Volume 16 Number 17 1988 Nucleic Acids Research

Truncated variants of apolipoprotein B cause hypobetalipoproteinaemia

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Received June 21, 1988; Revised and Accepted August 10, 1988

ABSTRACT

Familial hypobetalipoproteinaemia is a rare autosomal dominant disorder in which levels of apo-B-containing plasma lipoproteins are approximately half-normal in heterozygotes and virtually absent in homozygotes. Here we describe mutations of the apo-B gene that cause two different truncated variants of apo-B in unrelated individuals with hypobetalipoproteinaemia. One variant, apo-B(His_{1795} → Met-Trp-Leu-Val-Thr-Term) is predicted to be 1799 amino acids long and arises from deletion of a single nucleotide (G) from leucine codon 1794. This protein was found at low levels in very low density and low density lipoprotein fractions in the blood. The second, shorter variant, apo-B(Arg_{1306} → Term), is caused by mutation of a CpG dinucleotide in arginine codon 1306 converting it to a stop codon and predicting a protein of 1305 residues. The product of this allele could not be detected in the circulation. The differences in size and behaviour of these two variants compared to apo-B100 or apo-B48 point to domains that may be important for the assembly, secretion or stability of apo-B-containing lipoproteins.

INTRODUCTION

Lipid is secreted into the circulation and transported as water-soluble macromolecular complexes called lipoproteins (1,2,3). The lipoprotein consists of a hydrophobic core, mainly composed of triglyceride and cholesteryl-ester, surrounded by a monolayer of polar phospholipid and free cholesterol. The integrity of the particle is maintained by specific proteins called apolipoproteins. Apolipoprotein (apo)-B is the largest of these proteins. Two different sized forms of apo-B circulate in human blood. The larger form, designated apo-B100 on the centile system, contains 4536 amino acid residues (4,5). Apo-B100 is synthesised in the liver and is necessary for the assembly and secretion of endogenously synthesised triglyceride and cholesterol as very low density lipoprotein (VLDL). VLDL transports triglyceride to the periphery where it is hydrolysed and the fatty acids are taken up by muscle and adipose tissue. After removal of triglyceride the much reduced particle, designated low density lipoprotein

(LDL), is relatively enriched in cholesterol and apo-B100 is its sole protein component. LDL is the main cholesterol- carrying particle in blood and apo-B100 is the ligand that mediates delivery of cholesterol to cells by the LDL receptor pathway.

The smaller form of apo-B, apo-B48, is synthesised in the intestinal absorptive cell. and corresponds to the amino-terminal 2152 residues of apo-B100 (6,7,8). It lacks the domain in the carboxyl half of apo-B100 that binds to the LDL receptor. Apo-B48 is essential for the absorption of dietary lipids and for their secretion into the circulation as lipoproteins called chylomicrons. In the periphery triglyceride is removed from the chylomicron. The remnant particle is cleared by the interaction of another apolipoprotein, apo-E, with lipoprotein receptors in the liver. Thus apo-B48 has an essential structural role in dietary lipid absorption and transport, but it apparently has no active role in chylomicron remnant clearance.

An inability to assemble and secrete apo-B-containing lipoprotein particles is seen in two inherited disorders abetalipoproteinaemia and hypobetalipoproteinaemia (9). Subjects with either condition suffer from malabsorption of fat and fat-soluble vitamins A and E, which causes spinocerebellar degeneration, retinopathy, and acanthocytosis. Classical abetalipoproteinaemia is a Mendelian recessive disorder in which heterozygotes are asymptomatic. Individuals with homozygous abetalipoproteinaemia are characterised by an almost total absence of plasma apo-B100 and apo-B48 although apo-B mRNA is detectable in the liver and small intestine (10). In some subjects it has been possible to demonstrate the presence of the protein in these tissues (11) but in other studies this has proved negative (12,13).

Persons with homozygous hypobetalipoproteinaemia are indistinguishable from those with homozygous abetalipoproteinaemia. However, heterozygotes with hypobetalipoproteinaemia exhibit apo-B plasma levels below the fifth percentile. They may be symptomatically normal or exhibit some of the features of homozygous hypobetalipoproteinaemia or abetalipoproteinaemia. Low levels of apo-B protein and apo-B mRNA have been demonstrated in the liver of two patients with homozygous hypobetalipoproteinaemia (14). Young et al (15,16) have recently described a family with this condition in which two defective apo-B alleles segregate. One allele produces a truncated protein designated apo-B37 (molecular weight approximately 203 kD) and the other allele directs the synthesis of low levels of apo-B100. The molecular basis for these changes has not yet been reported.

