in trans contains three components: 1) a 3′-nucleotide target sequence (NUX) where N represents any base and X represents A, C, or U; 2) a conserved catalytic domain, and 3) flanking sequences complementary to the substrate RNA. Such ribozymes can perform an enzymatic reaction in which a target substrate RNA is cleaved and the ribozyme itself is not altered during the reaction. Recently, substrate-specific hammerhead ribozymes have been used to down-regulate gene expression in vitro and in vivo (17). Target-specific ribozymes have been shown to cleave and lower HIV RNA (18, 19) as well as other target transcripts such as α-lactalbumin mRNA in mouse cells (20) and leukocyte-type 12-lipoxygenase in vascular smooth muscle cells (21). Hammerhead ribozymes have also been used to create target-specific transgenic flies to alter the phenotype (22) and transgenic mice to inhibit gene expression (23–25).

ApoB48, the amino-terminal 48% of apoB100, is the product of an edited apoB mRNA (3, 4). It is synthesized in the small intestine and is an important component for the assembly and secretion of chylomicrons. Both the LDL receptor binding domain (which is essential for the cellular uptake of LDL) and the attachment site of apolipoprotein(a) (which is essential for the formation of lipoprotein(a), a highly atherogenic lipoprotein) are located in the carboxyl-terminal portion of apoB100, which is missing in apoB48. Therefore, the absence of the carboxy-terminal half of apoB100 in apoB48 has profound functional consequences. A recent study provided evidence that the production of apoB48 limits the accumulation of cholesterol-enriched LDL, thus decreasing the formation of atherosclerotic lesions (26).

In this study, we designed hammerhead ribozymes targeted at apoB mRNA sequences of GUA\textsuperscript{6679} ↓ and AUA\textsuperscript{6665} ↓, flanking the edited base C\textsuperscript{6666}. The study demonstrates that the hammerhead ribozyme cleaves apoB mRNA at the precise target site and decreases the levels of apoB mRNA. Furthermore, ribozyme cleavage produces a truncated protein of the expected size.

**MATERIALS AND METHODS**

**Construction of Plasmid Vectors**

**ApoB mRNA-specific Hammerhead Ribozymes**

Oligonucleotides used for engineering apoB mRNA-specific hammerhead ribozymes were synthesized at the core facility at Baylor College of Medicine. Briefly, sense and antisense strands of oligonucleotides of apoB mRNA-specific hammerhead ribozyme were annealed and cloned into XhoI and ClaI sites of a pGem 7ZF(+) vector (Promega, Madison, WI). Each construct was sequenced with Sequenase II (Amersham Pharmacia Biotech) in the presence of single strand DNA-binding protein (Amersham Pharmacia Biotech). Constructs of target sequences of apoB mRNA-specific hammerhead ribozymes were cloned into pGem 7ZF(+) (pGem) and used to generate a U6 snRNA probe for the quantitation of U6 snRNA transcripts by an RNase protection assay. The anti-U6 snRNA probe of 143 nucleotides was produced by EcoRI-linearized pU6 using Sp6 RNA polymerase.

**Human ApoB Construct**

pB3, containing a human apoB cDNA fragment of nucleotides 4953–5602 in pGem7ZF(+) (pU6) and used to generate the antisense apoB probe for the quantitation of apoB transcripts by the RNase protection assay. The anti-B3 probe of 721 nucleotides was produced by XhoI-linearized pB3 using T7 RNA polymerase.

**In Vitro Transcription Reaction**

For synthesis of in vitro transcripts, a maxicistron kit from Ambion (Austin, TX) was used. Briefly, a linearized DNA template (2 μg) was incubated in 20 μl of transcription buffer containing 40 μM Tris-HCl, pH 7.5, 6 mM MgCl\textsubscript{2}, 20 mM dithiothreitol, 5 mM ATP, 5 mM CTP, 5 mM GTP, 5 μM UTP, 40 units of RNase inhibitor, and 10 units of RNA polymerase. The reaction was carried out at 37 °C for 1–2 h, the DNA template was removed by incubation with 2 units of RNase-free DNase I at 37 °C for 30 min, and the RNA was recovered after phenol/chloroform, followed by ethanol precipitation with ammonium acetate. For synthesis of the radiolabeled transcript, 50 μCi of [\textsuperscript{32P}]UTP (10 μCi/μl, Amersham Pharmacia Biotech) was used instead of UTP. At the end of the reaction, the free nucleotides were removed using the nick column method (Amersham Pharmacia Biotech).

