Two apolipoprotein B gene defects in a kindred with hypobetalipoproteinemia, one of which results in a truncated variant, apoB-61, in VLDL and LDL

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Abstract We report the presence of two distinct defects of the gene for apolipoprotein B, one resulting in a new truncated variant, apoB-61, in a kindred with familial hypobetalipoproteinemia (FHB). The proband (age 33) and a sister (age 36) are both compound heterozygotes with total cholesterol levels of 39 mg/dl and 50 mg/dl, and apoB levels of 1 mg/dl and 2 mg/dl in plasma, respectively. Both appear to be asymptomatic. The apoB-61 mutation, present in a total of five individuals and inherited from the proband's father, is a 37 bp deletion in exon 26 starting with nucleotide 8525. This results in an apoB of 2784 amino acids with 12 novel carboxy-terminal residues. The apoB-61 is present to a considerable degree, relative to apoB-100, in the proband's very low (VLDL) and low density (LDL) lipoprotein fractions. Both lipoprotein fractions have abnormal particle size distribution by electron microscopy. The LDL contain cuboidal particles. Total cholesterol, LDL cholesterol, and apoB levels in the family display three phenotypic patterns: normal, low, and extremely low. ApoB haplotyping indicates the presence of another defective apoB allele in a total of seven individuals. This allele leads to low levels of apoB-100. The second apoB gene-linked defect occurring together with the apoB-61 mutation explains the 3-phenotype pattern. The severe hypocholesterolemia seen in the proband and a sister result from the genetic compound state involving both alleles. This study shows that severe hypolipidemia in an individual heterozygous for a truncation in apoB is likely to involve a second genomic defect.—**Pullinger, C. R., E. Hillas, D. A. Hardman, G. C. Chen, J. M. Naya-Vigne, J. A. Iwasa, R. L. Hamilton, J-M. Lalouel, R. R. Williams, and J. P. Kane.** Two apolipoprotein gene defects in a kindred with hypobetalipoproteinemia, one of which results in a truncated variant, apoB-61, in VLDL and LDL. *J. Lipid Res.* 1992. **33:** 699–710.

Supplementary key words lipoproteins • haplotype • Lp[a]

Apolipoprotein B (apoB) is an essential structural component of chylomicrons, VLDL, LDL, and Lp[a] and is the ligand for receptor-mediated clearance of LDL. Normally there are two forms of apoB in the circulation, apoB-48 and apoB-100. ApoB-100 (550 kDa), the more abundant form, is found on lipoproteins of hepatic origin, VLDL, LDL, and Lp[a], which transport endogenous lipids. In humans, apoB-48 (264 kDa) is found exclusively on the chylomicrons, lipoproteins of intestinal origin responsible for the transport of exogenous (dietary) lipids.

ApoB-100 is composed of a single polypeptide chain 4536 residues long, the product of a single apoB gene (1, 2). ApoB-48 comprises the N terminal 2152 residues of apoB-100 (3, 4) and is produced as the result of editing of the apoB RNA transcript when Gln codon 2153 (CAA) is converted to a stop translation codon (TAA) (5).

At least two genetic disorders exist that cause decreased levels of apoB-containing lipoproteins. One, recessive abetalipoproteinemia, is characterized in the homozygous state by extremely low levels of cholesterol and apoB in plasma. This is accompanied by severe fat malabsorption that can lead to deficiency of fat-soluble vitamins, spinocerebellar and retinal degeneration, and acanthocytosis. Heterozygotes with this trait are asymptomatic. Another inherited disorder is familial hypobetalipoproteinemia (FHB). Whereas the genetic defect (or defects) responsible for abetalipoproteinemia have not yet been determined, some cases of FHB have been shown to be due to mutations in the apoB gene (6). Some of these FHB mutations result in the presence of truncated variants of apoB in the plasma of the individuals concerned (7–15). These

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; Lp[a], lipoprotein[a]; FHB, familial hypobetalipoproteinemia; TTBS, Tris-buffered saline containing 0.2% Tween 20; HDL, high density lipoprotein; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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are the result of mutations that cause premature termination of translation. Other mutant alleles exist that result in decreased concentrations, or the absence of, normal full-length apoB-100 (6, 8). FHB homozygotes (or compound heterozygotes) that have two of these null alleles are clinically very similar to those individuals with abetalipoproteinemia (6, 16, 17). Those individuals with one null allele and one that produces a truncated apoB, or with two alleles that each produces a truncated apoB species, display a range of clinical features. Simple heterozygotes for FHB have reduced levels of plasma apoB and LDL cholesterol, but are generally asymptomatic (17).

We report here a kindred with hypobetalipoproteinemia in which the proband, her father, sister, and two brothers have an apoB allele that produces a new truncated species, apoB-61. In addition, the pattern of cholesterol and apoB levels in plasma and haplotypes of the apoB gene in this family indicate that there is an as yet unidentified defect in another apoB allele. Two of the individuals with the circulating apoB-61 species, the proband and her sister, have inherited this other apoB-linked trait as well and have extremely low levels of apoB in plasma. None of the members of this family, however, has any of the symptoms that are associated with abetalipoproteinemia.

Simple heterozygotes for FHB, in a number of cases, have truncated apoB variants present at low levels in plasma together with a relatively much higher level of apoB-100. However, the proband reported here, a compound heterozygote, has a very low concentration of particles containing apoB-100 in those ultracentrifugal fractions that contain apoB-61. This has allowed an examination of the chemical composition, morphology, and size distribution of the lipoproteins that contain apoB-61 in the proband's plasma. Because there have been reports (15, 18, 19) that FHB is sometimes associated with abnormal levels of HDL, we have measured levels of HDL cholesterol and total apoA-I in this kindred as well.

METHODS

Identification of proband

As a result of family studies into genetic abnormalities that affect plasma lipoprotein profiles, an asymptomatic 33-year-old woman was found to have total cholesterol and triglyceride levels in plasma of 39 and 22 mg/dl, respectively, and an LDL-cholesterol level of 6 mg/dl. Six of her seven siblings, in addition to her parents and maternal grandmother, also had total cholesterol levels in plasma below the fifth percentile (**Fig. 1**). These individuals and her normocholesterolemic brother were studied further.