We here define two naturally-occurring mutations of the apo-B gene that predict truncated variants of apo-B in two unrelated individuals with familial hypobetalipoproteinaemia.

METHODS

Individuals studied

The individual DD was a patient under investigation for fat malabsorption. Examination showed no neurological or retinal disease. She had apo-B plasma levels that were less than 2% of normal and > 50% acanthocytes. The diagnosis of homozygous hypobetalipoproteinaemia was made on DD, because both parents AD and PD had total plasma cholesterol and apo-B levels below the 5th percentile for their age and sex on each of several occasions on which they were measured (table 1). These are the accepted criteria for diagnosis of heterozygous hypobetalipoproteinaemia. AD and PD had 10% acanthocytes. Plasma HDL levels were normal. CP was the mother of a young child (ChP) under investigation for fat malabsorption. The father (MP) and second child were normal. Plasma cholesterol and triglyceride levels for CP were below the 5th percentile. Acanthocytes were not observed. These studies have been approved by the Ethical Committee at Northwick Park Hospital and the MRC Clinical Research Centre.

Cholesterol, triglyceride apo-B and total protein assays

Cholesterol and triglyceride assays of the lipoprotein fractions were carried out using colourimetric enzymatic kits according to manufacturer's instructions (Boehringer Mannheim Diagnostics, cholesterol kit no. 240319 and triglyceride kit no. 701882). Apo-B was assayed immunochemically using a polyclonal antiserum (Orion Diagnostics, Espoo, Finland). The total protein content of the fractions was assayed by the method of Lowry et al (17).

Lipoprotein fractionation

Venous blood samples were obtained from patients and control subjects in a non-fasted state. The blood was mixed immediately with an inhibitor mixture (18) to give final concentrations of EDTA 7.5 mg/ml, polybrene 25 μg/ml, benzamidine 2 mM, aprotinin 100 KIU/ml, soya bean trypsin inhibitor 75 μg/ml, sodium azide 175 μg/ml and phenyl methyl chloroketone 20 μg/ml. Plasma was collected after low speed centrifugation and PMSF was added to 75 μg/ml. Plasma density was adjusted with KBr and lipoproteins were fractionated by ultracentrifugation (19) to yield a combined chylomicron, VLDL and IDL (intermediate density lipoprotein) fraction ($p < 1.019$ g cm^{-3}), an LDL fraction ($1.019 < p < 1.063$ g cm^{-3}), an HDL fraction ($1.063 < p <$

1.25 g cm^{-3}) and an infranatant (p > 1.25 g cm^{-3}). The isolated fractions were centrifugated a second time and dialysed against phosphate buffered saline. The fractions were delipidated by dropwise addition to 20 volumes of a mixture of ethanol and diethyl ether (3:1). The resulting precipitates were washed with ether, dried under nitrogen and dissolved to a concentration of 2 - 3 mg/ml in 10% SDS, 5% β-mercaptoethanol.

SDS-polyacrylamide gel electrophoresis

Apolipoproteins, 50 µg per lane, were separated on 5 - 10% or 8 - 17% gradient gels, using the system of Laemmli (20). Gels were either fixed and stained with Coomassie Brilliant Blue or further analysed by immunoblotting.

Immunoblotting

Proteins were transferred electrophoretically to nitrocellulose paper from SDS-PAGE gels (21). The replicas were blocked with a solution containing 5% BSA / 0.25% gelatin / 0.15 M NaCl/50 mM Tris-HCl pH 7.4 / 5 mM EDTA / 0.05% Triton X-100. Filters were probed with a mouse Mab, anti-B Sol 9 (22) whose epitope lies between residues 454 and 586 of human apo-B (Pease et al, in preparation). ^{125}I protein A (Amersham, UK) was used as the second reagent.

