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Research Article

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Familial Hypobetalipoproteinemia Caused by a Mutation in the Apolipoprotein B Gene That Results in a Truncated Species of Apolipoprotein B (B-31)

A Unique Mutation That Helps to Define the Portion of the Apolipoprotein B Molecule Required for the Formation of Buoyant, Triglyceride-rich Lipoproteins

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Abstract

Apolipoprotein B-100 has a crucial structural role in the formation of VLDL and LDL. Familial hypobetalipoproteinemia, a syndrome in which the concentration of LDL cholesterol in plasma is abnormally low, can be caused by mutations in the apo B gene that prevent the translation of a full-length apo B-100 molecule. Prior studies have revealed that truncated species of apo B [e.g., apo B-37 (1728 amino acids), apo B-46 (2057 amino acids)] can occasionally be identified in the plasma of subjects with familial hypobetalipoproteinemia; in each of these cases, the truncated apo B species has been a prominent protein component of VLDL. In this report, we describe a kindred with hypobetalipoproteinemia in which the plasma of four affected heterozygotes contained a unique truncated apo B species, apo B-31. Apolipoprotein B-31 is caused by the deletion of a single nucleotide in the apo B gene, and it is predicted to contain 1425 amino acids. Apolipoprotein B-31 is the shortest of the mutant apo B species to be identified in the plasma of a subject with hypobetalipoproteinemia. In contrast to longer truncated apo B species, apo B-31 was undetectable in the VLDL and the LDL; however, it was present in the HDL fraction and the lipoprotein-deficient fraction of plasma. The density distribution of apo B-31 in the plasma suggests the possibility that the amino-terminal 1425 amino acids of apo B-100 are sufficient to permit the formation and secretion of small, dense lipoproteins but are inadequate to support the formation of the more lipid-rich VLDL and LDL particles. (*J. Clin. Invest.* 1990. 85:933–942.) hypolipidemia • polymerase chain reaction • monoclonal antibody • cholesterol

Introduction

Familial hypobetalipoproteinemia is a syndrome in which the concentrations of apo B and LDL cholesterol in plasma are abnormally low (1). Recent studies have demonstrated that the syndrome can be caused by a variety of mutations in the apo B gene (2–10). Familial hypobetalipoproteinemia heterozygotes, who possess one copy of a mutant apo B allele, are usually asymptomatic, and have apo B and LDL cholesterol levels about one-quarter to one-half of those found in normal subjects (3, 10, 11). Because of their low LDL cholesterol levels, heterozygotes may actually be protected from the development of atherosclerotic coronary artery disease (12). Familial hypobetalipoproteinemia homozygotes, who possess two mutant apo B alleles, have extremely low LDL cholesterol levels, and they may manifest a variety of clinical problems resulting from intestinal malabsorption of dietary fats and fat-soluble vitamins (1).

Many different kindreds with familial hypobetalipoproteinemia have been reported (3, 5–14). In several of the kindreds, the mutation in the apo B gene has been identified (4, 5, 8, 10). In each case, the mutation has created a premature stop codon that prevents the translation of a full-length apo B-100 molecule (4536 amino acids). For example, Collins and co-workers (5) have described a family with hypobetalipoproteinemia in which a single nucleotide substitution created a nonsense mutation in the codon for apo B-100 amino acid 1306. Interestingly, no truncated apo B species was detectable in the plasma lipoproteins or in the lipoprotein-deficient fraction of plasma ($d > 1.21$ g/ml), even with sensitive immunoblotting techniques, leading the authors to suggest the possibility that a mutant apo B species containing only 1305 amino acids (apo B-29) may not be secreted from cells into the plasma. In contrast, mutations leading to premature stop codons in more 3′ portions of the apo B gene have resulted in the production of a variety of truncated apo B species: apo B-37 (1728 amino acids, references 2–4), apo B-39 (1799 amino acids, reference 5), apo B-40 (9), apo B-46 (2057 amino acids, reference 10), apo B-87 (Young, S. G., V. Pierotti, and S. T. Hubl, unpublished observations), and apo B-90 (9). In subjects heterozygous for these mutations, a truncated apo B species is detectable in the plasma, although invariably at a low absolute concentration compared with the concentration of the full-length apo B-100 produced by their normal allele (2–5, 10). The density distributions of the various mutant apo B species within the different lipoprotein density fractions have differed in several respects, particularly in the amount of the truncated apo B species detectable within the HDL fraction (2–5, 9, 10). However, the density distributions of the truncated apo B species have shared two important similarities. First, each of the truncated mutants has been present within the triglyceride-rich VLDL fraction, strongly suggesting that each of the truncated apo B species can support the formation of a triglyceride-rich lipoprotein. Second, none of the truncated apo B species has been detectable in the lipoprotein-deficient fraction of plasma ($d > 1.21$ g/ml).

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In this study, we report a unique apo B gene mutation that results in the formation of a short, truncated apo B species, apo B-31. The mean LDL cholesterol level in four family members who are heterozygous for this mutation is quite low, 29 mg/dl. Apolipoprotein B-31 is the product of a mutant apo B allele that contains a deletion of a single guanine residue, and it is predicted to contain only 1425 amino acids. Apolipoprotein B-31 was detectable in the plasma of all four apo B-31 heterozygotes, but its distribution within the plasma lipoprotein density fractions was quite distinct from that reported for the other truncated apo B species. Apolipoprotein B-31 was present within the HDL fraction (d = 1.063–1.21 g/ml) and the lipoprotein-deficient fraction of plasma (d > 1.21 g/ml), but was absent from the VLDL and LDL fractions. These data suggest that the amino-terminal 1425 amino acids of apo B-100 may be adequate for the formation of the small, dense lipoproteins, but insufficient to support the formation of large, triglyceride-rich lipoproteins.

Methods

Human subjects and blood samples. The proband of the apo B-31 kindred (subject 1 of the family pedigree shown in Fig. 3 *A*) is a healthy 22-yr-old man from central Illinois. He was originally identified during the study of another kindred with familial hypobetalipoproteinemia, the apo B-46 kindred (10). Subjects 1 and 2 of the apo B-31 kindred (Fig. 3 *A*) are identical to subjects 1 and 2, respectively, of the apo B-46 kindred (10). Subject 2 is the mother of subject 1 and is heterozygous for an apo B gene mutation that results in the synthesis of a truncated apo B species, apo B-46. Subject 1 did not inherit the apo B-46 mutation, although the concentration of LDL cholesterol in his plasma was very low, 19 mg/dl (10). An analysis of subject 1's lipoproteins revealed a different truncated apo B species, which was designated apo B-31. The family study was therefore extended, and fasting blood samples were obtained from all available relatives of subject 1. In addition, blood samples from subjects 1 and 4 (Fig. 3 *A*) were obtained 2 h after a meal rich in fats. Blood samples were also obtained from normolipidemic control subjects (unrelated subjects who had low plasma levels of cholesterol) and well-characterized familial hypobetalipoproteinemia heterozygotes whose lipoproteins contained apo B-37 (2, 3) or apo B-46 (10). Blood was collected into sterile tubes containing EDTA (1.5 mg of EDTA/ml of blood). Blood cells were stored for the subsequent isolation of genomic DNA (15). A mixture of protease inhibitors and antimicrobial agents was added to the plasma samples that were to be used for preparing purified lipoprotein fractions (2). All studies involving human subjects were approved by the Human Use Committee of the University of California, San Francisco, and by the Institutional Review Board of the Kaiser Permanente Medical Care Plan.

