ApoB gene nonsense and splicing mutations in a compound heterozygote for familial hypobetalipoproteinemia

Li-Shin Huang,* Herbert Kayden,† Ronald J. Sokol, and Jan L. Breslow***

Laboratory of Biochemical Genetics and Metabolism,* The Rockefeller University, 1230 York Avenue, New York, NY 10021; Department of Medicine,† New York University School of Medicine, New York, NY 10016; and Department of Pediatrics,** University of Colorado School of Medicine, Denver, CO 80262

Abstract Two novel apoB gene mutations were identified in a patient (CM) with phenotypic homozygous hypobetalipoproteinemia. Haplotype analysis of the apoB alleles from this patient and his family members revealed him to be a genetic compound for the disease. In contrast to previous studies of other hypobetalipoproteinemic patients, no clues existed as to where in the apoB gene the molecular defects resided. Therefore, it was necessary to characterize the apoB genes of the patient by sequence analysis. The apoB gene contains 29 exons and is 43 kb in length. The gene encodes a 14.1 kb mRNA and a 4563 amino acid protein. Both apoB alleles from the patient were cloned via 26 sets of polymerase chain reactions (PCR). These clones contained a total of approximately 24 kb of apoB gene sequence, including regions 5' and 3' to the coding region, 29 exons, and the intron/exon junctions. Complete DNA sequence analysis of these clones showed that each apoB allele had a mutation. In the paternal apoB allele, there was a splicing mutation. The first base of the dinucleotide consensus sequence (GT) in the 5' splice donor site in intron 5 was replaced by a T. It is likely that this base substitution interferes with proper splicing and results in the observed absence of plasma apoB. In the maternal apoB allele, there was a nonsense mutation. The first base of the Arg codon (CGA) at residue 412 in exon 10 was replaced by a T, resulting in a termination codon (TGA). The nonsense mutation is likely to terminate translation after residue 411 resulting in a severely truncated protein only 9% of the length of B-100. The inheritance of these defective apoB alleles cosegregated with low total cholesterol levels observed in family members. One of the siblings, MM, who also presented with phenotypic homozygous hypobetalipoproteinemia, had both defective apoB alleles. Of the two other siblings, both of whom were phenotypical heterozygotes for the disease, one (GM) had the allele with the splicing mutation and the other (JM) had the allele with the nonsense mutation. In summary, a strategy is presented for identifying apoB gene mutations by PCR cloning and sequencing. This is useful for analysis of defects in patients where there is no clue as to the location of the mutation. The technique has resulted in the identification of two novel apoB gene mutations.—**Huang, L-S., H. Kayden, R. J. Sokol, and J. L. Breslow.** ApoB gene nonsense and splicing mutations in a compound heterozygote for familial hypobetalipoproteinemia. *J. Lipid Res.* 1991. **32:** 1341–1348.

Supplementary key words haplotype analysis • polymerase chain reaction

Apolipoprotein B (apoB) is the major protein constituent of low density lipoprotein (LDL) and is the ligand for the LDL receptor, which mediates cellular uptake of LDL (1). The apoB gene, on chromosome 2p, contains 29 exons and is 43 kb long (2–4). ApoB exists in two forms, B-100 and B-48, which differ in size and are synthesized in the liver and intestine, respectively (1). ApoB mRNA, which is 14 kb in length (5, 6), encodes a 4,563-amino acid protein containing a 27-amino acid signal peptide (7, 8). B-100 and B-48 are products of the same gene, with B-48 consisting of the 2,152 amino-terminal residues of B-100. Specific processing of apoB mRNA in the intestine introduces a stop codon at residue 2,153 accounting for the production of B-48 (9, 10).

Homozygous hypobetalipoproteinemia (HBLP) is a rare autosomal disease characterized by fat malabsorption, retinitis pigmentosa, ataxia, and acanthocytosis (1). Affected individuals have little or no plasma apoB or apoB-containing lipoproteins, such as chylomicrons, very low density lipoproteins (VLDL), and LDL. Individuals with heterozygous HBLP are asymptomatic but have about 30–50% of normal plasma apoB levels. Specific structural mutations of the apoB gene have been shown to be the cause of the reduced levels of plasma apoB in these patients (11–17). In this report, we determined the molecular defects in the apoB alleles of a compound heterozygote for HBLP in the absence of clues as to where the defects resided. Both apoB alleles were clones via products from 26 different sets of polymerase chain reactions (PCR) (18). These clones contained the entire coding region, all intron/exon junctions, the regions 5' and 3' to the gene.