**In Vitro Ribozyme Cleavage Reaction**

Control ribozyme RNA corresponding to sense apoB RNA was synthesized from the ribozyme plasmid vectors pRB15 and pRB16 (XhoI-linearized) using T7 RNA polymerase. Synthetic ribozyme RNA corresponding to antisense apoB RNA (HindIII-linearized) was produced using Sp6 RNA polymerase. The concentration of synthetic ribozyme RNA was determined by measuring optical density at 260 nm. pGem-CAA, containing a human apoB cDNA fragment (nucleotides 6506–7335) (29), was used to transcribe a \textsuperscript{32P}-radiolabeled 829-nucleotide synthetic apoB RNA (pGem-CAA linearized with HindIII) using T7 RNA polymerase in the presence of \textsuperscript{32P}UTP (10 μCi/ml, Amersham Pharmacia Biotech). In the in vitro ribozyme cleavage reaction was performed using 1 × 10\textsuperscript{5} cpm \textsuperscript{32P}-labeled apoB RNA as substrate and 2 μg of ribozyme RNA in a buffer containing 50 mM Tris, pH 7.5, 20 mM MgCl\textsubscript{2}, and 1 mM EDTA. The reaction was carried out for 1–2 h at either 37 or 50 °C as indicated. The products were analyzed using 5% polyacrylamide urea gel electrophoresis. The gel was autoradiographed and quantitated using a PhosphorImager SF scanner (Molecular Dynamics, Inc., Sunnyvale, CA).

**Reverse Ligation-mediated PCR (RL-PCR)**

**Synthesis of the RNA Linker**

The following two synthetic oligonucleotides were annealed and used for synthesis of the RNA linker using T7 DNA polymerase: pro-B3 5′-AGCTATTTTCAAATCATGTAA), corresponding to human apoB cDNA nucleotides 6699–6719, was used as the downstream template. Primers 1 and 2 (P1, P2, 5′-TACAAGTATTTTCAATCATAAAT), corresponding to human apoB cDNA nucleotides 6699–6719, were used as the downstream primer for PCR amplification. Primer 2 (P2, 5′-AGCTATTTTCAATCATAAAT), corresponding to human apoB cDNA nucleotides 6699–6719, was used as the downstream primer for PCR amplification.

**DNA Primers**

Three DNA primers 3′ of the RB15 ribozyme cleavage site GU\textsuperscript{6679} ↓ were designed, as suggested by Bertrand et al. (30). Primer 1 (P1, 5′-TCAATGATTTTCAATCATAAAT), corresponding to human apoB cDNA nucleotides 6728–6748, was used for reverse transcription. Primer 2 (P2, 5′-AGCTATTTTCAATCATAAAT), corresponding to human apoB cDNA nucleotides 6699–6719, was used as the downstream primer for PCR amplification. Primer 4 (P4, 5′-GCAAATGATTTTCAATCATAAAT), corresponding to human apoB cDNA nucleotides 6681–6711, was used for sequencing.
Apolipoprotein B mRNA-specific Hammerhead Ribozyme

24163

**RL-PCR**

Total cellular RNA (0.7 mg) after ribozyme cleavage was phosphorylated at the 5′-OH end using T4 polynucleotide kinase (Amersham Pharmacia Biotech). The phosphorylated RNA was ligated with RNA linker (100 μg/ml) using T4 RNA ligase (Roche Molecular Biochemicals). After ligation, cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Life Technologies, Inc.) with the P1 primer, followed by PCR using the downstream primer (P2) and the upstream primer (DNA linker primer, 5′-GGGCATAGCTGACCTGCGT) that is complementary to the RNA linker. This PCR product can be detected either by using 32P-end-labeled primer P2 or by ethidium bromide visualization after analysis with 8% polyacrylamide-urea gel electrophoresis.

**Sequencing**

Nonradioactive primers, P2 and DNA linker primer, were used to produce the PCR product for sequencing. After amplification, the PCR product was treated with 15 units of exonuclease (10 units/μl) and 3 units of shrimp alkaline phosphatase (2 units/μl) to remove the primers and free nucleotides. The purified PCR product was sequenced using the sequencing primer P4 with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia Biotech). The sequenced product was analyzed on 8% polyacrylamide-urea gel and detected by autoradiogram.

**Construction of Recombinant Adenoviral Ribozyme Vectors**

ApoB mRNA-specific hammerhead ribozymes, RB15, RB16, and RB15 mutant, were cloned into the adenoviral shuttle vector, pAvS6, which contains a Rous sarcoma virus promoter as described by Teng et al. (31). The recombinant adenovirus was prepared by co-transfection of pAvS6 containing apoB mRNA-specific hammerhead ribozyme and pJM17 into 293 cells. Adenoviral vectors containing apoB mRNA-specific ribozymes were plaque-purified on these cells. High titer recombinant adenovirus was amplified on 293 cells and purified by CsCl gradient centrifugation as described previously by Teng et al. (31). Recombinant adenovirus AvLacZ4 was supplied by Genetic Therapy Inc. (Gaithersburg, MD). It has the same structure as AvRB15 except that it contains a 3.1-kb nuclear targeted β-galactosidase cDNA insert instead of ribozyme.