Quantification of lipoproteins and apolipoproteins. The concentrations of plasma triglycerides and of total, LDL, and HDL cholesterol were measured according to Lipid Research Clinics techniques (16) in the Lipid Analytical Laboratory of the University of California, San Diego, which is under the continuous standardization program of the Centers for Disease Control in Atlanta. The concentrations of apo B-100 and apo A-I in plasma were measured in monoclonal antibody-based immunoenzymometric assays (17, 18). Apolipoprotein E phenotyping was performed on frozen serum samples (19).

Isolation and characterization of the plasma lipoproteins. The VLDL fraction (d < 1.006 g/ml) was isolated from fresh plasma by an 18-h ultracentrifugation at 40,000 rpm at 15°C in a Beckman 40.3 rotor (Beckman Instruments, Inc., Fullerton, CA). The VLDL fraction was then "washed" by an additional 18-h ultracentrifugation in the same rotor. The intermediate density lipoproteins (IDL) (d = 1.006–1.025 g/ml) and LDL fractions (d = 1.025–1.063 g/ml) were isolated by sequential 24-h ultracentrifugation spins at densities of 1.025 and 1.063 g/ml at 40,000 rpm at 15°C in a 40.3 rotor. The LDL fraction and the 1.063-g/ml infranatant fraction were washed by identical 24-h ultracentrifugation spins at d = 1.063 g/ml. The 1.063-g/ml infranatant fraction was then adjusted to d = 1.21 g/ml; the HDL fraction (d = 1.063–1.21 g/ml) was isolated by a 48-h ultracentrifugation at 40,000 rpm at 15°C in a 40.3 rotor. The HDL fraction was then washed by an additional 48-h ultracentrifugation at d = 1.21 g/ml. Each lipoprotein fraction was dialyzed against either PBS (154 mM NaCl, 21 mM Na_2HPO_4, 15 mM Na_2HPO_4, 0.3 mM EDTA, pH 7.35) or 1 mM EDTA, pH 7.4. The protein content of the lipoprotein fractions was assessed by a protein assay in which BSA was the standard (20). The apolipoprotein content of each of the lipoprotein fractions was assessed by electrophoresis on 3–12% gradient SDS-polyacrylamide gels (21, 22). The gels were stained with 0.1% Coomassie brilliant blue R-250 or silver (Bio-Rad Silver Stain Kit, 161-0443; Bio-Rad Laboratories, Richmond, CA) or were electrophoretically transferred to nitrocellulose membranes for Western blotting (22). For the Western blots, we used a variety of previously characterized apolipoprotein-specific antibodies (21, 23–25).

In one set of experiments, the d = 1.085–1.21-, the d = 1.085–1.25-, the d > 1.21-, and the d > 1.25-g/ml fractions were prepared from each subject heterozygous for the apo B-31 mutation (subjects 1, 3, 4, and 6). [The atypical density interval for the HDL density fraction (d = 1.085–1.21 g/ml) was chosen to minimize the amount of apo B-100 in the HDL fraction. There is very little apo B-31 in the d = 1.063–1.085-g/ml fraction.] The d = 1.085–1.21-g/ml and the d = 1.085–1.25-g/ml density fractions were isolated from 1 ml of plasma by using the methodology outlined above. The lipoproteins contained within these density fractions were dialyzed against PBS and then adjusted to a final volume of 1 ml with PBS. The d = 1.21-g/ml and d = 1.25-g/ml infranatant fractions were then subjected to an additional ultracentrifugation at d = 1.21 and 1.25 g/ml, respectively, at 15°C and 40,000 rpm for 48 h in a 40.3 rotor. The infranatant fractions from these tubes (the d > 1.21-g/ml fraction and the d > 1.25-g/ml fraction) were washed with PBS, concentrated on membrane cone filter (Centriflow type CF 25; Amicon Corp., Danvers, MA), and then adjusted to a final volume of 1 ml with PBS. The density of each fraction was ascertained at each step of the ultracentrifugation process. Identical procedures were followed to isolate the d > 1.21-g/ml fraction from selected control subjects. To determine whether apo B-31 was present within the HDL fractions and d > 1.21-g/ml and d > 1.25-g/ml fractions, we analyzed the ability of these fractions to compete with LDL apo B-100 for binding to the apo B-specific monoclonal antibody MB3 (21) in a solid-phase RIA as previously described (24, 26).

The density distribution of apo B-31 within the d > 1.075-g/ml fraction of plasma was analyzed by discontinuous salt gradient (1.075–1.25 g/ml) ultracentrifugation as previously described (2). After ultracentrifugation, the salt gradient was unloaded into 16 fractions; the density of each fraction was measured, and the content of apo B-31 within each fraction was determined by RIA (24, 26).

The size of the apo B-31-containing lipoproteins within the HDL fraction was analyzed by 4–30% gradient polyacrylamide gels electrophoresed under nondenaturing conditions (2, 23, 27). The gels were stained with silver or were electrophoretically transferred to sheets of nitrocellulose membrane for Western blot analysis using monoclonal antibodies specific for apo A-I and apo B (21, 23). The migration of the apo B-31-containing HDL particles on agarose gels was assessed by using 1% agarose gels (No. AC470100; Fisher Scientific Co., Pittsburgh, PA) and the Corning electrophoresis system (Corning Medical, Palo Alto, CA), according to the manufacturer's instructions.

A plasma sample from subject 3 was fractionated by molecular sieve chromatography on an agarose column (A-5M, Medium Gel; Bio-Rad Laboratories) as described by Gibson and co-workers (28). 5 ml of plasma was loaded onto the column and fractionated into 90 3.5-ml fractions. A sample of each fraction was analyzed for cholesterol. Another sample was dialyzed against water and lyophilized, and the apoprotein content was then analyzed by SDS-PAGE and Western blots, using apo B- and apo A-I-specific antibodies.