Abbreviations: PCR, polymerase chain reaction; apoB, apolipoprotein B; LDL, low density lipoprotein; HBLP, hypobetalipoproteinemia; VLDL, very low density lipoprotein; RFLP, restriction fragment length polymorphism; VNTR, variable number of tandem repeats; ASO, allele-specific oligonucleotide.

Sequence analysis revealed different mutations in the paternal and maternal alleles. The paternal allele had a base substitution in the dinucleotide consensus sequence for the 5' splice donor site in intron 5 and the maternal allele had a nonsense mutation in amino acid codon 412 in exon 10. These mutations cosegregated with the low total cholesterol observed in family members of the patient and are likely to be the causative mutations for the disease in this family.

MATERIALS AND METHODS

Clinical information

The hypobetalipoproteinemia family M consists of an unrelated father and mother and four offspring, as shown in the top panel of **Fig. 1.** The family is Caucasian and resides in the United States. The parents and two of the offspring (GM and JM) have been asymptomatic. The proband, CM, demonstrated malabsorption as early as 4 months of age, with vomiting after meals, a protuberant abdomen, and steatorrhea. At his next hospitalization at 10 months of age, the diagnosis of homozygous HBLP[1] was made and treatment with medium chain triglycerides was initiated, resulting in weight gain and a decrease in steatorrhea as described in a case report in 1974 (19). Abnormalities in liver function were also noted and a liver biopsy documented the earliest stages of portal fibrosis, as well as the presence of fat-filled hepatocytes. At 30 months of age, a repeat liver biopsy documented the rapid development of micronodular cirrhosis which was speculated to be a result of treatment with medium chain triglyceride (19). Progression of the liver disease continued, with portal hypertension, which necessitated a splenectomy, and esophageal varices, which led to multiple endoscopic scleral therapy procedures and finally an esophageal devascularization procedure at age 11. Severe neurologic deficiencies were present including ataxia, dysarthria, severe impairment of position and vibratory sensation, and absent deep tendon reflexes. These abnormalities were associated with vitamin E deficiency, and after intramuscular and intravenous vitamin E therapy the neurologic symptoms stabilized. At age 15 the proband had hepatitis A and upon hospitalization was noted to have cholelithiasis. The last 3 years of his life were punctuated by seizures, a cerebrovascular accident, recurrent massive gastrointestinal hemorrhages, and finally cardiac arrest at the age of 18.

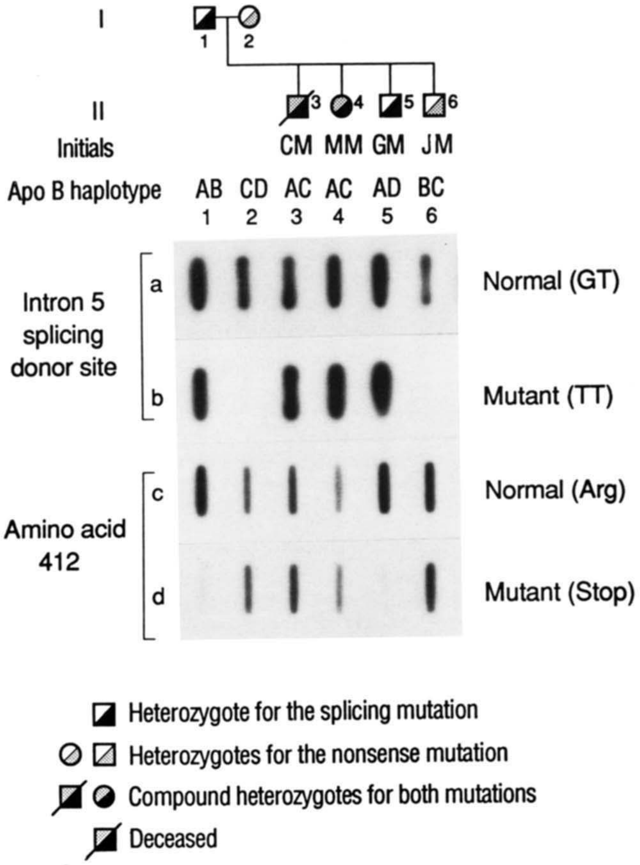

Fig. 1. Pedigree of the M family with familial hypobetalipoproteinemia and the inheritance of mutant apoB alleles. The haplotypes of the apoB alleles are below the initial of each individual. ApoB haplotypes A, B, C, and D are as listed in Table 3 and the analysis is described in Methods. The autoradiograms shown below the pedigree are slot blots used for ASO hybridization, which is described in Methods. Rows a and b are slot blot assay for the intron 5 slicing mutation. Rows c and d are slot blot assays for the nonsense mutation in amino acid 412. Probes used are wild type sequences for rows a and c, and mutant sequences for rows b and d.