Southern blot analysis

Genomic DNA was isolated (23) from 10 ml of peripheral blood and 2 µg DNA was digested overnight to completion with 10 units of restriction enzyme. Gel electrophoresis was performed in 0.8% agarose slabs and transfer to nylon membranes (BioRad Zeta-Probe) was carried out by alkaline Southern blotting in 0.4 M NaOH following depurination of DNA in 0.25 M HCl (24). Southern blots were hybridised with [^{32}P] oligolabelled cDNA inserts from plasmids pABF, pSB9 and pAB1 (4) or with 1.6 kb HindIII/XbaI and 0.75 kb XbaI genomic fragments (fig. 2)). The latter probes were obtained by a HindIII/XbaI digest of plasmid pBS6 which carries genomic sequences from intron 20 to exon 26 of the apo-B gene.

Oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems 380A synthesiser using β-cyanoethyl phosphoramidites, purified on Applied Biosystems OPC columns and dissolved at 1 mg/ml in water. The synthetic oligonucleotides used for DNA amplification and their positions in the apo-B coding sequence are:

CP1	21mer	CTGTTAGGACACCAGCCCTCC	4072 → 4092
CP2	23mer	GCCACCACTGTAGGAGGCGGACC	4256 → 4234
DD1	23mer	CCCTCACCTCCACCTCTGATCTG	4756 → 4778

DD2	22mer	CTTAAGTCCTTCTTGACTGACC	5339 → 5318
DD3	24mer	CAGGCCATGATTCTGGGTGTCGAC	5271 → 5294
DD4	24mer	CCCATTGCCATTTGTATGTGCATC	5873 → 5852
DD5	24mer	GTAATGGCCCCGTTTACCATGACC	5823 → 5846
DD6	23mer	GGCATGTGAAACTTGTCTCTCCC	6542 → 6520

Polymerase chain reaction

Samples (5 μg) of human genomic DNA were subjected to 30 cycles of amplification in 10 mM Tris-HCl pH 8.5, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each dNTP and 1 μg of each oligonucleotide. After an initial denaturation step at 95°C for 10 minutes, 2 units of Taq polymerase (Cetus) were added. Cycles consisted of 1.25 min @ 95°C, 1.5 min @ 55°C and 2 min @ 70°C with a final extension of 10 min.

M13 cloning and sequencing

Fragments of amplified DNA were purified from agarose gels by electroelution on to DE81 paper. The recovered DNA was blunt-ended with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase as described (25). Approximately 50 ng of DNA was ligated to 10 ng of EcoRV-digested, dephosphorylated M13tg131 vector. One tenth of the ligation was used to transform competent E. coli DH5αF'. Recombinant phage plaques were picked and M13 templates prepared. DNA sequencing was performed using Sequenase (USB) and [^{35}S]dATPαS in accordance with the manufacturers' instructions and the reactions were run on 6% field gradient gels.

RESULTS

Detection of an apo-B gene mutant by Southern blotting

The subjects studied were a patient (DD) with homozygous hypobetalipoproteinaemia, her mother (PD) and father (AD) both heterozygous for the disease and an unrelated heterozygous individual (CP) from a second kindred (Methods and table 1). To detect potential mutations or rearrangements of the apo-B gene in these individuals genomic DNA was digested with a panel of restriction enzymes, blotted and probed with [^{32}P]-labelled cDNAs spanning the entire coding sequence. Restriction patterns for DD, AD and PD were indistinguishable from normal controls but CP was heterozygous for a novel TaqI restriction site change detectable with probe pABF (fig. 1). This mutation was localised to exon 25 by the hybridisation pattern on TaqI genomic Southerns of two probes consisting of a 1.6 kb HindIII/XbaI and a 0.75 kb XbaI fragment (fig. 2) isolated from a clone spanning intron 20 to exon 26. When the TaqI site in exon 25 is

Table 1 Plasma cholesterol, triglyceride and apo-B concentrations.

Subject	Age	Total Cholesterol (4-6.5 mmol/1)+	Triglyceride (0.3-1.8 mmol/1)+	Apo-B (60-140 mg/dl)+
DD* (daughter)	21	1.27	0.30	1.5
PD (mother)	44	2.57	0.59	17
AD (father)	48	2.82	0.32	28
CP* (mother)	37	2.61	0.36	9
MP (father)	40	5.30	1.42	80
ChP (daughter)	3	1.20	0.50	28
LP (daughter)	2	3.70	3.90$^{\Delta}$	83

+ Normal ranges are 5 - 95 percentile.