Blood collection and analysis of lipids

Two samples of blood were taken from each individual, one in EDTA-containing tubes for lipid determinations and one in citrate-containing tubes for the preparation of genomic DNA (20). In addition, a sample of blood was drawn from the proband for analysis of the apoB species present in her lipoprotein fractions. In order to prevent degradation of apoB (21), the following inhibitors were added immediately: sodium azide (0.05%, w/v); EDTA (0.05%, w/v); benzamidine (0.03%, w/v). Total cholesterol, lipoprotein cholesterol, and triglyceride levels were assayed as previously described (22, 23).

Preparation of lipoprotein fractions

VLDL ($d < 1.006$ g/ml), LDL ($1.006 < d < 1.063$ g/ml), and HDL ($1.063 < d < 1.21$ g/ml) were prepared from plasma by sequential ultracentrifugation (24). Inhibitors of proteolysis were present during each step of the

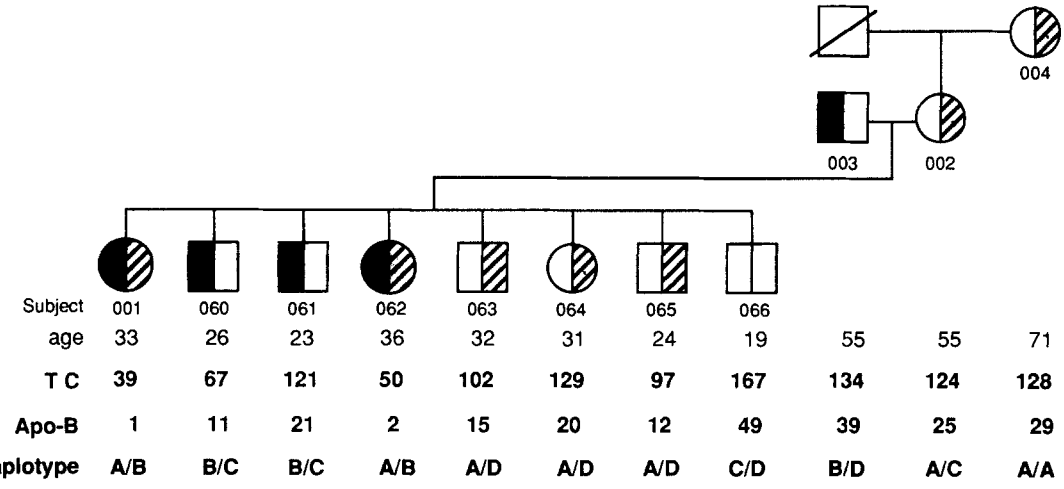

Fig. 1. Kindred 2005. Only members from whom blood samples were obtained are shown. The proband (B.A.S.) is subject number 001. Plasma total cholesterol (TC) and apoB (measured by ELISA) are expressed in mg/dl. ApoB haplotypes are as described in Table 2. ■, indicates apoB-61 mutation; ◪, unidentified mutation causing hypocholesterolemia and segregating with the apoB haplotype A inherited from subject number 004.

isolation as above. The fractions were then stored in the dark at 4°C. These lipoprotein fractions were examined by electron microscopy and the diameters were measured as previously described (25).

Limited protease digestions of lipoproteins

VLDL from the proband and from a control subject were subjected to limited proteolysis with cathepsin D (26). VLDL (0.05 mg apoB/ml) were incubated at an enzyme to substrate ratio of 1:200 at 37°C for 10 min in 40 mM Na acetate, 120 mM NaCl, pH 4.8. After this time Tris-HCl (pH 8.4) was added to a final concentration of 100 mM.

Control LDL (0.05 mg apoB/ml) were incubated with *Staphylococcus aureus* V8 protease at 37°C for 60 min in 125 mM Tris-HCl, 150 mM NaCl, pH 8.4, at an enzyme to substrate ratio of 1:150 (27).

Digestions by these two enzymes were stopped by boiling for 1 min in the presence of SDS (2% final concentration).

Electrophoresis and immunoblotting

VLDL, LDL, and HDL from the proband were analyzed, after delipidation with ethanol–ether 3:1 (v/v), on linear gradient (3–7%) SDS polyacrylamide gel electrophoresis (PAGE) using a discontinuous buffer system (27, 28). In control lanes, fragments of apoB produced by the limited digestion of normal LDL with *Staphylococcus aureus* V8 protease (SP) together with VLDL containing only apoB-48 (264 kDa) and apoB-50 (276 kDa) from a patient with homozygous FHB (10) were run as molecular weight markers to determine the size of the truncated apoB variant in the proband's plasma. The SP digest contains the following molecular weight species: apoB-100, 550 kDa; SP5, 406 kDa; SP1, 374 kDa; SP3, 230 kDa; SP2, 176 kDa; SP4, 144 kDa. VLDL digested with cathepsin D were also examined by SDS-PAGE. Gels were stained with Coomassie brilliant blue R-250.

Immunoblotting was carried out by a modification of the method of Towbin, Staehelin, and Gordon (29). Apolipoproteins from the gels were electrotransferred to zeta-probe membranes (Bio-Rad, Richmond, CA) in a buffer containing Tris (25 mM) and glycine (192 mM), pH 8.3. The membranes were blocked with 5% (w/v) nonfat dried milk in Tris-buffered saline, pH 7.5 (TBS) for 2 h at 23°C and then washed in TBS containing 0.2% (v/v) Tween 20 (TTBS). They were then incubated for 2 h at 23°C, with one of three anti-apoB antibodies, in TTBS plus 1% nonfat dried milk. These antibodies were: a goat antiserum to human LDL, a focal polyclonal rabbit antiserum against a synthetic apoB peptide corresponding to residues 890-908 (a gift from Dr. T. L. Innerarity of the Gladstone Foundation Laboratories), and an anti-apoB monoclonal antibody, 4G3 (30). After washing with TTBS, the blots were incubated for 1 h at 23°C with either ^{125}I-labeled swine anti-goat IgG, ^{125}I-labeled goat anti-rabbit IgG, or ^{125}I-labeled rabbit anti-mouse IgG as appropriate. The blots were washed with TTBS and subjected to autoradiography at −70°C using Kodak X-Omat RP film and an intensifying screen.