Enzymatic amplification of DNA, and DNA sequencing. The size of the apo B-31 and its reactivity with antibodies suggested that it must terminate near apo B-100 amino acid 1400 (see Results). To determine the precise mutation in the apo B gene responsible for apo B-31, a 759-bp portion of the apo B gene (apo B cDNA nucleotides 4369–5127, coding for apo B-100 amino acids 1388–1639) was enzymatically amplified (29) by using *Thermus aquaticus* DNA polymerase, oligonucleotides B31-1 (5′ ACAAGAATACGTTCACACTATCATGTGATG 3′, apo B cDNA nucleotides 4369–4398), B31-2 (5′ GCACCtGcAGACTACACTTCAAGTTGGTCG 3′, complementary to apo B cDNA nucleotides 5127–5098), and 0.5 μg of genomic DNA. Two base mismatches (lower case letters) were included in B31-2 in order to create a Pst I site. Enzymatic amplification was performed for 30 cycles at denaturation, annealing, and extension temperatures of 96°, 60°, and 74°C, respectively. The amplified DNA from subject 1, the proband of the apo B-31 kindred, was purified from a polyacrylamide gel, digested with restriction endonucleases, and then force-cloned into M13 for DNA sequencing (30, 31). After the mutation responsible for apo B-31 had been determined by DNA sequencing, the presence of this mutation in other family members was assessed by testing the binding of allele-specific oligonucleotide probes to the 759-bp portion of the apo B gene amplified from the genomic DNA of each family member. For these studies, one-tenth of the amplification reaction was loaded onto a Nytran membrane (No. 01860; Schleicher & Schuell, Keene, NH) by using a slot blot apparatus and was probed with ^{32}P-labeled oligonucleotides specific for the normal or the mutant allele. Oligonucleotides B31-3 (5′ TCAAAAGGTTTACTA 3′) and B31-4 (5′ CTCAAAAGTTTACTA 3′) are specific for the normal and mutant alleles, respectively. The slot blots were hybridized with the labeled oligonucleotides for 16 h at 22°C as described (30), washed at 35°C for 30 min in 2× standard saline citrate (30) containing 0.1% SDS, and then placed on x-ray film for 16 h.

Results

In a recent study (10), we documented the existence of a truncated species of apo B, apo B-46, in the plasma lipoproteins of affected members of a kindred with familial hypobetalipoproteinemia. Apolipoprotein B-46 was shown to be the result of a premature stop codon in the apo B gene that was created by a single nucleotide substitution. One member of that kindred, subject 1, had a very low concentration of LDL cholesterol in his plasma, 19 mg/dl, a level consistent with heterozygous familial hypobetalipoproteinemia, yet he had not inherited the mutant apo B-46 allele. On SDS-PAGE, the VLDL and LDL fractions of subject 1 were normal. However, the HDL fraction contained an abnormal protein band with an estimated molecular weight of 165,000 (Fig. 1 *A*). The abnormal protein was smaller than another mutant apo B species, apo B-37, previously observed in the HDL of another kindred with hypobetalipoproteinemia (2, 3). Western blots using apo B-specific antibodies demonstrated that the abnormal band in the HDL from subject 1 was a mutant apo B species, and it was designated apo B-31, according to the system of nomenclature suggested by Kane and co-workers (32). Two apo B-specific monoclonal antibodies helped us to estimate the length of apo B-31 (Fig. 1 *B*). Antibody MB3 (21), which binds to an epitope between apo B-100 amino acids 995 and 1082 (33), reacted with apo B-31. Antibody 2D8 (25), which binds to an epitope between apo B-100 amino acids 1403 and 1480 (33), did not react with apo B-31 (Fig. 1 *B*). Because of the size of apo B-31, and its reactivity with these antibodies, we originally estimated that it contained the amino-terminal 1400 amino acids of apo B-100.

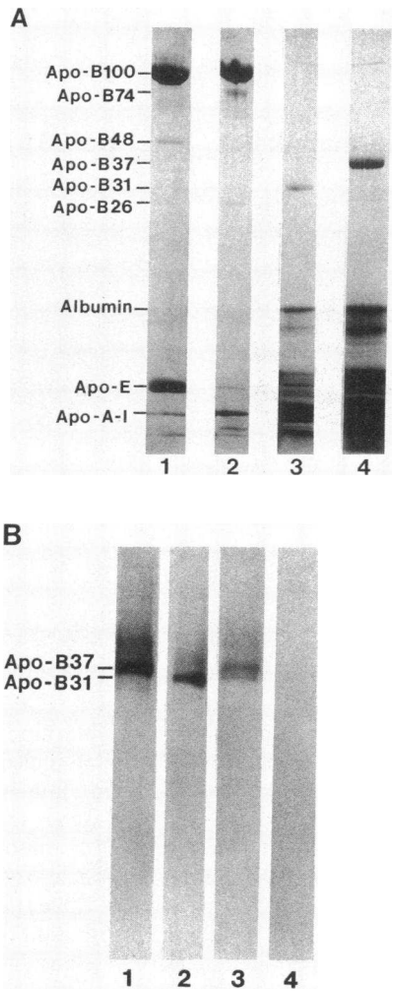


Figure 1. (*A*) Apolipoprotein B-31 in the HDL fraction of subject 1. The delipidated VLDL (lane *1*), LDL (lane *2*), and HDL (lane *3*) proteins from subject 1 and the HDL protein from an apo B-37 heterozygote (lane *4*) were electrophoresed on a 3–12% SDS-polyacrylamide slab gel. 50 μg of protein was loaded in lanes *1–3*, 100 μg in lane *4*. The gel was stained with 0.1% Coomassie brilliant blue R-250. The apo B-37 heterozygote was subject 1 of the H.J.B. kindred (3). The apo B-74 and apo B-26 bands observed in lane *2* represent proteolytic breakdown products of apo B-100 (31). (*B*) The ability of the apo B-specific monoclonal antibodies MB3 and 2D8 to bind to apo B-31. Approximately 20 μg of HDL protein from subject 4 (lanes *2* and *4*) and the same amount of HDL protein from an apo B-37 heterozygote (lanes *1* and *3*) were electrophoresed on a 3–12% SDS-polyacrylamide slab gel. The separated apolipoproteins were then electrophoretically transferred to a sheet of nitrocellulose membrane for immunoblotting with the apo B-specific monoclonal antibodies MB3 (lanes *1* and *2*) and 2D8 (lanes *3* and *4*). The apo B-37 heterozygote was subject 2 of the H.J.B. kindred (3).

To determine the molecular basis for this truncated apo B, the relevant portion of exon 26 of the apo B gene was enzymatically amplified from the genomic DNA of subject 1 and then was subcloned into M13 for sequencing. Because we suspected that subject 1 was heterozygous for a mutant apo B allele producing apo B-31, we expected to find subclones containing the normal apo B sequence and mutant subclones containing a premature stop codon. Indeed, about half of the subclones sequenced had the previously reported normal apo B sequence (34–38); the other subclones contained a deletion of a single guanine residue, apo B cDNA nucleotide 4480 (Fig. 2). As a result of this frameshift mutation, apo B-31 is predicted to contain 1425 amino acids; its calculated molecular weight, excluding glycosylation, is 159,645.