The daughter, MM, was first hospitalized at age 10. Her history documented malabsorption as an infant, evidenced by vomiting and steatorrhea, but this was controlled by a low-fat diet. The diagnosis of homozygous HBLP was established on this hospitalization, with evident neurologic involvement including absent deep tendon reflexes and severely depressed vibratory sensation. Vitamin E treatment was begun at age 10 and her symptoms have stabilized since then. She is now 15 years of age and attends school.

The lipid and lipoprotein profiles of the M family are shown in **Table 1.** Total cholesterol and triglyceride measurements were performed by an Autoanalyzer using the method specified by the laboratory manual of the Lipid Research Clinics Program (20). The results showed that the parents, who are obligate heterozygotes, have less than 50% of normal levels of LDL-cholesterol. Two of the siblings of the proband (GM and JM) are heterozygotes for

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[1] The patient (CM) was diagnosed to have abetalipoproteinemia in the 1974 case report (19). It has been shown that obligate heterozygotes for abetalipoproteinemia have normal levels of plasma LDL-cholesterol (1). The current data, showing that the parents of the patients have plasma LDL-cholesterol levels lower than normal levels, indicate that the proband (CM) and his sister (MM) are in fact phenotypic homozygotes for hypobetalipoproteinemia.

TABLE 1. Cholesterol (CHOL) and triglyceride (TG) levels in the M family

Individual No.[a]	Generation and Initials	Age[b]	Total CHOL	TG	LDL-CHOL	HDL-CHOL
		yr		*mg/dl*		
1	I (Father)	43	135	445	47	38
2	I (Mother)	40	90	40	NA[c]	NA[c]
3[d]	II (CM)	15	36	12	0	36
4	II (MM)	10	24	0	0	21
5	II (GM)	14	97	59	29	56
6	II (JM)	7	113	60	16	87

[a] Corresponds to the number in the pedigree shown in Fig. 1.
[b] Age at the time of blood drawing, 1985.
[c] NA, not available.
[d] CM was deceased in 1988 at the age of 18.

HBLP, as judged by their levels of LDL-cholesterol. No additional blood samples were available from this family at the time of this study. Therefore other biochemical analyses such as apoB measurements by immunoassays or detection of possible abnormal species of apoB protein by gel electrophoresis were not possible. However, the original case report of the proband showed no detectable plasma apoB by radial immunodiffusion (19).

Haplotype analysis

Genomic DNA from each member of the M family was isolated as described (3) and then used for both Southern blot analysis and PCR amplification. ApoB gene restriction fragment length polymorphism (RFLP) markers *Ava*II, *Hinc*II, *Pvu*II, *Xba*I, *Eco*RI and 3' VNTR (variable number of tandem repeats), were carried out by Southern blot analysis as previously described (3, 21-23). *Msp*I RFLP typing of the promoter region was carried out by PCR amplification followed by MspI digestion as described (24). The length polymorphism in the signal peptide was distinguished by electrophoresis of PCR-amplified products in a 12% polyacrylamide gel as described (25). In3G/T sequence polymorphism, *Apa*LI and *Alu*I RFLPs were carried out by PCR amplification and followed by allele-specific oligonucleotide (ASO) hybridization as described previously (21, 26).