Measurement of apolipoprotein levels

The total apoB concentration of plasma was determined by a modified competitive ELISA assay (31, 32) using a goat anti-human apoB polyclonal antibody covalently crosslinked to horseradish peroxidase (33). Normal LDL apoB-100 was used as a standard in this assay. No attempt was made to correct for the truncation in measuring the apoB protein mass in those individuals with circulating apoB-61. A radioimmunoassay was used to measure the apoE levels in plasma (34). Total apoA-I concentrations were determined using a commercial radial immunodiffusion assay with variability of ± 10% (Tago, Burlingame, CA). Lp[a] levels were measured by ELISA (35). This is a sandwich type assay that uses two monoclonal antibodies specific for Lp[a]. One is used to coat the plate and the other, linked to horseradish peroxidase, is used to quantitate the amount of Lp[a] bound to the plate. The assay range for Lp[a] in plasma is 0.1–45.0 mg/dl.

Sequence and position in apoB cDNA of the oligonucleotides used

#5 CTGAATTCATTCAATTGGGAGAGACAAG - 6504 to 6531

#21 GGGAATCAAGGAGTCTTCTGGTTGAG - antisense, 11555 to 11529

#24 CTCACCATATTCAAAACTGAGTTGAGGG - 12234 to 12261

#25 ATTTGTTCCTCCTCCCCCAAGTTTAGC - anti-sense, 14095 to 14069

#26 CCACCAATCAGAAATGTAGGTGACAAG - anti-sense, 7790 to 7764

#29 GGCTCACATGAAGGCCgAATTCCGAGAG - 7643 to 7670

#30 GCTATGTGGCaAGCTTTCAACAGTGTC - anti-sense, 8887 to 8861

#36 GGCACAGCAAAACCTCTAGAACACATAGTG - 5' primer for 3' HVR

#37 CCTTCTCACTTGGCAAATAGAATTCCTGAG - 3' primer for 3' HVR

#44 GTCATCTACCAAAGGAGATGTCAAGG - 10607 to 10632

Polymerase chain reaction, subcloning and DNA sequencing

Because the size of the abnormal species of apoB in the proband's VLDL and LDL was found to be 338 kDa, and assuming that this was the result of a C-terminal truncation, the protein should terminate approximately at amino

acid residue 2790. To determine the nature of the mutation responsible for the production of the apoB-61 in this subject, a 1245 bp region of apoB exon 26 was amplified by PCR (36) using primers #29 and #30. The reaction mixture, total volume 50 μl, contained 500 ng genomic DNA, 25 ng each primer, 3 mM Mg^{2+}, and 1.5 units of Taq polymerase (Cetus, Emeryville, CA). After denaturation at 95°C for 10 min, 30 cycles of amplification were carried out, each for 15 secs at 95°C, 15 secs at 60°C, and 2 min at 72°C. Primer #30 contains a single base substitution forming a *Hind*III site. In order to subclone into M13 for DNA sequencing, the PCR product was digested with *Pst*I and *Hind*III and electrophoresed on a 1% agarose gel stained with ethidium bromide. This was expected to yield a 538 bp band plus the required 694 bp fragment (apoB cDNA nucleotides 8181–8874, codons 2657–2888). However, it was noted that in addition to the 538 bp band there was a closely spaced triplet of bands centered at 694 bp. These latter three bands were recovered from the gel using Geneclean (Bio 101, La Jolla, CA) and cloned into M13mp18 and M13mp19.

Dideoxy chain termination sequencing (37) was performed using Sequenase (USB, Cleveland, OH). As a result of the DNA sequencing, which revealed a deletion mutation, DNA from each of the other family members was amplified with primers #29 and #30, cut with *Pst*I and electrophoresed on a 1% agarose gel to detect the presence or absence of the mutation.

ApoB restriction fragment length polymorphisms

For each family member, four apoB RLFPs, involving the restriction enzymes *Xba*I (38), *Msp*I (39), *Eco*RI (38), and *Mae*I (40), were studied. In each case the relevant region of the apoB gene was amplified by PCR, the product was digested overnight with the restriction enzyme, and analyzed on an agarose gel stained with ethidium bromide. The oligonucleotide primers used in these amplifications were: *Xba*I RFLP, #5 and #26; *Msp*I RFLP, #21 and #44; *Eco*RI RFLP, #24 and #25; *Mae*I RFLP, #29 and #30. The PCR conditions were as described above. An apoB allele that lacks the enzyme-cutting site, producing the largest restriction fragment, is designated 1. Conversely, an allele possessing the site and producing the smallest fragment is designated 2.

Number of tandem repeats in the apoB 3' hypervariable region

For each family member the polymorphic sequence downstream of the apoB gene that is composed of 15 bp AT-rich tandem repeats (41) was amplified by PCR. The 5' oligonucleotide primer used was #36 and the 3' primer was #37. The amplified material was electrophoresed on 2% agarose gels. The size of the DNA bands was determined by comparison with the migration of four control PCR fragments of known size and with the fragments in a ΦX174 *Hae*III digest. The formula used to calculate the number of tandem repeats was based on the sequence data of Knott et al. (41) and Ludwig, Friedl, and McCarthy (42).

RESULTS

Isolation of a truncated apoB variant

The VLDL and LDL fractions from the proband (subject #001), when electrophoresed on SDS-PAGE, were found to contain an abnormal protein band with a molecular mass of about 338 kDa together with apoB-100 and, in the case of VLDL, apoB-48 (**Fig. 2**). The total amounts of the 338 kDa protein and apoB-100 in these two fractions were very low. The abnormal protein was shown by immunoblotting of VLDL using a polyclonal antibody to apoB-100 to be a truncated species of apoB (**Fig. 3**). Hence, from its size it was designated as apoB-61 using the nomenclature of Kane, Hardman, and Paulus (43). On parallel immunoblots, a monoclonal antibody to apoB, 4G3, recognized apoB-100 but no other species of apoB present, whereas rabbit antiserum to a apoB N-terminal synthetic peptide (residues 890–908) recognized apoB-48, apoB-61, and apoB-100 (data not shown). The epitope for 4G3 has been localized to the region between residues 2980 and 3084 (44). A trace of apoB-100, but no apoB-61, was observed in the HDL fraction on SDS-PAGE (data not shown).