**Cell Culture and Recombinant Adenovirus Infection**

**Cell Culture**

Human hepatoma cell line (HepG2) was cultured in Eagle’s minimum essential medium (EMEM) containing 10% fetal bovine serum, 2 mg/ml glutamine, 50 units/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids at 37 °C with 5% CO2. HepG2 cells were plated onto six-well culture dishes until the cells reached 80% confluency. The cells were washed three times with 2 ml of buffer (50 mM Tris, pH 7.5, 0.2% Nonidet P-40, and 5% sucrose). The cells were then homogenized using a Dounce homogenizer (pestle A, six gentle strokes) to release the nuclei. The nuclei were purified by centrifugation (3500 × g for 20 min) through a 10% sucrose cushion (buffer H without Nonidet P-40, containing 10% sucrose). Nuclear RNA was extracted using phenol/chloroform and precipitated with ethanol.

**Continuous Labeling or Pulse-Chase Labeling Experiment**

HepG2 cells were plated onto six-well plates until 80% confluent. Cells were infected with 2 × 105 pfu of AvRB15 or AvRB15 mutant in serum-free EMEM for 15 h. Control cells were without adenovirus infection. The cells were washed with PBS and methionine-free EMEM (ICN Biomedicals Inc.), followed by incubation with methionine-free EMEM for 30 min. The cells were then labeled with a [35S]methionine mix (100 μCi/ml, ICN, Costa Mesa, CA) in methionine-free EMEM. For the continuous-labeling experiment, the cells were labeled at 37 °C for 0, 10, 30, 45, 60, 120, and 180 min. For pulse-chase experiments, the cells were labeled for 15 min at 37 °C. After pulse labeling, the media were removed, and the cells were incubated with serum-free EMEM containing 2 mM methionine for 0, 10, 30, 45, 60, 120, and 180 min. At each time point, medium was collected, and cells were lysed with 2 ml of buffer (50 mM Tris, pH 9.0, 100 mM NaCl, 1% Nonidet P-40) containing a protease inhibitor mixture (Roche Molecular Biochemical). Both cell and medium samples were subjected to immunoprecipitation using human apoB monoclonal antibody 1D1 or 4G3 (Lipoproteins and Atherosclerosis Group, University of Ottawa Heart Institute, Ottawa, Canada).

**Quantitation of the Levels of RB15 RNA in Nucleus and Cytoplasm Fractions after AvRB15 Infection**

**Cytoplasmic RNA Extraction**—HepG2 cells were plated onto 10-cm culture dishes and infected with 5 × 10⁵ pfu of AvRB15 for 15 h. Cytoplasmic RNAs were extracted as described by Bertrand et al. (32). Briefly, the cells were pelleted by centrifugation at 1000 rpm for 5 min, rinsed with ice-cold PBS, and resuspended in RNA extraction buffer (140 mM NaCl, 1.5 mM MgCl2, 10 mM Tris, pH 8.0, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 40 units of RNasin). The samples were placed on ice for 5 min, and centrifuged at 12,000 × g for 90 s. The supernatant was pelleted at 37 °C for 30 min with 1 μg/ml of RNase A and RNase T1 for 30 min. After digestion, the protected fragment of 640 nucleotides was analyzed with 6% polyacrylamide-urea gel electrophoresis.

**Quantitation of RB15 RNA and U6 snRNA by RNase Protection Assay**

Cytoplasmic RNAs (10 μg) and nuclear RNAs (10 μg), prepared as described above, were used to quantitate RB15 RNA and U6 snRNA by RNase protection assay (Ambion). The method for the quantitation of RB15 RNA has been described already. To measure the levels of U6 snRNA, a 32P-labeled anti-U6 snRNA probe of 143 nucleotides was produced from the pU6 vector (linearized with XhoI) using T7 RNA polymerase in the presence of [32P]UTP. After RNase digestion, a protected fragment of 110 nucleotides was analyzed with 6% polyacrylamide-urea gel electrophoresis and quantitated using the PhosphorImager SF scanner. The distributions of nucleocytoplasmic RB15 RNA or U6 snRNA were expressed as a percentage of the total amount of RB15 or U6 RNAs in the fraction of cytoplasm plus nucleus.