* Receiving vitamin E supplement.

$^{\Delta}$ Non-fasting level.

present the 1.6 kb probe hybridises with 2.8 kb and 0.8 kb restriction fragments and the 0.75 kb probe hybridises to a 0.8 kb band. If the TaqI site in exon 25 is lost both the 1.6 kb and 0.75 kb probes hybridise to a new 3.6 kb band composed of the 2.8 kb and 0.8 kb fragments. The result (fig. 1) confirms that CP is heterozygous for the loss of the single TaqI site in exon 25. The spouse (MP) and one daughter (LP) of CP both gave a normal TaqI restriction pattern when probed with pABF but the second child ChP, currently undergoing investigation for fat malabsorption and diagnosed as having hypobetalipoproteinaemia, was also heterozygous for the loss of the exon 25 TaqI site (table 1). TaqI site mutations commonly occur through spontaneous deamination of the 5-methyl-cytosine in methylated CpG dinucleotides within the TCGA recognition sequence resulting in a C → T transition. Thus arginine codon 1306 (CGA) in exon 25 could mutate either to CAA (glutamine) or UGA (stop). The latter would terminate translation and give rise to a truncated apo-B protein 1305 amino acids long.

Identification of truncated apo-B by SDS-polyacrylamide gel electrophoresis and immunoblotting

Plasma triglyceride-rich lipoproteins (chylomicrons and VLDL), LDL and HDL fractions were prepared by sequential ultracentrifugation (19)

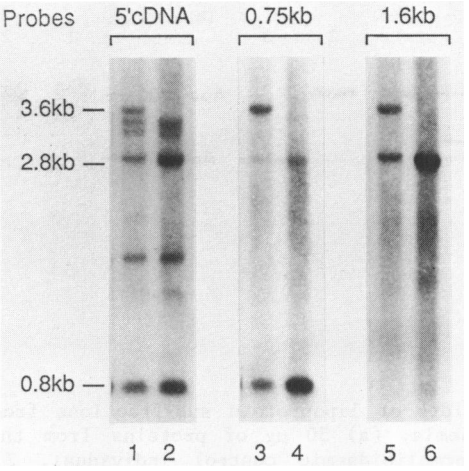

Figure 1: Genomic Southern blots of TaqI digested DNA. Lanes 1, 3 and 5 DNA from patient CP. Lanes 2, 4 and 6 control DNA. Lanes 1 and 2 were hybridised with pABF (4), a 6.5 kb cDNA from the 5' end of the apo-B message. Lanes 3 and 4 were probed with a 0.75 kb genomic XbaI fragment (fig. 2). Lanes 5 and 6 were probed with the 1.6 kb HindIII/XbaI genomic fragment.

Lipoprotein fractions were delipidated and their proteins separated by SDS-polyacrylamide gradient gel electrophoresis. Immunoblots were performed with an apo-B monoclonal antibody Sol 9 which reacts strongly with an epitope near the amino terminal of apo-B (Pease et al in preparation).

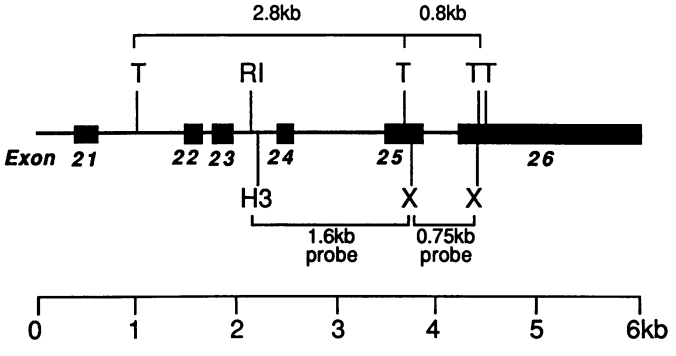

Figure 2: Restriction map of the apo-B gene around the TaqI mutation in patient CP. The positions of two genomic probes and the TaqI fragments which they detect are shown. Loss of the TaqI site in exon 25 joins the two TaqI fragments to give a 3.6 kb band (fig. 1). Abbreviations: T = TaqI, RI = EcoRI, X = XbaI.