When VLDL from the proband was subjected to limited proteolysis with cathepsin D and the products were electrophoresed on SDS-PAGE under reducing conditions,

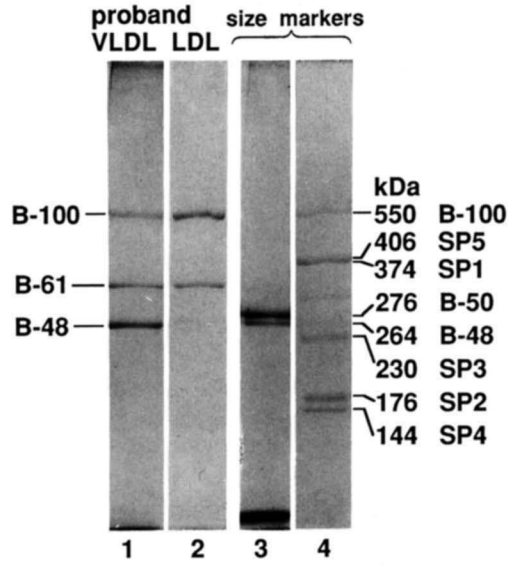

Fig. 2. SDS polyacrylamide slab gel (3–7%) showing: VLDL (lane 1) and LDL (lane 2) from the proband (subject 001). Lanes 3 and 4 contain apoB molecular weight standards: VLDL containing apoB-48 and apoB-50 from a patient with homozygous FHB (lane 3); a digest of control LDL with *Staphylococcus aureus* V8 protease, lane 4.

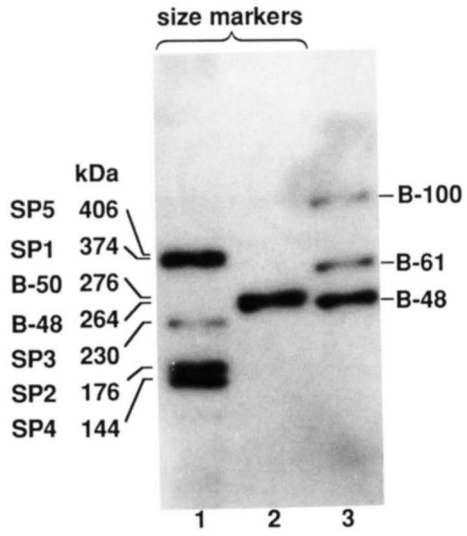

Fig. 3. Immunoblot that shows the binding of a goat anti-human apoB specific antiserum to the abnormal apoB-61 species in the proband's VLDL (lane 3). Lanes 1 and 2 are apoB molecular weight standards: a digest of control LDL with *Staphylococcus aureus* V8 protease (lane 1); VLDL containing apoB-48 and apoB-50 from a patient with homozygous FHB (lane 2).

the apoB-61 was decreased in size along with the apoB-100 to a common protein band slightly smaller than apoB-61 (**Fig. 4**). Cathepsin D has been shown (26) to cleave VLDL apoB-100 preferentially between amino acids 2701 and 2702, yielding a protein of about 310 kDa (residues 1–2701) and one of about 240 kDa (residues 2702–4536). The apoB-48 was not affected by cathepsin D.

The immunoblotting data, together with the cathepsin D results and the molecular mass of 338 kDa, suggest that the apoB-61 is a carboxyl-terminal truncated variant extending beyond residue 2701 and ending approximately at amino acid 2800.

Characterization of the apoB-61 mutation

To identify the mutation that is responsible for the production of this variant, the relevant region of the apoB gene was amplified from the proband's genomic DNA using the polymerase chain reaction (PCR) and subsequently subcloned and sequenced. Oligonucleotide primers #29 and #30 were used that amplify a 1245 bp portion of exon 26 between codons 2478 and 2893 (cDNA nucleotides 7643 and 8887). This region contains only one site for the restriction endonuclease *Pst*I, at nucleotide 8180. Primer #30 contains a single base change, creates a recognition sequence for *Hind*III. The amplified material was digested with *Pst*I and *Hind*III and an aliquot was electrophoresed on a 1% agarose gel. This was expected to produce bands of 694 bp and 538 bp as did control genomic DNA. However, with the material from the proband, in addition to these two bands there were two other bands forming a triplet centered at 694 bp. Such a pattern would be seen in PCR-derived DNA if a significantly large deletional mutation were present on one allele. The lower band in the triplet represents a homoduplex from the mutant allele and the upper band heteroduplex material containing a section of unpaired, single-stranded DNA. Such heteroduplex DNA displays slower than usual mobility for its size through the agarose gel.

The presence of a deletional mutation was confirmed when the triplet of bands was recovered from the gel using Geneclean, cloned into M13mp18 and M13mp19, and sequenced. **Fig. 5** shows a normal sequence ladder and one produced from the mutant allele, which reveals a 37 bp deletion of nucleotides 8525 to 8561. This mutation was seen in clones from both the coding and noncoding strands. In all, seven clones were sequenced, four were found to have the deletion and three were normal.