After the mutation responsible for apo B-31 was identified, we performed a family study in which we interviewed all available family members of subject 1 and obtained blood samples from them. All family members (Fig. 3 *A*) reported normal growth and development, and none reported any clinical

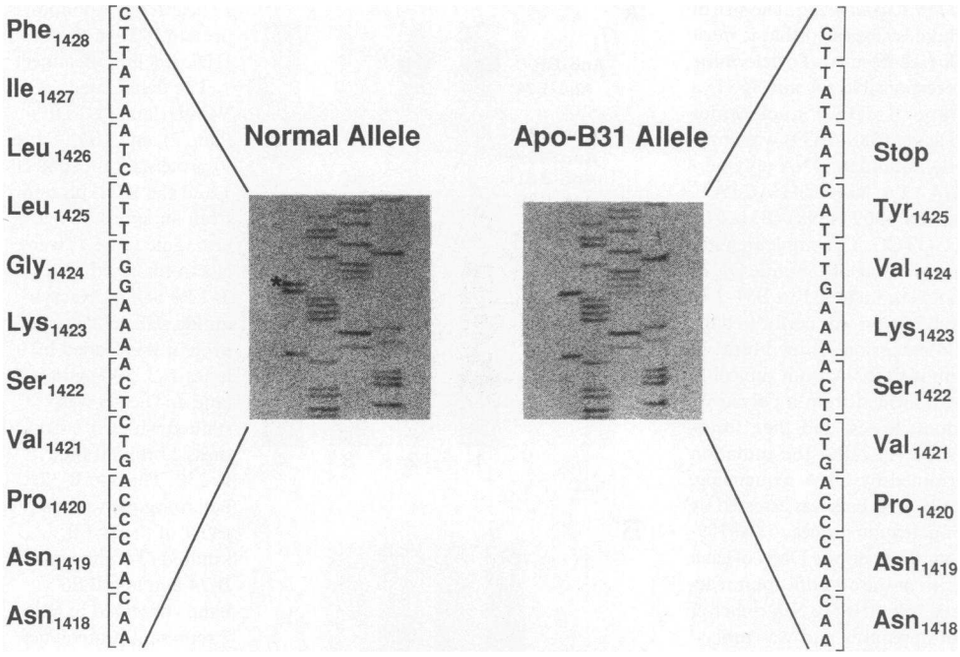


Figure 2. Autoradiograms of DNA sequencing gels demonstrating the mutation responsible for apo B-31. The region of the apo B gene suspected to contain the mutation was enzymatically amplified from the genomic DNA of subject 1 and then was subcloned into M13 for sequencing (see Methods). Approximately half of the subclones contained the normal apo B sequence (*left*), and half contained a deletion of apo B cDNA nucleotide 4480 (*right*). The single nucleotide whose deletion defines the mutant allele is marked by an asterisk in the normal allele. This frameshift mutation is predicted to yield two novel amino acids, valine and tyrosine, followed by a premature stop codon (TAA). No other changes from the consensus apo B sequence were noted in the mutant subclones.

symptoms of fat malabsorption. Experiments testing the binding of allele-specific oligonucleotide probes to the amplified DNA of various family members identified four family members (subjects 1, 3, 4, and 6) who were heterozygous for the apo B-31 mutation (Fig. 3 *B*). The mean concentrations of apo B-100 and LDL cholesterol in the four apo B-31 heterozygotes were very low: 22 and 29 mg/dl, respectively (Table I).

Next, we sought to characterize further the distribution of apo B-31 within the plasma lipoprotein density fractions. On Coomassie blue-stained SDS-polyacrylamide gels, apo B-31 was easily detectable within the HDL fractions of each of the four apo B-31 heterozygotes, but none was observed in the VLDL or LDL fractions (Fig. 1 *A*). In order to be able to detect very small amounts of apo B-31, these experiments were repeated and the gels were stained with silver. Even on overloaded silver-stained gels, apo B-31 was not detectable in the VLDL, IDL, or LDL fractions isolated from any of the four apo B-31 heterozygotes (Fig. 4). This was true regardless of whether the lipoproteins were isolated from blood drawn under fasting conditions or after a fat-rich meal.

The apo B-31-containing particles within the HDL density fraction were easily detectable on gradient polyacrylamide gels electrophoresed under nondenaturing conditions (Fig. 5). The apo B-31-containing HDL particles tended to be somewhat smaller than the apo B-37-containing HDL particles, which, in turn, were smaller than the apo B-46-containing HDL particles (Fig. 5). The apo B-31-containing HDL particles did not contain apo A-I. Like the previously characterized apo B-37-containing HDL particles (2), apo B-31-containing particles within the HDL range had pre-beta mobility on agarose gels (see Fig. 7 *B* below).

The apo B-31 in the HDL of affected family members was easily detectable by a competitive RIA using an apo B-specific monoclonal antibody, MB3 (Fig. 6 *A*). Although apo B is typically undetectable in the $d > 1.21$-g/ml fraction of plasma of normolipidemic control subjects, we hypothesized that some apo B-31-containing particles might be present in the $d > 1.21$-g/ml fraction of affected family members. This hypothesis was initially tested by a competitive RIA using an apo B-specific antibody. The $d > 1.21$-g/ml fraction and the $d > 1.25$-g/ml fraction from the apo B-31 heterozygotes competed with LDL apo B-100 for binding to monoclonal antibody MB3 in a solid-phase RIA (Fig. 6 *B*), whereas the $d > 1.21$-g/ml fraction from an unaffected family member (subject 7) did not. The $d > 1.21$-g/ml and the $d > 1.25$-g/ml fractions from the apo B-31 heterozygotes did not compete in a parallel RIA using monoclonal antibody MB47 (24), an antibody that binds to the carboxy terminus of apo B-100 and does not bind to apo B-31. In the four apo B-31 heterozygotes, the concentration of apo B-31 in the $d > 1.21$-g/ml density fraction was from 20 to 80% less than the concentration of apo B-31 in the $d = 1.085$–1.21-g/ml fraction. In each subject, the concentration of apo B-31 in the $d > 1.25$-g/ml fraction was $< 25\%$ of that found in the $d > 1.21$-g/ml fraction. The concentration of apo B-31 in the plasma of the four apo B-31 heterozygotes, as judged by the total amount of apo B-31 in the $d = 1.085$–1.21-g/ml fraction and the $d > 1.21$-g/ml fraction, was < 2 mg/dl. The presence of apo B-31 within the $d > 1.21$-g/ml fraction was confirmed by Western blot analysis (Fig. 7).

To determine the density distribution of apo B-31 in the plasma more accurately, we subjected the $d > 1.075$-g/ml fraction of plasma from each apo B-31 heterozygote to discontinuous salt gradient ultracentrifugation. In each subject, the peak concentration of apo B-31 was in the $d = 1.18$–1.20-g/ml fractions. Similar experiments with the HDL of apo B-46 heterozygotes demonstrated that apo B-46 is contained only in the more buoyant subfractions of HDL ($d = 1.063$–1.090 g/ml). Previously, we found that apo B-37-containing HDL particles had peak densities of 1.10–1.15 g/ml (2). These data demonstrate that the apo B-31-containing HDL fraction particles are denser than the HDL particles containing the longer mutant apo B species. This information is consistent with the size of the apo B-31-, apo B-37-, and apo B-46-containing HDL particles (Fig. 5).