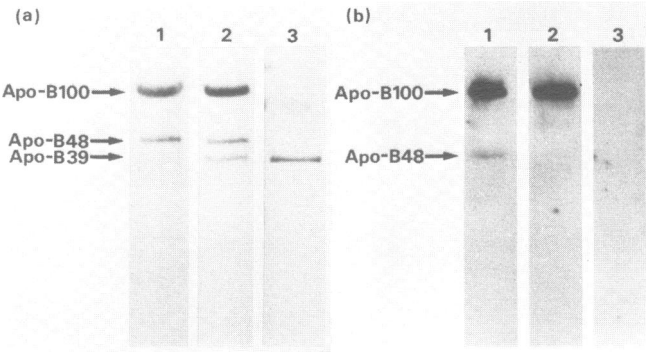

Figure 3: Western blots of lipoprotein sub-fractions from individuals with hypobetalipoproteinaemia. (a) 50 μg of proteins from the VLDL/chylomicron fractions of 1) normolipidaemic control individual, 2) patient PD, 3) patient DD. (b) 50 μg of proteins from 1) VLDL, 2) LDL, 3) HDL of patient CP. Blots were prepared and probed with anti-human apo-B Sol 9 antibody as described in Methods.

In the individual CP the abnormal apo-B species predicted on the basis of termination at amino acid 1305 could not be demonstrated on western blots (fig. 3) or on coomassie stained gels (not shown) in either VLDL/chylomicron, LDL, HDL or infranate fractions. This raised the possibility that codon 1306 changes to CAA (glutamine) and that a short protein is not produced.

In the individual DD an aberrant species of apo-B was found in the VLDL/chylomicron fraction (fig. 3). DD had virtually no LDL. This apo-B migrated close behind the myosin standard and was estimated to have a molecular weight greater than 205 kD. The protein was absent from the HDL or infranate fractions. The mother (PD) of DD also had the same truncated apo-B in both her VLDL and LDL lipoprotein fractions, but it was present at reduced levels compared to the apo-B100 arising from her one normal apo-B allele (fig. 3). Only apo-B100 and apo-B48 were detected in the father AD. A short protein could be demonstrated in both PD and DD despite an apparently normal gene structure, implying a point mutation or a small insertion/deletion event. The size of the protein suggested that this mutation would lie near the 5' end of exon 26.

Characterisation of the apo-B gene mutations by amplification of genomic DNA, cloning and sequencing.

To determine the precise nature of the mutation in each individual genomic DNA was amplified by the polymerase chain reaction (PCR) (26) and

sequenced. For DD and PD approximately 1.7 kb of exon 26 was amplifed as 3 nested segments encoding nucleotides 4756 to 6542 of the mRNA (see Methods). The three PCR fragments were blunt-ended with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase and cloned into dephosphorylated EcoRV-cut M13tg131 for DNA sequence analysis. In both DD and PD, the individuals with the truncated plasma form of apo-B, approximately equal numbers of clones containing either the normal apo-B sequence or the deletion of guanine nucleotide 5591 (fig. 4) were obtained. Within the 1786 bp segment sequenced, no other nucleotide changes were found. Deletion of G 5591 was not observed in PCR amplified DNA from a control, normal individual. This mutation removes the third base of leucine codon 1794 and predicts a translation frame shift, in which His_{1795} is replaced by Met. Translation continues in frame +1 adding four more amino acid residues (Trp-Leu-Val-Thr) and then terminates. This variant, apo-B(His_{1795} → Met-Trp-Leu-Val-Thr-$Term_{1800}$), has a predicted molecular weight of 201.6 kD before glycosylation which is consistent with the protein seen on SDS gels in these patients. Based on its migration relative to apo-B100, apo-B48 and apo-B26 the protein is equivalent to apo-B39.

In the individual CP, in whom the anticipated short form of apo-B could not be demonstrated in plasma, a 185 bp section of exon 25 encoding nucleotides 4072 to 4256 of the apo-B mRNA was amplified (see Methods). This spans the TaqI site which was absent from one allele of CP. The PCR fragment was cloned and sequenced as before. The TaqI site was present in approximately half of the clones examined. In the remainder TCGA was replaced by TTGA (fig. 4) confirming that arginine codon 1306 (CGA) is replaced by a stop codon UGA through mutation of the CpG dinucleotide. This mutation predicts that hypobetalipoproteinaemia in patients CP and ChP is caused by an in-frame termination codon arising from a C → T transition at nucleotide 4125 of the coding sequence. The truncated form of apo-B (apo-B(Arg_{1306} → Term)) generated by this mutation is eight amino acids longer than a plasma kallikrein cleavage product of apo-B100 (designated apo-B26) (27) and has an estimated molecular weight of 146.2 kD before glycosylation.