Cloning of the apoB alleles from an HBLP patient

To clone apoB alleles, genomic DNA from patient CM (individual II.3 in Fig. 1) was amplified by PCR (18). The 5' upstream region (−341 bp), 29 exons, intron/exon junctions, and 3' untranslated region of the apoB gene were cloned through 26 sets of PCR (**Table 2**). In each set of PCR, with the exception of exon 26, the region for amplification included sequences in intron/exon junctions. Due to its large size, five sets of PCR were used to amplify exon 26 which is 7572 bp. In some sets of PCR, two exons were contained in one reaction product. All primers contained restriction enzyme sites which were either engineered or present in the original apoB gene sequences (27). In the PCR reaction, 1 μg of genomic DNA was amplified with primers as listed in Table 2 in a reaction mixture containing 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.1% gelatin, and 2 units of Taq DNA polymerase (Perkin-Elmer Cetus). In most sets of PCR, the process consisted of denaturation at 92°C for 1 min, reannealing at 55°C for 2 min, and extension at 70°C for 3 min. The process was repeated for another 29 cycles in a DNA thermal cycler (Perkin-Elmer Cetus). In the reactions amplifying exon 1 and the 5' upstream region, 30 cycles of 95°C (1 min), 57°C (1 min), and 70°C (2 min) were carried out. In the reactions amplifying exons 9, 13, and 14, reannealing was at 50°C, while in the reactions amplifying exon 21, reannealing was at 58°C. For cloning, the PCR reaction mixtures were precipitated and resuspended in the appropriate buffer for restriction enzyme digestion. The digested reaction mixtures were centrifuged through Centricon 30 filters (Amicon Corp.) to remove deoxynucleotides, then extracted with phenol chloroform, and ethanol-precipitated. The digested mixtures were ligated to pUC vectors and used to transform *E. coli* DH5α competent cells. ApoB recombinants were identified by screening the transformants with specific oligonucleotide probes as listed in Table 2. All oligonucleotides used were synthesized by phosphoramidite chemistry on a DNA synthesizer (Applied Biosystems, Inc., model 381A).

DNA sequence analysis

For sequencing, 12 apoB recombinants derived from each set of PCR-amplified DNA were selected to present both parental alleles from the patient. Sequencing of multiple clones also permitted recognition of artifactual mutations derived from PCR. For clones with large inserts such as those derived from exons 26-B, 26-C, and 29 PCR sets, multiple sequencing reactions were required to obtain the entire sequence of the clone. In the clones derived from these regions, two parental alleles were distinguishable due to the presence of polymorphisms in these regions (Table 3). In this case, only two clones (each representing the paternal or maternal allele) are needed for sequencing. However, two clones of each allele were selected for further sequencing to permit recognition of artifactual mutations. Double-stranded plasmid DNA isolated from these clones was denatured with an equal volume of 0.4 N NaOH for 5 min, neutralized with 100 mM ammonium acetate and 200 mM sodium acetate (pH 7.0), and precipitated by addition of ethanol. The resuspended sample was sequenced with modified T7 DNA polymerase (Sequenase, United States Biochemical Co.) (28) according to the dideoxy-termination method of Sanger, Nicklen, and Coulson (29). PCR primers were also used as sequencing primers. For clones with large inserts, specific sequencing primers were synthesized.