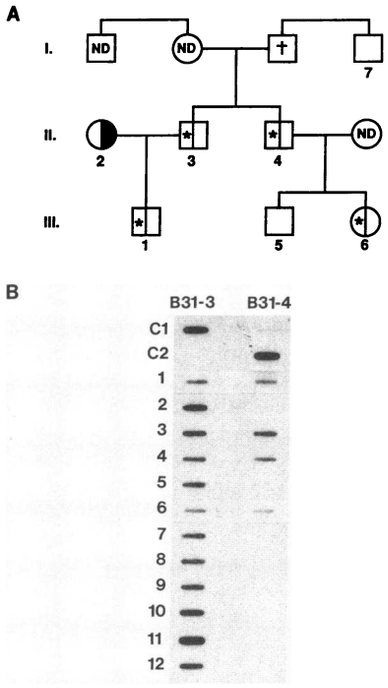


Figure 3. (*A*) Pedigree of the apo B-31 kindred. Each member of the kindred is shown in this pedigree. The proband is subject 1. Subjects possessing the apo B-31 allele are indicated by a circle or square containing an asterisk. The apo B-46 allele is indicated by a half-shaded circle; subject 2 is an apo B-46 heterozygote. Subjects 1 and 2 of the apo B-31 kindred are identical to subjects 1 and 2, respectively, of the apo B-46 kindred (10). ND indicates that the subject was not available for phlebotomy; a dagger indicates that the subject is deceased. I, II, and III denote the three generations of this kindred. (*B*) Binding of allele-specific oligonucleotide probes to a 759-bp portion of the apo B gene amplified from the genomic DNA of members of the apo B-31 kindred. The slot blots were performed according to the procedures outlined in the Methods. The slot blot in the left panel was probed with oligonucleotide B31-3, which is specific for the normal allele; the slot blot shown in the right panel was probed with B31-4, which is specific for the mutant allele. Slot C1 contains single-stranded DNA from an M13 subclone containing the normal apo B sequence; C2, single-stranded DNA from an M13 subclone containing the mutant sequence; slots 1–7, the DNA amplified from the genomic DNA of subjects 1–7 of the apo B-31 kindred; slots 8–12, the amplified DNA of five unrelated hypolipidemic control subjects (whose total cholesterol levels were less than the 5th percentile for age- and sex-matched controls).

An experiment with plasma that had been size-fractionated on an agarose column showed that the absence of apo B-31 in the VLDL and LDL density fractions was not an artifact of ultracentrifugation. 5 ml of plasma from subject 3 was fractionated by molecular sieve chromatography on an agarose column. No apo B-31 eluted in the VLDL fraction (≈ fraction 35). The LDL cholesterol peak was found in fractions 40–43, and the HDL cholesterol peak in fractions 52–61. The apo B-100 peak was found in fractions 40–43. A small amount of apo B-31 was found in fraction 43, but the apo B-31 peak was in fractions 46–52. The apo A-I peak extended from fraction 49 to fraction 61. The observation that the apo B-31 peak eluted somewhat earlier than the apo A-I peak is not surprising, based on the size of the apo B-31- and apo A-I-containing HDL particles demonstrated by nondenaturing gel electrophoresis (Fig. 5). Although the apo B-31 and apo A-I particles were both found in the HDL fraction, the apo B-31 particles were significantly larger.

Discussion

In this paper, we demonstrate that a unique frameshift mutation in the apo B gene (deletion of apo B cDNA nucleotide 4480) yields the clinical syndrome of familial hypobetalipoproteinemia. The plasma of each affected heterozygote contained a truncated species of apo B (apo B-31) and a very low concentration of LDL cholesterol (range, 19–35 mg/dl; see Table I). Recent studies have established that a variety of defects in the apo B gene that prevent the synthesis of full-length apo B-100 can result in familial hypobetalipoproteinemia (2–10). The responsible mutations, each of which results in a premature stop codon, comprise a large deletion in the apo B gene, very short deletions causing frameshifts, and single nucleotide substitutions (Fig. 8). Some data suggest that familial hypobetalipoproteinemia may be as common as 0.1 to 0.8% of the population (40, 41). Therefore, further biochemical inves-

Table I. Lipoprotein and Apolipoprotein Concentrations in the Apo B-31 Kindred

Subject No.	Age/Sex	Triglycerides	Total Chol*	LDL Chol*‡	HDL Chol*	Apo B-100	Apo A-I	Apo E phenotype
				mg/dl				
Apo B-31 heterozygotes								
1§	22/M	81	101	19 (<5)	66	10	156	4/3
3	49/M	18	87	28 (<5)	55	29	145	4/3
4	50/M	21	160	35 (<5)	121	27	167	3/3
6	27/F	28	119	32 (<5)	81	22	99	3/3
Apo B-46 heterozygote								
2§	45/F	89	162	98 (<25)	46	77	190	4/3
Unaffected subjects‖								
5	26/M	38	154	83 (<25)	63	44	118	3/2
7	75/M	94	199	139 (<50)	41	107	141	4/3

* Total Chol, total plasma cholesterol; LDL Chol, LDL cholesterol; HDL Chol, HDL cholesterol. ‡ The LDL cholesterol concentration percentile according to age- and sex-matched controls in the LRC Prevalence Study (39) is shown in parentheses. § Subjects 1 and 2 in the apo B-31 kindred are identical to subjects 1 and 2 in the apo B-46 kindred; the lipoprotein and apolipoprotein levels shown here are the same levels reported in the description of that kindred (10). All members of the kindred who donated blood samples were healthy and free of significant medical problems, except for subject 3, who has adult-onset diabetes mellitus, for which he takes insulin therapy. ‖ Family members whose lipoproteins did not contain either apo B-46 or apo B-31.