DISCUSSION

The data presented here demonstrate that familial hypobetalipoproteinaemia can be caused by mutations of the apo-B gene. The two different mutations (fig. 5) are the first to be fully characterised. In one family,

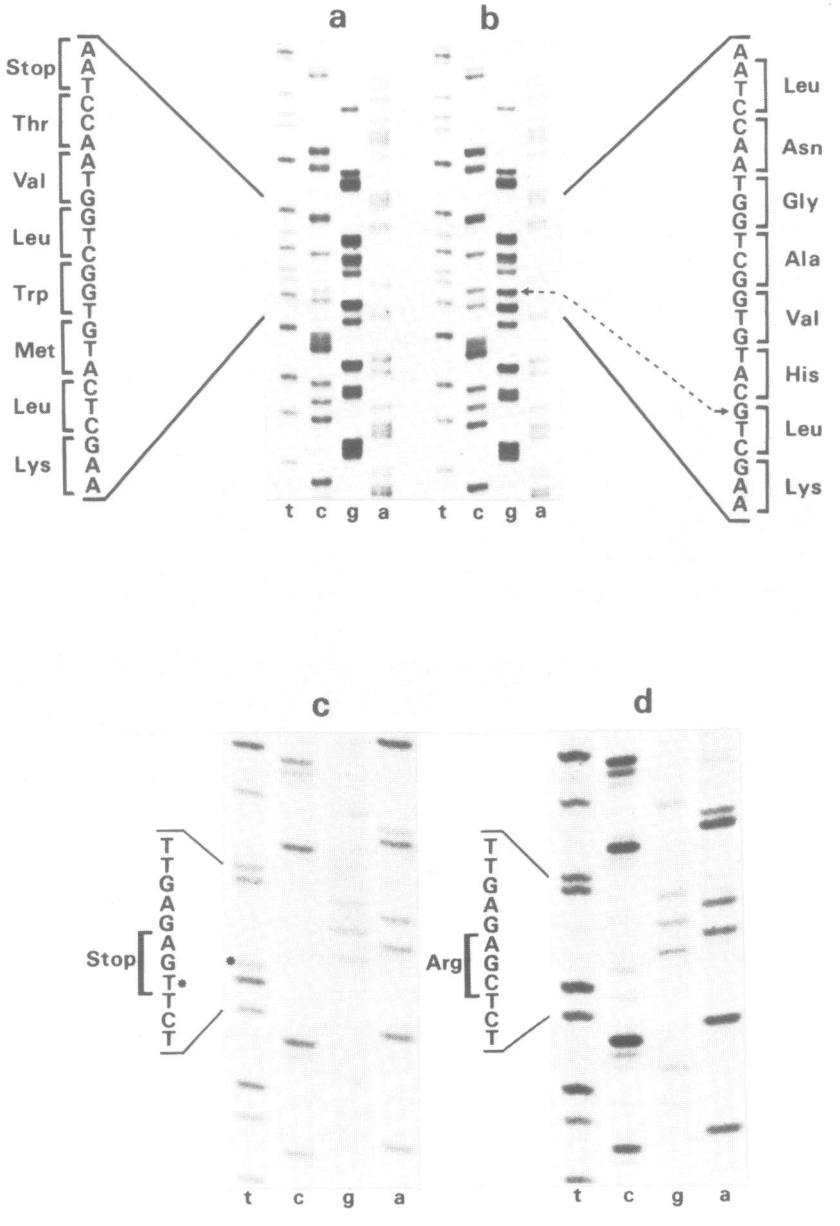

Figure 4: DNA sequence ladders of apo-B gene mutations. (a) mutant and (b) normal sequences from patient DD. The G nucleotide which is deleted from leucine codon 1794 in the defective allele is arrowed. The frame-shifted and normal translations are displayed alongside the ladders. (c) mutant and (d) normal sequences from patient CP showing a C → T transition (asterisk) converting arginine 1306 to a termination signal.