TABLE 2. Primers used for PCR amplifications and cloning

Exon no.[a]	5' Primer (5' → 3')[b] sense strand	3' primer (5' → 3')[b] antisense strand	Probe sequence (5' → 3') Sense (S) or antisense strand (AS)	Size of PCR product[c]
1	TCCGAATTCAGTCCAGGAGAA	CCATCCTGAGCCTGCAG	TTGAGCCTTGAAGAGCCTCG (AS)	720 bp
2	GGAAGCTTACAGAATTTCTTTCTC	GTAGAATTCAGTTGGCATCCCTT	TGTCAGCCTGGTCTGTCCAA (S)	250 bp
3	CCAGAATTCGCTGTCCTTGGGAG	TAATCTAGATCATGATTCCTATT	CCTAAGCTTCAGATGAGATATTC (S)	452 bp
4	TTGGAGCTCTGATTAGAGATTAA	CACGAGCTCATACCTCAGCGGAC	GGTGTATGGCTTCAACCCTG (S)	300 bp
5 & 6	GATAAGCTTATTTCCGTGACCAT	AGTTCTAGAATTGTATTAATAAG	ATGGCCAGCTGGAGCTCATAC (AS)	1100 bp
7 & 8	TAAAAGCTTTTAACAGAGATACA	TTGTCTAGATTTTCCAGCAACTA	CACTGGACGCTAAGAGGAAG (S)	1000 bp
9	AGAAGCTTTAGCAGGCATTGAA	TTGAAGCTTCAGTCAGTTACCAT	ATTTTGCTCAGAGATGGTTA (AS)	370 bp
10	TTCTGAGCTCCAAGTTGGGTT	AAGAATTCAATTTGTGTTTGCTGA	TTGTTGACCGCGTGGCTCAGCG (AS)	560 bp
11 & 12	TTCGAATTCACAAGTTTGGGTTTT	GATGAAATCTAGAGTCTCATTCC	CCGCAGAATCAAATAGGTGTAA (AS)	600 bp
13	AGAAGCTTCAATACCAGCCATTT	GGTCTAGATCTGCTACATATTT	GTTGGAATTCCCAGCTCAGG (AS)	469 bp
14	AGATCTAGACCCAAAGACTTA	TCTAAGCTTCCTCTGGGTAGCT	AGAAGGGAATCTTATATTTG (S)	472 bp
15	ACATCTAGAGTGGGACTACTAGG	AAAGAATTCTTTTTGCATTGAGA	CTTTGTACTGGGTTAATGGT (S)	317 bp
16	TGGGAATTCAAAGGTGTTTGACA	AGTGAATTCAAGGCAAACCTC	CATCTGGGGGATCCCCTGCAGA (AS)	398 bp
17	ATAAAGCTTAATTACTCTCCAATG	CATTCTGGTGGAAGCTTGAAGTT	TGGAGAATTCCTTTGAACTC (S)	431 bp
18	AACTCTAGAGAACTGAGAACTCG	TGATCTAGATCAACTGTTTAGCC	AACCTGGGAAGCTGAAGTTT (S)	450 bp
19 & 20	GAGAATTCGTGATGTCCATTTTGA	CATGAATTCTGAACCTGAGACT	TGAAGCTTGCTAGGTGGCCAT (AS)	1000 bp
21	TGGAATTCCACAAACAAGCTAAGTG	TCGAATTCGACATGCGCAGAGGGTTAAA	AGAGAATTCAGAGATGTGTGGTA (AS)	1500 bp
22 & 23	TTTAAGCTTGCATATCTCCATTG	CAAGAATTCCTGGGGGAAGGAAG	ATGGAATTCACACCCTTGATTTT (AS)	700 bp
24 & 25	GTCAAGCTTAATAATTAACTTGTC	AAAGCTTCCAAGTAGCAAGGAAG	CTTTGCAAGACCACCTCAAC (S)	1700 bp
26-A	AGGGATCCTAAACAAAGCTGT	TTAGGATCCCTCTGACAAGACA	ATTTCTAGATTCGAATATCAAAT (S)	474 bp
26-B	ATTTCTAGATTCGAATATCAAAT	CTTCTAGAGTCTCTCGGAAT	TCAGAATTCATTTCAGCATATGA (AS)	3277 bp
26-C	AAGAAGCTTAATGAATTATCTTT	TGGGGAGCTCGTCGTGAGATTT	GAGCTGCAGTGGCCCGTTCCAGA (S)	2526 bp
26-D	AAGGATCCCTTGAAAACGACAAA	TAAGGATCCTGCAATGTCAAGGT	CTCAAGCTTTCTCTTCCAGATTT (S)	1520 bp
26-E	GCAGGATCCTTAGAAGGACAC	CATGGATCCACACATAAATACATA	ACACTCCCCGAGGTAAAATT (S)	894 bp
27 & 28	ATAGAATTCTCCCAGTTTTCACA	ACTTCTAGAGAATATTTCCTCAGA	GATGCCTTTCTTGTCTTTCT (AS)	646 bp
29	TTCTCTAGAAGTATTACATGAGTCCTTGATTGA	TTTTCTAGATCCTTCTGAGTTCAGAGA	GCCAGTTTCCAGGGACTCAA (S)	2630 bp

[a] Exon 26 was cloned by five sets of PCR reaction, A to E.
[b] The restriction enzymes sites (underlined) for cloning are either engineered or original sequences.
[c] The sizes of PCR products are estimates.