Nucleotide 8525 is the third base of TCC codon for $serine_{2772}$. The 37 bp deletion causes this codon to become TCG, which also codes for serine. There is then a frame shift producing amino acid residues His, Asn, Ser, Gln, Thr, Leu, Arg, Leu, Ile, Arg, Trp, Leu, before a stop translation codon is reached. Thus, the resulting protein has 2784 amino acids, which is 61.4% of the number of apoB-100 and would have an unglycosylated molecular mass of about 312 kDa, consistent with the observed migration on SDS-PAGE. A protein terminating at residue 2784 is also consistent with the observation that the

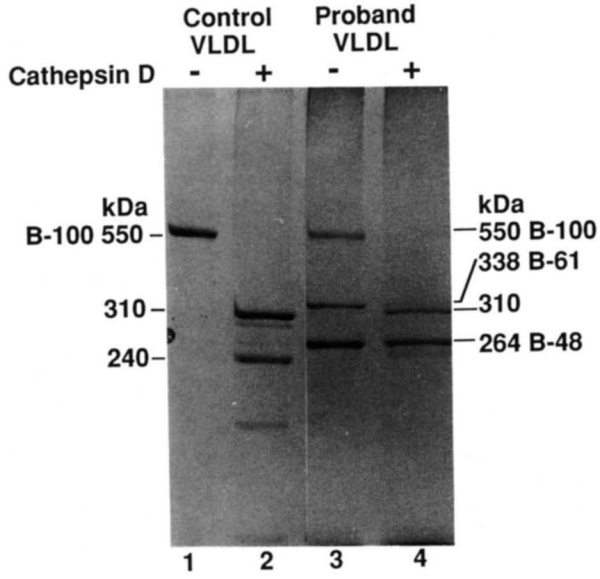

Fig. 4. SDS-polyacrylamide gel showing limited digests of VLDL with cathepsin D; control VLDL before and after proteolysis (lanes 1 and 2, respectively); VLDL from the proband before and after proteolysis (lanes 3 and 4, respectively). Cathepsin D, which cleaves apoB between residues 2701 and 2702, cut both the apoB-61 and apoB-100 to give a 310 kDa band. The other fragment from apoB-100, not clearly seen from the proband's VLDL, is the 240 kDa band. ApoB-48 is not affected by cathepsin D. These results are consistent with the apoB-61 terminating at residue 2784 as deduced from the DNA sequence.

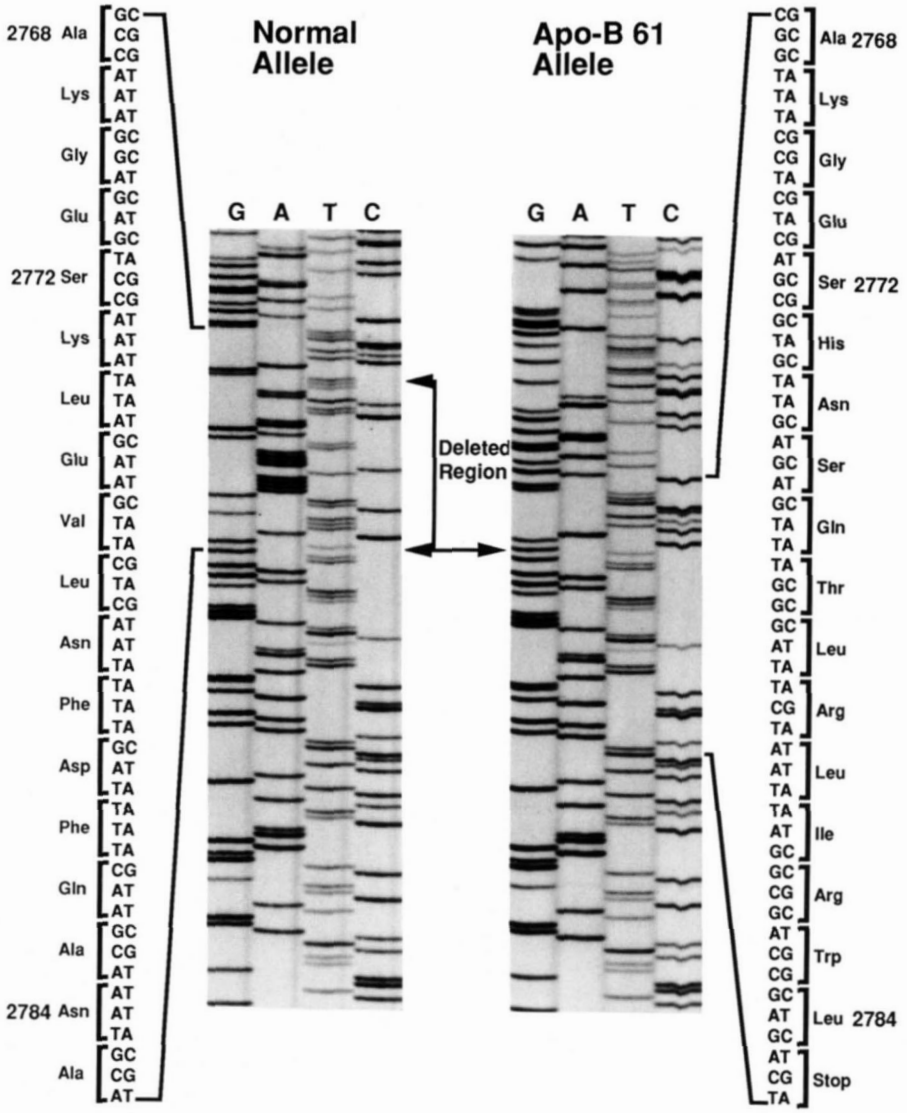

Fig. 5. DNA sequencing gel of negative strand of part of apoB exon 26 from the proband showing the normal (left) and apoB-61 allele (right). The region of the 37 bp deletion in the apoB-61 allele is indicated. This mutation gives rise to a frameshift and the translation of 12 novel amino acids before a stop codon is reached, and results in the production of a 2784 amino acid protein.

size of the apoB-61 was decreased slightly by digestion with cathepsin D, which cleaves between amino acids 2701 and 2702 (26).

In order to study the inheritance of this mutation and the lipoprotein profiles of related family members, blood samples were obtained from the proband's seven siblings, from her parents, and from her maternal grandmother. To determine whether the apoB-61 allele was present in each case, genomic DNA was amplified with primers #29 and #30, cut with *Pst* I, and run on an agarose gel. The observation of triplet bands centered at 707 bp revealed those family members who, like the proband, were apoB-61 heterozygotes (**Fig. 6**). A total of five subjects, including the proband, were observed to carry this mutant apoB allele. However, in addition to these five individuals, five other family members had a total cholesterol less than the fifth percentile for their age and sex. An examination of total cholesterol, LDL cholesterol, and total apoB levels in this family (**Table 1**) revealed three phenotypic patterns. The first is within the normal range (subject 066). The second group consisting of eight members had low levels for all three measurements (subjects 002, 003, 004, 060, 061, 063, 064, and 065). Two family members, the proband (001) and a sister (062), display the third phenotype with extremely low values. It would appear, therefore, that there is another genetic defect that is causing decreased cholesterol and apoB levels besides the apoB-61 mutation because five subjects (002, 004, 063, 064, and 065) of the eight with the intermediate phenotype do not carry this allele.