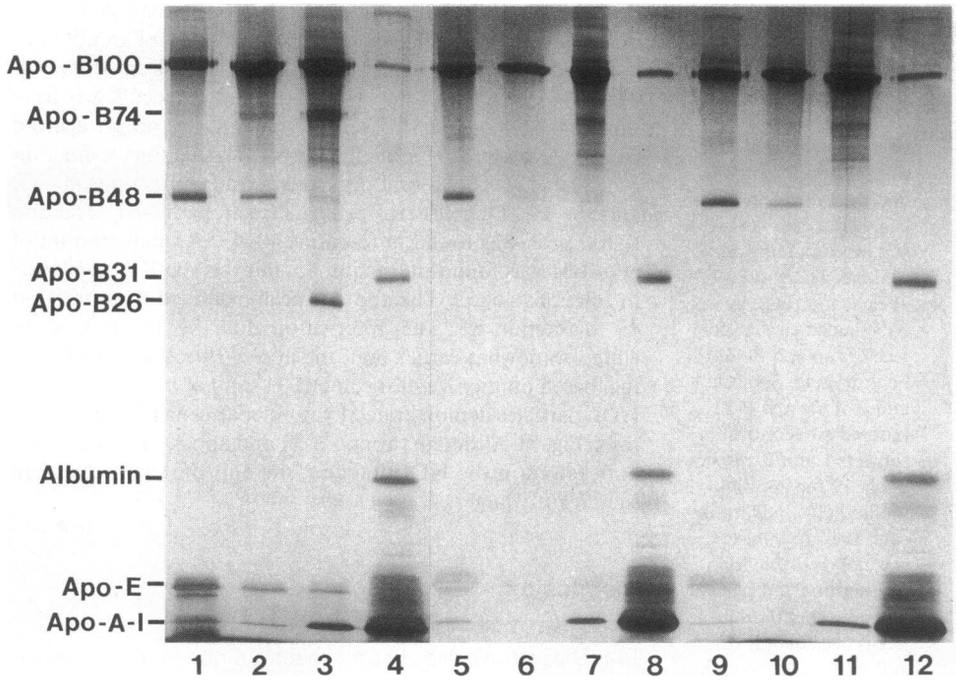


Figure 4. Silver-stained SDS-polyacrylamide gel demonstrating that apo B-31 is contained exclusively within the HDL fraction. The delipidated proteins from the VLDL (lanes *1, 5, 9*), IDL (lanes *2, 6, 10*), LDL (lanes *3, 7, 11*), and HDL (lanes *4, 8, 12*) were electrophoresed on a 3–12% SDS-polyacrylamide slab gel, and the gel was stained with silver. Lanes *1–4* show the lipoproteins isolated from plasma of subject 1, which was obtained 2 h after a fat-rich meal; lanes *5–8*, the lipoproteins isolated from fasting plasma of subject 4; lanes *9–12*, the lipoproteins isolated from plasma of subject 4 obtained 2 h after a fat-rich meal. Identical results were obtained with the lipoproteins from subjects 3 and 6 (not shown). A large amount of protein (3 μg) was deliberately loaded onto each lane to enable us to detect a small amount of apo B-31.

tigations of families with low cholesterol levels will undoubtedly lengthen the list of apo B gene mutations that cause familial hypobetalipoproteinemia. It is also possible that further biochemical studies on families with hypobetalipoproteinemia may ultimately disclose that the hypobetalipoproteinemia phenotype may occasionally be caused by defects in gene products other than apo B.

Fig. 8 summarizes much of this recent research. An apo B gene mutation predicted to result in the synthesis of apo B-29 (1305 amino acids) has been reported (5), but no apo B-29 was detectable in the patient's plasma. In contrast, apo B-31 (1425 amino acids) was easily detectable in the plasma, as were each of the longer truncated mutants. Based on these observations, it is tempting to hypothesize that apo B-29 (1305 amino acids) falls below the critical length of the apo B molecule required for the formation and secretion of an apo B-containing lipoprotein from cells, whereas apo B-31 (1425 amino acids) satisfies the length requirement. This hypothesis seems plausible, and if it were shown to be correct, then the mutation described in this paper would constitute an extremely informative ex-

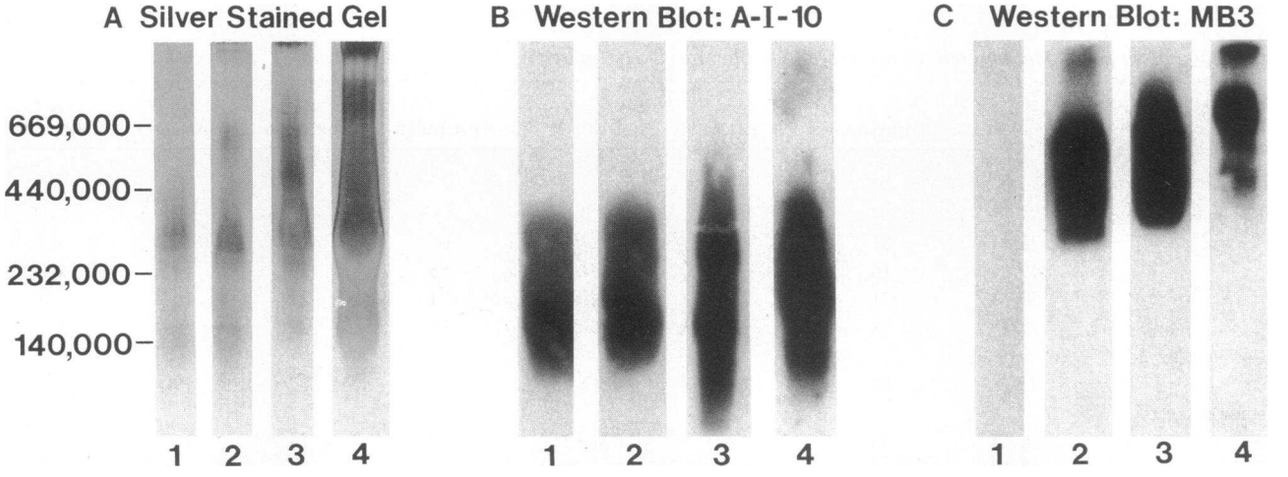


Figure 5. A gradient polyacrylamide gel demonstrating the size of apo B-31-containing HDL particles in relation to apo B-37-containing HDL particles and apo B-46-containing HDL particles. Approximately 4–8 μg of HDL protein from a normolipidemic control subject (lane *1*), an apo B-31 heterozygote (lane *2*), an apo B-37 heterozygote (lane *3*), and an apo B-46 heterozygote (lane *4*) were electrophoresed on a 4–30% polyacrylamide gel under nondenaturing conditions (2, 23, 27). (*A*) Gel stained with silver; (*B*) A Western blot using an apo A-I-specific monoclonal antibody, A-I-10 (23); (*C*) A Western blot using an apo B-specific monoclonal antibody, MB3 (21). The migration of the size standards is indicated: thyroglobulin (669,000), ferritin (440,000), catalase (232,000), and lactate dehydrogenase (140,000).