Detection of apoB mutations and polymorphism by ASO hybridization

To determine the inheritance of the mutations in members of the M family, oligonucleotides were synthesized as primers for PCR amplification of regions found by sequencing to contain mutations. Allele-specific oligonucleotides were synthesized for hybridization. For the splicing mutation in intron 5, primers used for PCR were: #237 (5'-GATAAGCTTATTTCCGTGACCAT-3') and #240 (5'-CATGAATTCGAGTTTCAAGGGCC-3'). ASO probes used were: #959 (5'-TGTTTCTGGTGAGGATT-3'), specific for the normal sequence, and #960 (5'-TGTTTCTGTTGAGGATT-3') specific for the mutant sequence. For the nonsense mutation in amino acid 412, primers used for PCR were: #380 (5'-TTCTGAGCTCCAAGTTGGGTT-3') and #466 (5'-AAGAATTCAATTTGTGTTTGCTGA-3'). ASO probes used were #954 (5'-CGCAGCCGAGCCA-3') for the normal sequence and #946 (5'-CGCAGCTGAGCCA-3') for the mutant sequence. For ASO hybridization, the PCR-amplified DNA sample was denatured with 0.5 M NaOH/1.5 M NaCl, neutralized with 1 M Tris-HCl, pH 7.5, 1.5 M NaCl, and applied to Zeta-Probe membranes (Bio-Rad) in a slot blot apparatus (Bio-Rad). The membranes were hybridized with the ASO probes at 42°C overnight. The membranes were washed twice with 6× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate), and 0.05% sodium pyrophosphate at room temperature for a total of 1 h and were washed once at 37°C for 30 min. They were then washed for 5 min at 51°C for probe #954, 52°C for probe #946, 54°C for probe #960, and 56°C for probe #959. The membranes were exposed to X-ray film for several hours.

To determine the inheritance of a new sequence polymorphism in the codon for amino acid 2285 as listed in Table 3, ASO probes were synthesized for hybridization. The probes are #961 (5'-ATAAATGACGTTCTTGA-3'), which is specific for codon GAC, and #980 (5'-AATAAATGATGTTCTTGAG-3'), which is specific for codon GAT. The hybridization and washing conditions were as described above; the final washing temperature was 51°C for probe #961 and 57°C for probe #980.

RESULTS

Haplotype analysis in an HBLP family

Haplotyping of the apoB alleles in the M family using 12 polymorphic apoB gene markers is shown in **Table 3** and Fig. 1. As shown in the top panel of Fig. 1, four different apoB haplotypes were distinguishable in the parents of the proband. The haplotypes of the father (I.1) are designated A and B and the haplotypes of the mother (I.2) are designated C and D. Both CM (II.3) and MM (II.4) had no detectable LDL-cholesterol and therefore are phenotypic homozygotes for HBLP, whereas the other two siblings, GM and JM (II.5 and II.6), had very low LDL-cholesterol and therefore are phenotypic heterozygotes for HBLP. As shown in Fig. 1, proband CM and sibling MM each have the same two apoB haplotypes, A and C, indicating that they are compound heterozygotes for the disease. It could be deduced from these results that the defective apoB allele in the father is haplotype A, and the defective apoB allele in the mother is haplotype C. Of the two heterozygous siblings, GM has the defective allele with haplotype A from the father, whereas JM has the defective allele with haplotype C from the mother.

DNA sequence analysis of the apoB alleles from patient CM

The apoB alleles from the proband, CM (II.3), were first examined by Southern blot analysis using probes spanning the entire apoB gene. No gross abnormality was observed (data not shown). Both apoB alleles from CM were then cloned and sequenced. Sequence analysis confirmed the base differences between the two apoB alleles, A and C, assigned to CM by haplotyping. Sequence data also revealed two new mutations in these two apoB alleles as described below.

Identification of a splicing mutation in patient CM

Sequence analysis of clones containing exons 5 and 6 (Table 2) revealed a base substitution at the junction of exon 5 and intron 5. As shown in **Fig. 2,** the normal allele contained the dinucleotide consensus sequence GT at the 5' splice donor site. In the mutant allele, the first base of this dinucleotide was replaced by a T. The inheritance of this mutant allele was assessed by ASO hybridization. The bottom panel of Fig. 1 shows that this mutant sequence was inherited from the father and therefore by haplotype analysis, must be present on the haplotype A allele (Fig. 1 and Table 3). This allele is present in sibling GM (II.5) as well as CM and MM and these individuals all had low total cholesterol. The mutation is therefore likely to be responsible for the reduced amount of total cholesterol in the individuals possessing the A allele.