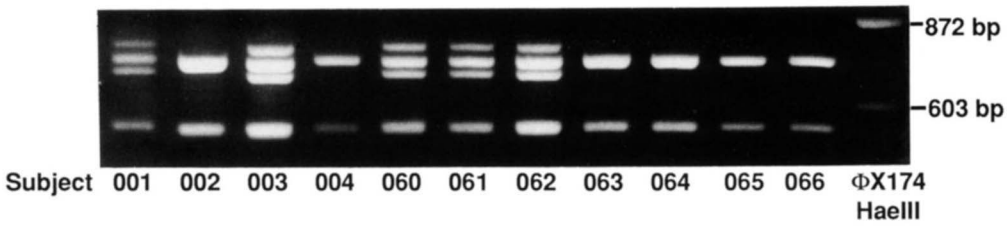

Fig. 6. An agarose gel stained with ethidium bromide showing PCR products cut with *Pst*I. A normal allele gives 538 and 707 bp bands. Subjects 001, 003, 060, 061, and 062 have a triplet centered at 707 bp. The lower band of the triplet is from the allele containing the 37 bp deletion. The middle band is from normal allele and the top band is heteroduplex DNA.

ApoB gene haplotypes

It was possible to ascertain apoB haplotypes on each family member from a study of four RFLPs by determining the number of tandem repeats in the 3' hypervariable region and from a knowledge of the presence or absence of the apoB-61 allele. Only in the case of one person (004), the maternal grandmother, was it not possible to resolve the apoB alleles into two different haplotypes. Four haplotypes were identified in all and are defined in **Table 2.** The individual with the normolipidemic phenotype (066) has haplotypes C and D, while the two sisters with severe FHB (001 and 062) have haplotypes A and B. Haplotype B is the apoB-61 allele. Those other subjects with either haplotype A or B have the intermediate phenotype.

HDL cholesterol, apoA-I, apoE and Lp[a] levels

Most of the total cholesterol in plasma is carried in the HDL of the proband and her sister (062) with the same phenotype and apoB genotype, that is 87% and 84%, respectively. In addition, the proband and a brother (060) have HDL cholesterol levels below the fifth percentile for their age and sex, as well as low total apoA-I and apoE concentrations (Table 1). However, the other subject, besides the proband, with the compound heterozygous FHB state (062) does not have such a low HDL concentration. Overall, this family displays normal HDL cholesterol, apoA-I, and apoE levels.

Along with the extremely low amount of apoB-containing lipoproteins, the proband and her sister (062) have no detectable Lp[a] (Table 1). Two individuals (004 and 063) with intermediate levels of apoB also have no Lp[a].

Triglyceride concentrations

Plasma levels of triglycerides are generally low throughout this family. Six individuals have values below the fifth percentile and two others (061 and 062) have values between the fifth and tenth percentiles.

Electron microscopy of lipoprotein fractions

The electron photomicrographs of negatively stained VLDL ($d < 1.006$ g/ml fraction) from the proband show particles with diameters that are bimodally distributed and with fewer of the smaller ~300 Å normal size particles, whereas there are more larger than 400 Å (**Fig. 7**). The mean and median diameters were 409 Å and 393 Å, respectively, compared to a normal VLDL sample with diameters of 344 Å and 325 Å, respectively.

The diameters of the particles in the LDL fraction from

TABLE 1. Data on individuals from kindred 2005

Subject ID	Age	Sex	Ht	Wt	TC	TG	VLDL-C	LDL-C	HDL-C	ApoA-I	ApoE	ApoB	Lp[a]	ApoB Haplotype
	yr		*cm*	*kg*										
001*	33	F	166	86	39L	22L	2	6L	34L	126	1.2	1	<0.1	A/B
002	55	F	164	76	124L	52L	7	41L	72	201	4.1	25	3.8	A/C
003*	55	M			134L	54L	9	92	39	167	2.8	39	3.8	B/D
004	71	F			128L	87	17	59L	50	160	4.5	29	<0.1	A/A
060*	26	M			67L	74	8	35L	30L	129	1.3	11	6.1	B/C
061*	23	M			121L	48	6	55L	61	184	2.5	21	3.4	B/C
062*	36	F	165	82	50L	42	5	2L	42	134	1.2	2	<0.1	A/B
063	32	M			102L	24L	3L	41L	63	193	1.5	15	<0.1	A/D
064	31	F			129L	31L	4	75	67	195	3.3	20	6.8	A/D
065	24	M			97L	25L	3	48L	50	182	3.5	12	4.8	A/D
066	19	M			167	67	8	122	44	176	5.5	49	7.3	C/D

The lipid, apolipoprotein, and Lp[a] levels in plasma are expressed in mg/dl. Plasma levels of apoB and Lp[a] were determined by ELISA, apoA-I by radial immunodiffusion, and apoE by radioimmunoassay. Values below the fifth percentile (age and sex-matched with the Lipid Research Clinic Population Studies Data Book) are flagged (L). ApoB haplotypes are as defined in Table 2. Subjects with apoB-61 are indicated by an asterisk.