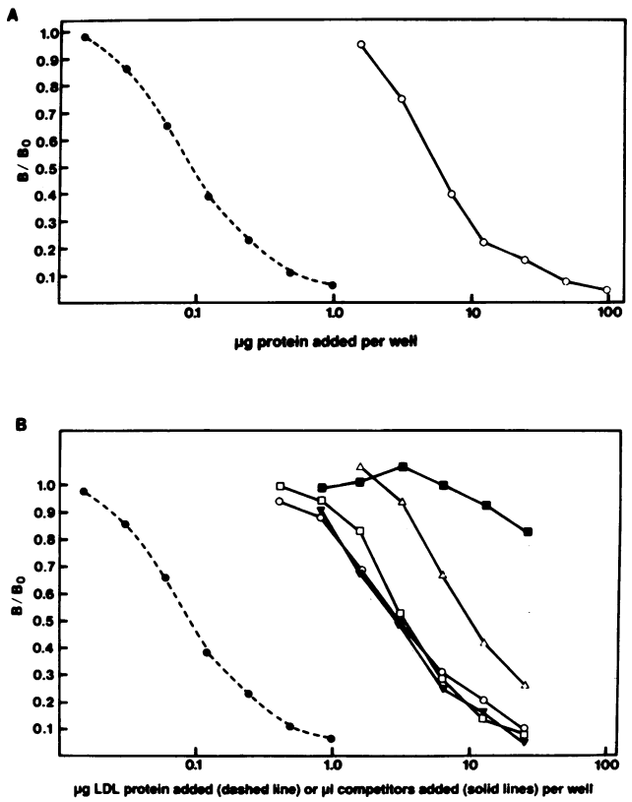


Figure 6. (*A*) The ability of the HDL fraction of an apo B-31 heterozygote to compete with LDL apo B-100 for binding to an apo B-specific monoclonal antibody in a solid-phase RIA. The LDL isolated from a normolipidemic control subject (●) and the HDL of subject 1, an apo B-31 heterozygote (○), were added to the RIA according to their protein content. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the HDL of subject 1 is shown in lane *3* of Fig. 1 *A*. This RIA was performed using monoclonal antibody MB3 as previously described (24, 26), and the results were plotted as B/B_0 vs. log protein concentration, where B and B_0 are specific counts bound in the presence and absence of competitor, respectively. Similar results were obtained using monoclonal antibody MB20, which binds within apo B-100 amino acids 1-1297 (2). A parallel RIA was performed to test the ability of subject 1's HDL to compete in an RIA using monoclonal antibody MB47 (which binds to apo B-100 but not apo B-31). The results of the MB47 RIA indicated that over 95% of the competition observed with subject 1's HDL in the RIA using antibody MB3 was due to the presence of apo B-31 and not to small amounts of apo B-100 in the HDL fraction. (*B*) The ability of the $d > 1.21$-g/ml fraction and the $d > 1.25$-g/ml fraction of an apo B-31 heterozygote to compete with LDL apo B-100 for binding to monoclonal antibody MB3 in a solid-phase RIA. The LDL standard (●) was added to the assay on the basis of protein content. The $d = 1.085$–1.25-g/ml fraction (▼), the $d = 1.085$–1.21-g/ml fraction (○), the $d > 1.21$-g/ml fraction (□), and the $d > 1.25$-g/ml fraction (△) from subject 1, as well as the $d > 1.21$-g/ml fraction of subject 7, an unaffected family member (■), were isolated from 1 ml of plasma and their final volumes adjusted to 1 ml, as outlined in Methods. Each of these density fractions was added to the assay according to volume. Except for the LDL standard, none of these fractions competed significantly in a parallel assay using monoclonal antibody MB47. Virtually identical results were obtained with the $d > 1.21$-g/ml and $d > 1.25$-g/ml fractions of subjects 3, 4, and 6. Based on these RIAs, the concentration of apo B-31 in the plasma of the four apo B-31 heterozygotes was estimated to be less than 2 mg/dl.

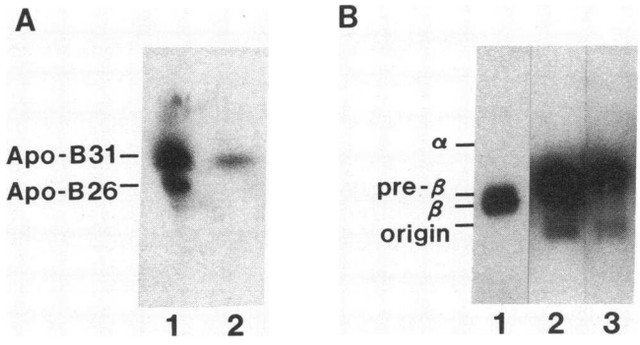
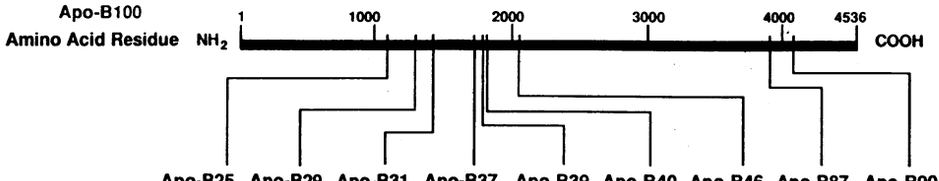


Figure 7. (*A*) Western blot of a 3–12% SDS-polyacrylamide gel demonstrating the presence of apo B-31 in the $d > 1.21$-g/ml fraction of an apo B-31 heterozygote. The same lipoprotein fractions prepared for the RIA in Fig. 6 *B* were used in this experiment. 20 μl of the $d = 1.085$–1.21-g/ml fraction of subject 4 was loaded in lane *1*. (Because no proteolytic inhibitors had been added to this sample, a proteolytic breakdown product of apo B-31, apo B-26, was observed in addition to apo B-31). 3 μl of the $d > 1.21$-g/ml fraction of subject 1 was loaded in lane *2*. After the samples had been electrophoresed on the SDS-polyacrylamide gel, the separated proteins were then electrophoretically transferred to a sheet of nitrocellulose membrane. The blot was reacted with the apo B-specific antibody, MB3. Identical results were obtained with the $d > 1.21$-g/ml fractions of subject 3, 4, and 6. The apo B-31 band in the $d > 1.25$-g/ml fraction was occasionally detectable, but was very faint (data not shown). (*B*) Western blot of a 1% agarose gel demonstrating the presence of apo B-31 particles in the $d > 1.21$-g/ml fraction of the plasma of an apo B-31 heterozygote. The 1% agarose gels were electrophoresed by using the Corning electrophoresis system, and a Western blot was performed using the apo B-specific antibody MB3 as previously described (2). Lane *1* shows normal plasma; lane *2*, the $d > 1.075$-g/ml fraction of plasma of subject 1; lane *3*, the $d > 1.21$-g/ml fraction of plasma of subject 1. The origin is shown, as are the locations of the β, pre-β, and α bands. A very small amount of pre-β-migrating apo B-31 was also observed in the $d > 1.25$-g/ml fraction (data not shown).

periment of nature in that it would help to define the minimum length of apo B required for lipoprotein formation and secretion. However, there are several reasons to be cautious about accepting this hypothesis as fact. First, the number of apo B mutations described to date are few, so any such hypotheses are very preliminary. Second, factors other than the length of the protein (such as the stability of the mutant messenger RNA, the integrity of each apo B disulfide bond, the exact amino acid sequence of the carboxy-terminal portion of the truncated protein, etc.) may greatly influence or even determine the protein's synthetic rate and its ability to be processed, transported intracellularly, and secreted. Finally, the presence or absence of a mutant apo B species in the plasma may have at least as much to do with its metabolism in the plasma as it does with its rate of secretion from cells. As Collins and coworkers (5) pointed out, the absence of apo B-29 in the plasma of their patient may be caused by extremely rapid catabolism of the protein in the plasma rather than by a slow secretion rate from cells.