TABLE 3. Haplotyping of the apoB genes in the M family

		Haplotype[a]				
Polymorphism	Region	A	B	C	D	Ref.
*Ava*II	5′ Upstream	–	+	+	+	21
*Msp*I	Promoter	–	+	+	+	24
9bpID	Signal peptide	I	I	I	D	25
In3G/T	Intron 3	T	G	G	T	21
*Apa*LI	Exon 4	+	+	+	–	26
*Hinc*II	Intron 4	+	+	–	–	21
*Pvu*II	Intron 4	–	–	–	–	21
Splicing mutation	Intron 5	M	N	N	N	this report
Nonsense mutation	Exon 10 (aa412)	N	N	M	N	this report
*Alu*I	Exon 14	–	–	–	+	21
aa2285	Exon 26	GAT	GAT	GAC	GAC	this report
*Xba*I	Exon 26	–	–	+	+	22
*Eco*RI	Exon 29	–	–	+	+	3
3'VNTR	3' Downstream	1	1	2	2	23

[a] Presence (+) or absence (–) of a restriction enzyme site; insertion (I) or deletion (D) of nucleotides; normal (N) or mutant (M); 1/2: 1 contains more copies of tandem repeats.

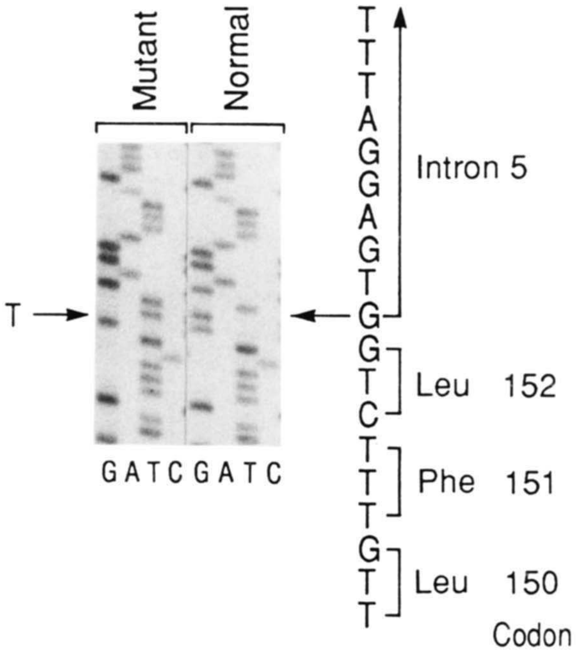

Fig. 2. DNA sequence of splicing mutation in intron 5. Two clones representing normal (right panel) and mutant (left panel) apoB alleles from the patient, CM, are shown. The normal splice consensus dinucleotide GT is replaced in the mutant allele by the sequence TT which is indicated by an arrow.

Identification of a nonsense mutation in patient CM

Sequence analysis of clones containing exon 10 (Table 2) revealed a substitution in the codon for amino acid 412. As shown in **Fig. 3,** the normal allele contains the sequence CGA for Arg at residue 412. At this location the mutant allele contains the sequence TGA, which is a termination codon, resulting in a nonsense mutation. The inheritance of this mutant allele was demonstrated by the ASO hybridization and is shown in the bottom panel of Fig. 1. The mutation was inherited from the mother and therefore residues on allele C. Among the siblings, JM (II.6), as well as CM and MM, have the nonsense mutation. Individuals who have this mutant sequence also had low total cholesterol, suggesting that the mutation in the codon 412 is responsible for this phenotype in the individuals possessing the C allele. The proband CM and his sister MM had both mutations and consequently had no detectable LDL-cholesterol. The C to T base substitution creates the following restriction enzyme sites: *Alu*I, *Dde*I and *Pvu*II.

A new silent sequence polymorphism

An additional novel sequence polymorphism in the apoB gene was observed in the proband, CM. There was a base substitution (C→T) in the third base of codon 2285, which codes for Asp (data not shown). The substitution does not result in an amino acid change and is unlikely to have functional significance. It does, however, serve as an additional apoB gene marker for haplotype analysis.

DISCUSSION

We have developed a PCR-cloning strategy to elucidate molecular defects in the apoB gene in a compound heterozygote for familial HBLP. To characterize both apoB alleles, 26 pairs of PCR primers were synthesized and used for amplification of the entire coding region, exon/intron junctions, 5' upstream, 5' and 3' untranslated regions. This was followed by cloning and sequencing. Two mutations, a splicing mutation in intron 5 and a nonsense mutation in amino acid codon 412, were identified to be the likely causative mutations in the patient.