TABLE 2. ApoB haplotypes observed in individuals from kindred 2005

Subject ID	3'HVR	Xba1	Mae1	Msp1	EcoR1	ApoB-61 Mutation	Haplotypes
001	34/36	1/2	1/2	2/2	2/2	+ / –	A/B
002	34/36	1/2	1/2	2/2	2/2	– / –	A/C
003	34/48	1/1	1/2	2/2	2/2	+ / –	B/D
004	36/36	2/2	1/1	2/2	2/2	– / –	A/A
060	34/34	1/1	2/2	2/2	2/2	+ / –	B/C
061	34/34	1/1	2/2	2/2	2/2	+ / –	B/C
062	34/36	1/2	1/2	2/2	2/2	+ / –	A/B
063	36/48	1/2	1/1	2/2	2/2	– / –	A/D
064	36/48	1/2	1/1	2/2	2/2	– / –	A/D
065	36/48	1/2	1/1	2/2	2/2	– / –	A/D
066	34/48	1/1	1/2	2/2	2/2	– / –	C/D
Haplotype							
A	36	2	1	2	2	–	
B	34	1	2	2	2	+	
C	34	1	2	2	2	–	
D	48	1	1	2	2	–	

To study the four RFLPs, four regions of exon 26 of the apoB gene in each individual were amplified by PCR, digested with the appropriate restriction endonuclease, and analyzed on agarose gels. The section spanning the hypervariable region (HVR), 3' to the apoB gene, was amplified by PCR and the number of 15 base pair tandem repeats was determined from the size on agarose gels. A plus sign indicates alleles carrying the apoB-61 mutation.

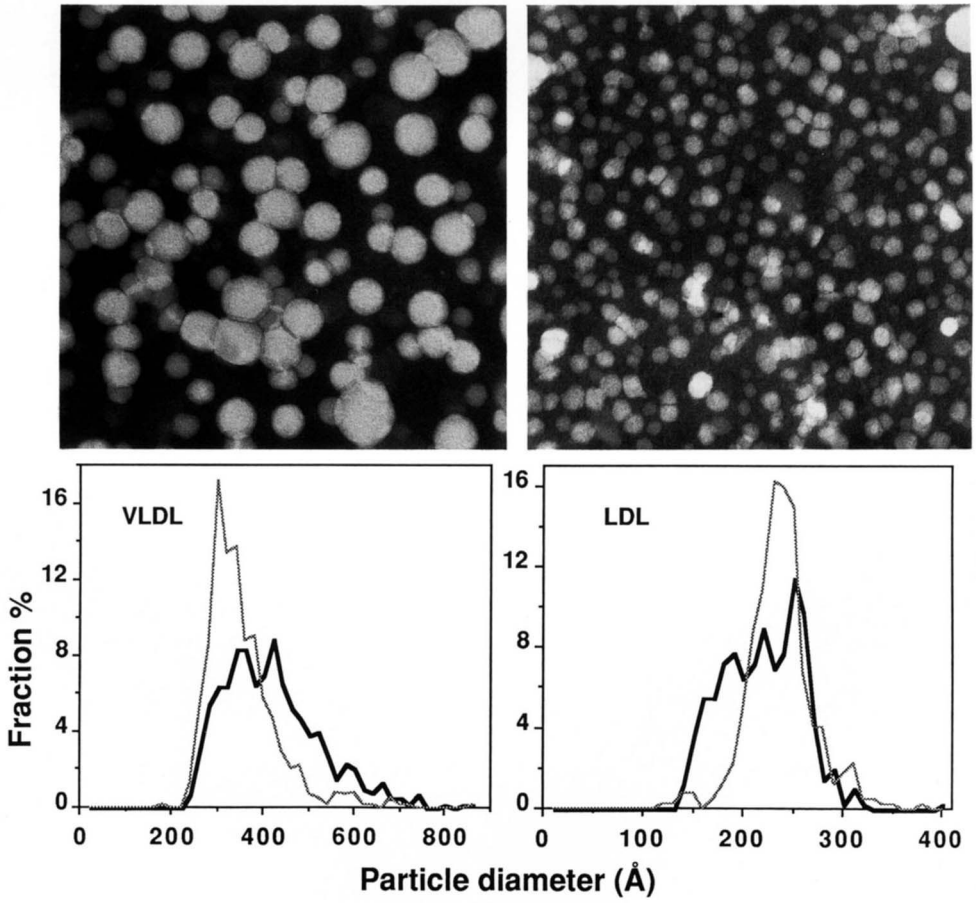

Fig. 7. Electron photomicrographs (117,000 × magnification) and size profiles (solid line) of VLDL and LDL prepared from the proband's plasma, with normolipemic profiles superimposed (broken line) for comparison.

the proband are unevenly distributed across an abnormally wide range (Fig. 7). A significant proportion are smaller than in normal LDL. As can be seen from the photograph, a number of these smaller particles are unusual in appearance, being somewhat cuboidal in shape. The mean and median diameters of LDL were 233 Å and 231 Å, respectively, compared to 214 Å and 216 Å for normal LDL. The size and shape of the particles seen in the HDL electron photomicrographs (not shown) were similar to that of normal HDL.

DISCUSSION

In a kindred with FHB we have found a new truncated variant, apoB-61. VLDL and LDL from the proband contained the abnormal protein of 338 kDa. Western blots using apoB-specific antibodies showed that the protein was a carboxyl-terminal truncated apoB species and did not extend beyond residue 3084. When cut with cathepsin D this protein was decreased in size, consistent with it terminating beyond the cathepsin D cleavage site at residues 2701 to 2702. Using PCR and M13 subcloning and sequencing, we have characterized the responsible mutation as a moderately large 37 bp deletion in exon 26 of the apoB gene (cDNA nucleotides 8525 to 8561). This mutation results in a translational frameshift and the addition of a novel 12 amino acid peptide starting at residue 2773, yielding a 2784 residue peptide, 61.4% of apoB-100.

A number of cases of FHB have been shown to be linked to the apoB gene and, in a proportion of these, truncated species of apoB have been found in the plasma (6, 17). The associated mutations in most instances are single base substitutions or single base deletions. One, apoB-37, was found to be due to a 4 bp deletion in exon 26 (45). Huang and co-workers (16) have reported an individual with FHB who was homozygous for a deletion of 694 bp, including all of apoB exon 21. This mutation was thought to be the result of an Alu-Alu recombination event. The 1085 amino acid protein predicted from the introduction of a premature stop codon was not seen in the plasma of this patient.

In the present study DNA from all family members was amplified and run on an agarose gel to detect the presence of the 37 bp deletion mutation. The proband, her father (subject 003), a sister (062), and two brothers (060 and 061) were shown to be heterozygous for the apoB-61 mutation. Four apoB RFLPs and the 3' hypervariable region were studied in the family. In this way it was only possible to haplotype three of the four parental apoB alleles. However, the presence of the apoB-61 mutation enabled all four to be distinguished from one another.