**Immunoprecipitation of ApoB**

Briefly, monoclonal antibody 1D1 or 4G3 was incubated with protein A-Sepharose (RepliGen) in binding buffer (1.5 mM glycine, 3.0 mM NaCl, pH 8.9) for 1 h. The beads were then blocked with 10% nonfat dry milk to reduce nonspecific background binding. Immunoprecipitation was carried out by incubating 1 ml of medium or cell lysate with antibody-protein A complex at 4 °C overnight. Immunocomplex beads were washed twice with wash buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS), once with a 1:1 mixture of wash buffer and 1M NaCl, and once with wash buffer only. The beads were suspended in a sample buffer containing 8% urea and 2% SDS. ApoB proteins were resolved on 12% Polyacrylamide gel (FMC, Rockland, ME). The gel was fixed and enhanced with fluorescent technology. The migration bands of apoB100 and truncated apoB were quantitated by using the PhosphorImager SF scanner.
Fig. 1. Schematic diagram of human apoB mRNA-specific hammerhead ribozyme. The structure of RB15 consists of three stems; stems I and III are nucleotide sequences complementary to the apoB mRNA, and stem II is nucleotide sequences of the hammerhead ribozyme catalytic domain. Conserved nucleotides of the catalytic domain are in _boldface italic_ type. The conserved catalytic nucleotide G⁵ mutates to A and is designated as the RB15 mutant. The cleavage target site of apoB mRNA is shown as GUA⁶⁶⁷⁹.

**Characterization of Secreted Apolipoprotein B-containing Lipoproteins**

In some experiments, after the pulse-chase experiment, the media were subjected to sequential ultracentrifugation at densities of 1.006, 1.063, and 1.210 g/ml. The salt density of the media was adjusted by adding appropriate amounts of NaCl/KBr salt solutions. After centrifugation, lipoproteins were collected and dialyzed extensively. Each lipoprotein fraction was immunoprecipitated with the monoclonal antibody 1D1 or 4G3. The method for immunoprecipitation of apoB was the same as described above.

**RESULTS**

**Hammerhead Ribozymes Targeted at Sites Flanking C⁶⁶⁶⁶⁶ of ApoB mRNA**

A schematic diagram of the hammerhead ribozyme targeted at GUA⁶⁶⁷⁹ of apoB mRNA is shown in Fig. 1. The conserved catalytic domain (stem II) is flanked by 13 nucleotides complementary to the apoB mRNA targeted at GUA⁶⁶⁷⁹ (stems I and III). The nucleotide sequences in _boldface_ type are essential nucleotides required for ribozyme cleaving activity. Mutation of these nucleotides will result in partial or complete loss of ribozyme function. The RB15 mutant was constructed by substituting the G⁵ with an A (G⁵A). In _in vitro_ ribozyme activity was assayed using ribozyme RNAs of RB15, RB16, and RB15 mutant. As shown in Fig. 2, apoB RNA of 829 nucleotides was cleaved by ribozyme RB15 to produce two fragments of 656 and 173 nucleotides, whereas cleaving by ribozyme RB16 generated two fragments of 670 and 159 nucleotides. Ribozyme RB15 RNA targeted at sequences GUA⁶⁶⁷⁹ cleaved 49 ± 6.6% (n = 5) of apoB RNA at 37 °C in 1 h. As expected, the proportion of cleaved apoB RNA increased to 91 ± 4.1% (n = 5) at 50 °C. Ribozyme RB16 RNA targeted at sequences AUA⁶⁶⁶⁵ cleaved apoB RNA more efficiently than ribozyme RB15. At 37 °C for 1 h, it cleaved 66 ± 12% (n = 5) of apoB RNA; the proportion of cleaved apoB RNA increased to 90 ± 2.9% (n = 5) at 50 °C. Ribozyme cleaving activity was time-dependent. The activity of both target sites (GUA⁶⁶⁷⁹ and AUA⁶⁶⁶⁵) at 37 °C after a 1- or 2-h incubation increased to 75 and 86%, respectively. The cleaving activities of both RB15 and RB16 reached maximum levels (>95%) at 50 °C after a 2-h incubation. Control experiments using either antisense ribozyme RB15 RNA or ribozyme RB15 mutant RNA had no detectable cleaving activity.

**Effect of Adenovirus-mediated Ribozyme Expression in HepG2 Cells**

To examine apoB mRNA-specific ribozyme activity in cells, ribozymes targeted at nucleotides 6665 (AUA) and 6679 (GUA) were used to construct the recombinant adenoviral vectors, AvRB16 and AvRB15, respectively. AvRB15 mutant was produced as the control for inactive ribozyme RB15.