The prevalence of phenotypic heterozygotes for hypobetalipoproteinemia has been shown to be about 0.1 to 0.8% in the population (30). In selected probands, structural mutations in the apoB gene have been found that result in the appearance of low levels of truncated proteins ranging from B-31 to B-89 in plasma (12–13, 15–17). It has also been reported that mutations leading to predicted protein sizes of B-25 or B-29 result in no corresponding polypeptides detectable in plasma (13, 14). The lack of these small apoB proteins in the plasma is suggested to be due to absence of lipid binding regions critical for lipoprotein assembly (13, 17). In some other HBLP patients, normal-sized apoB protein is observed (31). To determine the molecular defects in patients with either complete absence of apoB or normal-sized apoB, a systematic method for characterization of the apoB genes is needed. This report provides a complete set of primers and conditions for characterizing the apoB gene where no clues as to the nature of the mutation exist. Available polymorphic markers are useful in distinguishing the two apoB alleles of heterozygotes, thus reducing the number of clones to be

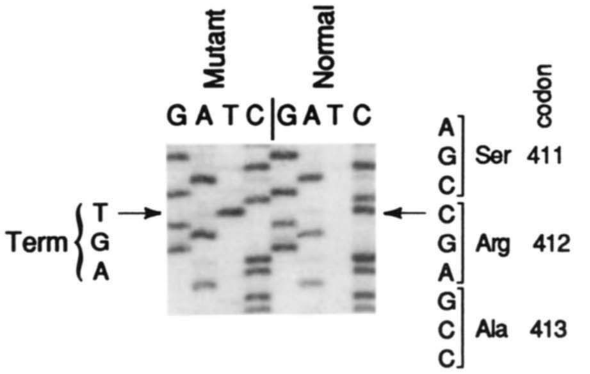

Fig. 3. DNA sequence of nonsense mutation in amino acid codon 412. Two clones represent normal (right panel) and mutant (left panel) apoB alleles from the patient, CM are shown. Codon for amino acid 412 changed from CGA (Arg) to TGA (termination) in the mutant allele. The mutant sequence is indicated by an arrow.

sequenced. PCR products obtained by the methods described here can be analyzed by direct sequencing, or screened for base substitutions or small deletions by denaturing gradient gel electrophoresis (DGGE) or other methods (32).

The first example of an apoB gene splicing mutation is reported here. It has been shown that the dinucleotide consensus sequences GT in the 5' splice donor site and AG in the 3' splice acceptor site, are important for correct splicing to occur (33). Mutations in these splice sites in the globin genes have been shown to greatly reduce the amount of normal mature mRNA and in some cases activate nearby cryptic splice sites, leading to the production of aberrant mRNAs which are often rapidly degraded (34, 35). Splicing mutations have been described in other apolipoprotein genes (36, 37). In a patient with apoC-II deficiency, a substitution of C for G in the 5' splice donor site of intron 2 of the apoC-II gene has been reported (36). The mutation results in markedly reduced levels of normal-sized apoC-II in this patient (36). In a patient with familial apoE deficiency, an A to G substitution in the first base of AG consensus dinucleotides in the 3' splice acceptor site of intron 3 has been shown (37). The mutation results in the use of cryptic splice sites and leads to production of two abnormal-sized mRNA species. Both mRNA species contain in-frame chain termination codons within intronic sequences and encode short apoE polypeptides that are not detectable in plasma. The mutation in the 5' splice site of intron 5 of the apoB gene reported here is likely to be a cause of the hypobetalipoproteinemia in the M family, although the exact effect of this splicing mutation is not clear.

In this report we have also described a novel nonsense mutation, i.e., a C to T transition in the CGA codon for Arg at residue 412. The resulting stop codon would result in a premature translation termination leading to a protein of 411 amino acids that would represent only 9% of the B-100 protein, i.e., B-9. Plasma samples from this family are no longer available and therefore it is not possible for us to look for abnormal apoB species in plasma. However, based on previous reports that neither B-25 nor B-29 are detectable in plasma (13, 14), it seems unlikely that B-9 would be present in the plasma of the patients studied. This mutation is the third example of nonsense mutations occurring in CGA codons for Arg in the apoB gene. One such mutation occurs at residue 1306 and accounts for the predicted B-29 species (13). The other occurs at residue 2058, leading to a prematurely terminated protein of B-46 (16). It has been shown that CpG dinucleotides are hot spots for mutation in other human diseases (38). As mentioned previously by Collins et al. (13), there are 12 CGA codons for Arg in apoB coding sequences. The fact that nonsense mutations have been reported in three of these suggests that these CpG dinucleotides are hot spots for mutation in the apoB gene. ■

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