In this kindred there appears to be another genetic defect that causes decreased cholesterol and apoB levels in plasma besides the 37 base pair deletion mutation in the apoB gene (haplotype B). It is probable that this additional trait is apoB gene-linked and is associated with haplotype A, although the establishment of definitive linkage is not possible since the family is not large enough. The plasma cholesterol and apoB levels in this family appear to fall into three phenotypic patterns. The first of these is within the normal range; subject 066 (age 19 years) has a plasma cholesterol level of 167 mg/dl and plasma apoB of 49 mg/dl. This individual has apoB haplotypes C and D. The second phenotype has a level of cholesterol in plasma below the fifth percentile as well as a low level of apoB. Eight individuals fall within this group with total cholesterol ranging from 67 to 134 mg/dl and apoB from 11 to 39 mg/dl. All have haplotypes A or B together with C or D. The third phenotype has very low levels of cholesterol and apoB in plasma. The two individuals with this phenotype, the proband and a sister (062), have total cholesterol levels of 39 and 50 mg/dl, respectively, and apoB of 1 and 2 mg/dl, respectively. They each have apoB haplotypes A and B. The maternal grandmother's haplotypes are both A. This does not, however, affect the conclusions and, if this hypothesis is correct, presumably only one of her apoB alleles, the one inherited by her daughter (002), is defective. We conclude that this apoB allele probably contains an as yet unidentified mutation and, because the proband has low amounts of apoB-100 along with the apoB-61 in her plasma, that this mutation results in decreased levels of apoB-100. The reason for the reduced levels of apoB (both apoB-100 and apoB-61) in the proband's plasma is not clear, as is so with the other cases of FHB reported. If the apoB-100 protein derived from the allele with haplotype A is normal, a mutation in the promoter region causing a decreased rate of transcription and hence lower levels of mRNA is possible. Alternatively, mutations in the 3' untranslated region that affect the stability of the mRNA or that affect the degree of, or site of, polyadenylation might lead to decreased rates of translation. However, the protein itself might be defective in some way, despite its normal size, due to a mutation in the coding region of the gene. This could result in its being cleared from the circulation at an unusually fast rate.

It is thought that decreased rates of synthesis of at least some of the truncated variants of apoB seen in FHB may be responsible for the low levels of these species in plasma (6), although these authors point out that the explanation remains elusive. It seems that these proteins are capable of a significant degree of lipid binding. This has been shown by transient or stable expression studies using cultured hepatoma cell lines (46, 47). Even apoB-13, smaller than those seen so far in individuals with FHB, is capable of being secreted into the medium, with species larger than apoB-17 forming buoyant lipoprotein particles. A number of proteolytically derived apoB peptides have been shown to possess lipid-binding properties in recombination experiments (25). This study showed that these

regions are widely distributed within the apoB-100 molecule (several are contained within apoB-61). We have recently found a normal rate of production, together with high rates of removal, of a VLDL species containing mainly apoB-50 (along with some apoB-48) in a patient with homozygous FHB (48). Variants such as this, and the apoB-61 seen in the present work, do not contain the putative LDL receptor-binding region, hence increased clearance would have to be via some other route, perhaps that which is responsible for chylomicron remnant removal or by a scavenger pathway.

The HDL cholesterol, total apoA-I, and apoE levels in this family fall within the normal range, overall, despite the proband and a brother (060) having low amounts of all three. The apoB-37 proband was reported to have a very low HDL cholesterol concentration (7, 19), whilst recently in one family the presence of apoB-67 was linked with hyperalphalipoproteinemia (15). It appears, nevertheless, that there is no clear pattern of association between FHB in general and HDL levels (17).

Lp[a] was below the detectable level in the plasma from the proband and her sister (062), both of whom have extremely low levels of apoB (Table 1). This, however, cannot be taken as evidence that the apo[a] antigen is unable to exist freely in plasma unattached to apoB, because three other family members, who have intermediate levels of apoB, also have no detectable Lp[a]. One of these is the maternal grandmother (004) who presumably carries two 'null' apo[a] alleles.

The size of the proband's VLDL particles is larger than normal and is bimodally distributed. The apoB composition of these VLDL is abnormal. In addition to a considerable proportion of apoB-61, much more apoB-48 is present than normal VLDL, where apoB-48 is often barely detectable (see Fig. 4). Hence, this VLDL preparation is enriched in apoB-48-containing chylomicrons due either to the low production or rapid removal of the smaller apoB-100-containing particles.

Although the mean and medium diameters of the LDL from the proband were only 19 Å and 15 Å, smaller, respectively, than control LDL, the distribution of sizes was uneven and broad. From the frequency profile it can be seen that these LDL contain a number of abnormally small particles between ~150 Å and ~200 Å. From the photomicrographs it can be seen that these smaller LDL are cuboidal in appearance. Fig. 2 shows that these LDL contain only apoB-100 and apoB-61 with somewhat more of the former. We previously observed such cuboidal particles of ~200 Å diameters in the LDL interval from a patient with normotriglyceridemic abetalipoproteinemia (48, 49). This individual we have now shown to be a rare case of homozygous FHB with only truncated apoB-50 and a small amount of apoB-48 in the LDL fraction (10). It is likely in the present case that the small cuboidal LDL are apoB-61-containing particles. It is thought that an abnormal phospholipid composition, together perhaps with a higher proportion of unesterified cholesterol in these particles, is responsible for the cuboidal appearance (17).

Some compound heterozygotes or homozygotes for FHB display overt clinical symptoms and others like the proband reported here do not. Nevertheless, the extremely low cholesterol and apoB levels in plasma is a signal indicating the presence of apoB-linked mutations. This has recently enabled the discovery of a number of these mutations that have subsequently been shown not to lead to overt clinical symptoms in the heterozygous state. While such simple heterozygotes have low cholesterol and apoB levels in plasma, the mutations would, in the normal course of events, remain undetected. It seems likely, therefore, that apoB truncation mutations, such as the apoB-61 reported here, may occur frequently and could explain, in part, the low end of the cholesterol spectrum in the general population.

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