**Ribozyme RB15 Gene Expression in HepG2 Cells**

HepG2 cells were infected with 2 × 10⁵ pfu of AvRB15 for 5, 10, 15, and 24 h. At each time point, total RNA was extracted from cells, and RB15 mRNA expression was determined by the RNase protection assay. As shown in Fig. 3, the expression of RB15 was time-dependent, increasing from 12 ± 4.6 (n = 4) to 21 ± 4.4 (n = 4) to 21 ± 5.0 (n = 4) to 98 ± 27 (n = 4) pg of RB15 RNA/10 μg of total RNA at 3, 5, 10, and 15 h, respectively. By 24 h, RB15 expression increased to 3090 ± 147 pg of RB15 mRNA/10 μg of total RNA. This markedly increased gene expression was probably the result of adenovirus replication, since HepG2 cells contain E1A-like proteins (33). All of the experiments described in this study were performed by infection of HepG2 cells with recombinant ribozyme adenovirus for 15 h.
Detection of 3' Ribozyme Cleavage Product in HepG2 Cells Using RL-PCR

Many investigators have reported that ribozyme cleavage products cannot be detected by classical techniques, such as Northern blot analysis or RNase protection assay. Bertrand et al. (30) developed a sensitive RL-PCR method that can detect the 3' cleavage product after ribozyme reaction. By using this technique, we were able to detect the 3' ribozyme cleavage product of apoB mRNA after AvRB15 treatment in HepG2 cells. We confirmed the precise cleavage site in apoB mRNA by direct sequencing. As shown in Fig. 4A, after AvRB15 treatment, a radioactive band of 65 nucleotides was detected only in the RNA that was ligated with RNA linker, transcribed with reverse transcriptase, and followed by PCR. There was no detectable band when the RNA was not ligated to RNA linker or not transcribed with the addition of reverse transcriptase. Control RNAs from Av1LacZ-treated (Fig. 4A) or AvRB15 mutant-treated cells (data not shown) after RL-PCR did not have any detectable bands. To confirm the exact cleavage site, we sequenced the PCR product. As shown in Fig. 4B, RB15 ribozyme cleaved human apoB mRNA precisely at the expected position of nucleotide 6679 in HepG2 cells.

Effect of AvRB15 Treatment on the Levels of ApoB mRNA in HepG2 Cells

To determine whether AvRB15 treatment has an effect on the levels of apoB mRNA, we used the RNase protection assay to quantitate apoB mRNA concentration after treatment. As shown in Fig. 5, a protected fragment of 640 nucleotides was shown in nontreated HepG2 cells and in cells treated with either AvRB15 mutant or AvRB15. The levels of apoB mRNA decreased ~80% (ratio of AvRB15-treated/non-treated RNA, 0.200 ± 0.014; n = 3) after AvRB15 treatment, compared with that of nontreated HepG2 cells (1.0, n = 3). In contrast, there was no effect on apoB mRNA levels of HepG2 cells treated with AvRB15 mutant (1.015 ± 0.115, n = 3). Human GAPDH transcript was measured and used as an internal control for the assay. As shown, a protected fragment of 316 nucleotides was identified. There was no change in the levels of the consecutively expressed GAPDH transcripts in nontreated HepG2 cells (1.0, n = 3), or cells treated with either AvRB15 mutant.
apoB mRNA and GAPDH are indicated. The concentration of GAPDH RNA was determined by RNase protection assay using the RPA II kit. The probe and protected products were analyzed with 5% polyacrylamide urea gel electrophoresis. The concentration of apoB RNA was determined by a PhosphorImager SF scanner. The expression levels of human GAPDH were determined in each sample. Antisense GAPDH RNA probe (403 nt) was synthesized from the Ambion pTRI-GAPDH template in a MAXscript kit for in vitro transcription reactions. GAPDH probe (~1 × 10^6 cpm) was incubated with 10 μg of total RNA, and the expression levels of apoB mRNA were determined by an RNase protection assay using an RPA II kit. After RNase digestion, a protected fragment of 640 nt of apoB mRNA was generated. The products were analyzed with 5% polyacrylamide urea gel electrophoresis. The concentration of apoB RNA was determined by a PhosphorImager SF scanner. The probes and protected fragments of apoB mRNA and GAPDH are indicated.

(1.004 ± 0.035, n = 3) or AvRB15 (0.91 ± 0.091, n = 3). Therefore, the apoB-specific hammerhead ribozyme greatly reduced apoB mRNA transcripts with high specificity.