The mutation described in this paper is also informative because of the unique distribution of the mutant apo B species, apo B-31, within the lipoprotein density fractions. Apolipoprotein B-31 is present in the HDL fraction and in the lipo-



	Apo-B25	Apo-B29	Apo-B31	Apo-B37	Apo-B39	Apo-B40	Apo-B46	Apo-B87	Apo-B90
Mutation	Deletion of 694 bp of the apo-B gene, including all of exon 21	C → T transition at apo-B cDNA nucleotide 4125	Deletion of apo-B cDNA nucleotide 4480	Deletion of apo-B cDNA nucleotides 5391-5394	Deletion of apo-B cDNA nucleotide 5591	Not yet reported	C → T transition at apo-B cDNA nucleotide 6381	Not yet reported	Not yet reported
Predicted Number of Amino Acids	1085*	1305	1425*	1728*	1799*	~1814‡	2057	~3900‡	~4082‡
Truncated Apo-B Species in Plasma	None	None	HDL and d>1.21g/ml fraction	§ VLDL, LDL, HDL	VLDL, LDL	VLDL, LDL, HDL	VLDL, LDL, HDL	VLDL, LDL	VLDL, LDL
Reference	8	5	This paper	2-4	5	9	10	Unpublished data, S. Young, V. Pierotti, S. Hubl	9

Figure 8. Apolipoprotein B gene mutations reported to cause hypobetalipoproteinemia. *The apo B-25, apo B-31, apo B-37, and apo B-39 mutations result in 73, 2, 1, and 5 out-of-frame amino acids, respectively, at the carboxy terminus. ‡The estimate of the number of amino acids in apo B-40 and apo B-90 was made by multiplying 4536 (the total number of amino acids in apo B-100) by 0.4 and 0.9, respectively. The number of amino acids in apo B-87 has been estimated from its size and its reactivity with a variety of apo B-specific antibodies. §In apo B-37 heterozygotes in the H.J.B. kindred, apo B-37 is found predominantly in the VLDL and HDL, with only trace amounts in the LDL. In the compound heterozygotes of the H.J.B. kindred, apo B-37 is also predominantly observed in the VLDL and HDL, but relatively more is observed in the LDL fraction (2, 3). The apo B-29 mutation was originally designated apo B-26 because the truncated protein was close in size to apo B-26, a proteolytic breakdown product of apo B-100. However, the number of amino acids in the truncated protein, 1305, makes its size closer to apo B-29.

protein-deficient fraction of plasma ($d > 1.21$ g/ml), but it is completely absent from the VLDL and the LDL fractions (Figs. 1, 4–7). This distribution was observed both in fasting plasma and in plasma obtained after a fat-rich meal. In contrast, apo B-37 (1728 amino acids), as well as each of the longer truncated apo B species listed in Fig. 8, has been easily detectable within the triglyceride-rich VLDL fraction. These data suggest the possibility that while the amino-terminal 1728 amino acids of apo B-100 contain adequate lipid-binding domains to participate in the formation of a VLDL particle, the amino-terminal 1425 amino acids do not. Yang and coworkers (42, 43) have recently provided important information about the structure of apo B-100 amino acids 1425–1728 on LDL particles (i.e., the region between apo B-31 and apo B-37). In prodigious studies, they performed extensive mapping of the LDL-apo B-100 tryptic peptides and were able to determine the portions of the apo B-100 molecule on native LDL particles that were "accessible" to trypsin and therefore readily releasable from the LDL particles (the "trypsin-releasable peptides") and the portions that were accessible only after the LDL particles had been delipidated and retrypsinized (the "trypsin non-releasable peptides"). Different regions of the apo B-100 molecule had markedly different amounts of trypsin-releasable peptides. The region of apo B-100 between amino acids 1425 and 1728 contained alternating regions of trypsin-releasable and trypsin non-releasable peptides. These investigators were careful to point out that the factors governing trypsin releasability were incompletely understood, and that trypsin releasability versus non-releasability did not necessarily imply a lipoprotein surface location versus a lipoprotein core location for the peptides. However, it is tempting to hypothesize that this region of apo B molecule (amino acids 1425–1728), which is present in apo B-37 but absent in apo B-31, dips in and out of the lipoprotein core and is therefore important for lipid binding. If this hypothesis is correct, it would be consistent with the alternating pattern of trypsin releasability and non-releasability within this region and consistent with the presence of apo B-37 (but not apo B-31) in the larger, more lipid-rich lipoproteins. Ultimately, it should be possible to study the size, density, and lipid-binding characteristics of lipoprotein particles containing various lengths of the apo B molecule in more detail by transfecting lipoprotein-producing cultured hepatoma cells with apo B expression vectors containing various lengths of the apo B cDNA.

Apolipoprotein B-31 was present in the lipoprotein-deficient fraction of plasma ($d > 1.21$ g/ml) in all four affected heterozygotes (Figs. 6, 7). Most of the apo B-31 in the $d > 1.21$-g/ml fraction was contained on particles with a buoyant density between 1.21 and 1.25 g/ml. The presence of apo B-31 in such a high density range is almost certainly due to the fact that apo B-31-containing particles have a very low lipid/protein mass ratio (44). To our knowledge, this is the first example of any apo B species detected in the $d > 1.21$-g/ml fraction of plasma. The unique density distribution of apo B-31 within plasma will have to be taken into account when devising a strategy to screen for truncated apo B species in subjects with hypobetalipoproteinemia. The screening process should not be confined to those lipoprotein density fractions that normally contain almost all of the apo B (the VLDL and LDL fractions). Instead, each of the lipoprotein fractions, including the HDL fraction and the $d > 1.21$-g/ml fraction, should be examined for the presence of a truncated apo B species.

Recent studies have demonstrated that some apo B gene defects causing hypobetalipoproteinemia do not produce a truncated apo B species but instead result in reduced concentrations of a full-length apo B-100 (2, 3, 5, 45, 46). This type of gene defect may prove to be analogous to β+ thalassemia, in which β globin is synthesized, but in reduced amounts. β+ thalassemia has been shown to be caused by promoter mutations that result in reduced gene transcription, intron-exon splicing errors, and a mutation in the polyadenylation signal

that reduces intracellular mRNA levels (47). The examination of apo B alleles yielding reduced amounts of a full-length apo B-100 may uncover a similar spectrum of molecular defects.

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