**Effect of AvRB15 and AvRB16 on ApoB Biosynthesis and Secretion in HepG2 Cells**

Next, we investigated the effect of AvRB15 and AvRB16 on apoB biosynthesis and secretion in HepG2 cells. HepG2 cells were infected with AvRB15 or AvRB16 (2 × 10^5 pfu) for 15 h; cells infected with Av1LacZ and AvRB15 mutant (2 × 10^5 pfu) were used as controls. After infection, cells were washed and labeled with [35S]methionine for 15 min and chased for 3 h. At the end of incubation, culture media and cell lysates were immunoprecipitated using human apoB-specific monoclonal antibody 1D1 (which recognizes residues 474–539) (34). As shown in Fig. 6A, media from nontreated and Av1LacZ-infected HepG2 cells contained apoB100 only. The same result was obtained from AvRB15 mutant-infected cells (data not shown). In contrast, media from AvRB15- and AvRB16-treated cells had apoB100 and a truncated apoB of the expected molecular weight. There was substantially more truncated apoB in the media from cells treated with AvRB15 (~50% of total secreted apoB) than cells treated with AvRB16 (~5% of total secreted apoB). Similarly, in cell lysates, apoB100 was the only protein detected in nontreated and Av1LacZ-treated cells, whereas both apoB-100 and a truncated apoB were detected in AvRB15- or AvRB16-treated cells. To confirm that the detection of truncated apoB was not the result of apoB degradation, culture media and cell lysates were immunoprecipitated using the human apoB-specific monoclonal antibody 4G3 (the C-terminal region-specific antibody that recognizes residues 2980–3084). Only apoB100 was detected in nontreated, Av1LacZ4-, AvRB15-, or AvRB16-treated cells (Fig. 6B). Therefore, the results suggest that apoB-specific hammerhead ribozymes cleave apoB mRNA in HepG2 cells, resulting in the production of a truncated protein that is secreted into the media.

The media from controls (nontreated and AvRB15 mutant-infected cells), AvRB15-infected cells, and AvRB16-infected cells were fractionated by sequential ultracentrifugation into VLDL (d < 1.006 g/ml), LDL (d = 1.006–1.063 g/ml), and HDL (d = 1.063–1.210 g/ml), followed by immunoprecipitation with monoclonal antibody 1D1. In nontreated HepG2 cells, only apoB100 was detected in VLDL, LDL, and HDL fractions (Fig. 6C). The same results were obtained from AvRB15 mutant-infected cells (data not shown). In contrast, after AvRB15 treatment, a truncated apoB was detected in the HDL fraction, but not in the fractions of VLDL or LDL. Similarly, the truncated apoB band was detected, but barely visible in the HDL fraction of cells treated with AvRB16. Therefore, the result suggests that the truncated apoB produced after apoB mRNA-specific ribozyme treatment in HepG2 cells was assembled and secreted as HDL-like lipoprotein particles. As noted, a band of ~120 kDa was observed in LDL and HDL fractions of samples treated with AvRB15 and AvRB16. The nature of this band is not clear. Interestingly, unlike the result demonstrated with the in vitro ribozyme cleavage experiment, under in vivo conditions, RB15 ribozyme targeted at GUA^{6679}↓ cleaved apoB mRNA more efficiently than RB16 ribozyme targeted at AUA^{6665}↓.

We expected Rous sarcoma virus-driven RNA to be localized in the cytoplasm; however, our results showed that apoB mRNA-specific ribozyme cleavage produced a truncated apoB, which was secreted as HDL particles. This is unusual, and it was necessary to confirm the location of the expressing ribozyme RNA. RNAs from the nucleus and cytoplasm fractions were extracted from HepG2 cells after AvRB15 treatment. The RNase protection assay was used to quantitate the distribution of ribozyme RB15 RNA in each fraction. To monitor leakage of nuclear contents into the cytoplasm fraction, we measured endogenous U6 snRNA, which is expected to be located in the nucleus only (35). The results showed that 80 ± 6.3% (n = 4) of U6 snRNA was found in the nuclear fraction, whereas 85 ± 3.5% (n = 4) of RB15 RNA was in the cytoplasm fraction. Therefore, by normalizing against the amount of U6 snRNA that leaked into the cytoplasm, we estimated that the relative amount of RB15 RNA in the cytoplasm was ~70%.

**Kinetics of ApoB100 and Truncated ApoB in HepG2 Cells after AvRB15 Treatment**

To understand more about the physiological effect of AvRB15 on the synthesis and secretion of apoB100 and truncated apoB in HepG2 cells under the supplement of oleic acid, we carried out the following studies.

**Continuous Labeling of HepG2 Cells after AvRB15 Treatment with [35S]Methionine—** HepG2 cells cultured in the presence of 3% BSA or 1 mM oleic acid/BSA (Sigma), were infected with AvRB15 for 15 h. After infection, cells were labeled with [35S]methionine, and cell media and lysates were collected at 0, 10, 30, 45, 60, 120, and 180 min. ApoB was immunoprecipitated with monoclonal antibody 1D1 and analyzed by SDS-PAGE. Each data point is an average of four experiments (Fig. 7). After AvRB15 treatment, both apoB100 and truncated apoB were synthesized in the cells (Fig. 7, A and B) and secreted into the
media (Fig. 7, C and D). In both conditions (Fig. 7, A and B), the synthesis rate of truncated apoB was faster than that of apoB100 (the unit of the result is expressed as PhosphorImager counts (PI)/2 h/mg of cell protein; under BSA conditions, truncated apoB was 151,640 and apoB100 was 36,351; under oleate/BSA conditions, truncated apoB was 340,020 and apoB100 was 224,983). In contrast, for both conditions (Fig. 7, C and D), there were more apoB100 molecules secreted into the media compared with truncated apoB; in the presence of oleate, 5-fold more full-length apoB100 molecules were secreted than truncated apoB. When we estimated the percentage of secreted radiolabeled apoB to that synthesized in the cells, only −5% of the radiolabeled truncated apoB was secreted into media compared with that of apoB100 (~20%). Therefore, the result suggests that compared with apoB100, only a small amount of the truncated apoB was secreted.

**Pulse-Chase Experiments**—To confirm that the synthesis of truncated apoB is not derived from post-translational degradation of apoB100, we examined protein synthesis after AvRB15 treatment with a 15-min pulse-labeling followed by a chase of 10, 30, 45, 60, 120, and 180 min in the presence of 1 mM oleate/BSA. ApoB was immunoprecipitated and analyzed from cell media and lysates at the indicated time points. Each data point is an average of three experiments. As observed in the continuous labeling experiment, after AvRB15 treatment, the cells secreted relatively more full-length apoB100 into the media than truncated apoB (Fig. 8A). During the first 60 min after the chase, in the presence of oleate, the amount of labeled intracellular apoB100 and truncated apoB decreased by ~80% (Fig. 8B). For apoB100, most of the radioactivity (>70%) was recovered in the media (Fig. 8C), whereas only ~30% of truncated apoB radioactivity was recovered in the media (Fig. 8D).
Therefore, these results suggest that compared with full-length apoB100, a substantial amount of the truncated apoB was degraded intracellularly.

**DISCUSSION**

ApoB100 is the major protein component of LDL and is responsible for the binding of this lipoprotein to the LDL receptor. Studies in humans showed that overproduction of apoB is positively correlated with premature coronary artery disease (5, 36), which suggests that elevated levels of apoB-containing lipoproteins in plasma play a causal role in the development of atherosclerosis. Studies using HepG2 cells show that apoB mRNA is constitutively expressed with a relatively long half-life of 16 h (37), and apoB mRNA levels do not change even in situations when apoB secretion from HepG2 cells is altered (38–40). Therefore, apoB is regulated mainly at a post-transcriptional level. In this study, we set out to determine whether the apoB mRNA-specific hammerhead ribozyme would cleave apoB mRNA, and if this in turn would result in decreased apoB100 mRNA levels and altered apoB production in vivo. To test our hypothesis, recombinant adenovirus-expressing hammerhead ribozymes targeted at nucleotide sequences AUA6665 and GUA6679 of apoB mRNA flanking the editing base C6666 were used to infect HepG2 cells. This study shows for the first time that hammerhead ribozymes successfully cleaved a 14-kb endogenous apoB mRNA at the expected target site. This reaction results in a reduction of apoB mRNA levels and the secretion of a truncated apoB product. Kinetic studies suggest that most of the truncated apoB was degraded intracellularly.

Several examples have been reported in which hammerhead ribozymes targeted at specific mRNA inhibit gene expression. A striking finding of the present study was the degree of specificity that achieved with ribozymes RB15 and RB16 RNA. The region of 29 nucleotides flanking the apoB mRNA edited base C6666 is conserved among mammals (41). The cis-acting elements required for apoB mRNA editing have been extensively characterized (42). It is known that the distal flanking sequences of the edited base are AU-rich, and this characteristic

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**Fig. 7. Continuous labeling experiment of [35S]methionine.** The effect of AvRB15 on the synthesis and secretion of apoB100 and truncated apoB in HepG2 cells is shown. HepG2 cells under the conditions of 3% BSA, or 1 mM oleic acid/BSA were subjected to AvRB15 infection for 15 h, followed by labeling with [35S]methionine for 0, 10, 20, 30, 45, 60, 120, and 180 min. Cell media and lysate were collected at indicated time points, and equal volumes (1 ml) of cell media (C and D) or lysates (A and B) were subjected to immunoprecipitation using the monoclonal antibody 1D1. The immunoprecipitated was analyzed with 6% ProSieve50 SDS-PAGE. ApoB100 and truncated apoB were quantitated by a PhosphorImager SF scanner. The result is expressed as phosphor image (PI) counts/mg of cell protein. Each data point is an average of four experiments. ApoB100 is represented as open circles, and truncated apoB is shown as closed circles.
affects the efficiency of RNA editing (43, 44). It has been suggested that the relatively AU-rich sequences flanking the edited base may result in a poorly defined RNA secondary structure, allowing protein factor(s) to interact more readily with the edited base. Using the predicted secondary structure program FOLD to analyze the whole apoB100 mRNA, the sequences of nucleotides 6000–7000 flanking the edited base C6666 of apoB mRNA had the lowest energy requirement, compared with other regions of apoB mRNA. Thus, it is possible that the region flanking the edited base has a kinetic advantage for the success in cleaving the transcript. Further investigation of other regions of apoB mRNA could elucidate the accessibility of apoB mRNA to a ribozyme.

The markedly decreased levels of apoB mRNA after AvRB15 treatment may have a significant impact on apoB gene regulation. Studies have demonstrated that the levels of apoB mRNA in HepG2 cells and in animals are resistant to dietary or drug manipulation; apoB is regulated primarily at a post-translational level (39). In this study, ribozyme RB15 RNA cleaved apoB mRNA, generating a truncated apoB product. Pulse-chase experiments showed that most of the truncated apoB product was degraded intracellularly. Thus, ribozyme treatment decreased apoB mRNA levels, decreased apoB100 production, and produced a truncated apoB that was prone to degradation. This presents a very efficient way to regulate apoB production. Therefore, this study demonstrates the potential use of AvRB15 as a gene therapy vector to reduce atherogenic apoB-containing lipoproteins in humans. Furthermore, the production of truncated apoB mimics the phenotype of hypobetalipoproteinemia. Studies using gene targeting to disrupt the apoB gene in mice result in embryonic lethality in homozygotes (11, 45, 46). In heterozygotes, disrupting of one apoB allele had significant consequences on lipoprotein metabolism. A study of apoB83 transgenic mice indicated that the levels of apoB83 mRNA decreased, apoB83 was synthesized, and 25% of apoB100 was secreted. This could be the result of increased apoB83 intracellular degradation (11). Therefore, our study had the similar results as heterozygotes of apoB gene knockout in mice. Ribozyme targets at the levels of mRNA; it does not affect the genomic DNA. It would be interesting to produce

![FIG. 8. Pulse-chase analysis of apoB100 and truncated apoB of HepG2 cells after AvRB15 infection. HepG2 cells under the condition of 1 mM oleic acid/BSA were infected with AvRB15 for 15 h, followed by pulse labeling with [35S]methionine for 15 min and chasing for 0, 10, 20, 30, 45, 60, 120, and 180 min. At the indicated time, cell media A and cell lysates B were collected for apoB immunoprecipitation. The products were analyzed with 6% ProSieve50 SDS-PAGE, and apoB100 and truncated apoB were quantitated by a PhosphorImager SF scanner. Each data point is an average of three separate experiments, and the data are expressed as phosphor image (PI) counts/mg of cell protein. C and D were results calculated as percentage of maximum immunoprecipitated apoB product in cell lysates.](image-url)
apoB mRNA-specific ribozyme transgenic mice to use as an animal model for hypobetalipoproteinemia.

The production of a truncated protein after ribozyme cleavage is unusual. In cells, RNAs do not appear to diffuse freely but are sorted to specific cellular locations (47, 48). Since colocalization of ribozyme and its target can substantially increase the effectiveness of the ribozyme (32, 49), we constructed apoB-specific ribozyme cassettes driven by the Rous sarcoma virus promoter, which has been shown to export capped and polyadenylated transcripts efficiently to the cell cytoplasm (32). We noted that the apoB-specific hammerhead ribozyme is located predominantly in the cytoplasmic compartment (~75%), where protein synthesis occurs. Thus, there are several possible reasons for the production of the truncated apoB after ribozyme cleavage. One possibility is that both the substrate and the ribozyme were co-localized in the same compartment of cytoplasm. Second, the cleavage site of ribozyme RB15 RNA is ~7 kb downstream from the translation start site; therefore, the cleaving reaction probably does not interfere with the initiation of translation of apoB mRNA. The third possibility is that apoB biosynthesis is unique; after the initiation of translation, the newly synthesized apoB is translocated into the cytoplasm. Thus, there are several possibilities for the production of the truncated apoB after ribozyme cleavage.

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