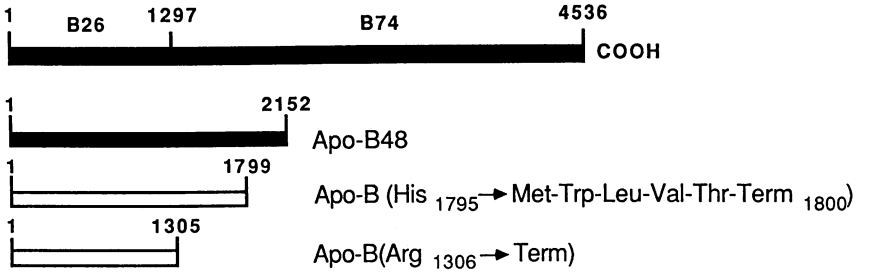

Figure 5: Truncated variants of apo-B which cause hypobetalipoproteinaemia. The normal plasma forms apo-B100 and apo-B48 are also shown. The kallikrein cleavage site (27) at residue 1297 in apo-B which generates apo-B26 and apo-B74 is marked.

hypobetalipoproteinaemia is caused by a single base change in arginine codon 1306. A C → T transition converts CGA to TGA introducing a translational termination signal and predicting a gene product of only 1305 amino acids, apo-B(Arg_{1306} → Term). CpG dinucleotides provide potential sites for spontaneous mutation. In humans 35% of single base changes in coding sequences which cause genetic disorders are due to C → T or G → A transitions within CpG dinucleotides (28). The presence of 12 CGA codons in the apo-B mRNA (table 2) suggests that more truncated proteins may be found as a result of CpG mutations.

In the second kindred studied, two affected individuals were shown to possess an apo-B allele with a deletion of a single G nucleotide corresponding to base 5591 of the mRNA. This introduces a translational frame shift and results in the addition of a short hydrophobic peptide Met-Trp-Leu-Val-Thr at Leu 1794. A truncated apo-B molecule of 1799 residues is predicted, apo-B(His_{1795} → Met, Trp-Leu-Val-Thr-Term). Subjects PD and DD both possess an abnormal plasma apo-B species, the mobility of which is entirely consistent with this mutation. Based upon its migration in SDS-PAGE, relative to apo-B markers, this protein represents apo-B39. Young et al (15,16) have documented the presence of a short apo-B protein, apo-B37, in one kindred with hypobetalipoproteinaemia. From its size and antibody crossreactivity apo-B37 is believed to consist of the amino terminal 1700 residues of apo-B100 (29) and is therefore about 100 amino acids smaller than apo-B39. Important differences exist between both of the variants we describe and apo-B37. Apo-B(Arg_{1306} → Term) could not be detected in lipoproteins from subject CP or in the lipoprotein-depleted fraction of plasma. Apo-B39 was present only in the VLDL/chylomicron and LDL

Table 2 CGA arginine codons in the apo-B coding sequence.

Nucleotide position in mRNA	Amino acid residue	Affected restriction site
261	18	-
1,407	40	-
1,443	412	PvuII
1,800	531	-
4,125	1,306	TaqI
6,162	1,985	-
6,380	2,058	TaqI
7,665	2,486	-
7,686	2,493	-
7,692	2,495	-
7,728	2,507	-
12,657	4,150	-

fractions of PD and in the VLDL/chylomicron fraction of DD. DD had insufficient LDL for analysis. No apo-B39 was present in the HDL density range or in the infranatant of either DD or PD. In contrast, only a small amount of apo-B37 was found in VLDL and LDL whereas the majority sedimented in the HDL fraction where it was the main apoprotein in an abnormal particle containing little or no apo-A1. These differences may indicate regions of apo-B100 which determine the pathway of lipoprotein assembly and secretion. The regions deleted from these variants contain lipid binding domains that are potentially important for these processes. Alternatively, differences in the genetic background of the individuals studied might explain this variation. Characterisation of more apo-B gene variants and the expression of apo-B cDNA constructs in human cell lines are in progress to address these issues.

The mechanism by which truncated variants of apo-B cause hypobetalipoproteinaemia remains unclear. It is possible that their rate of secretion is reduced relative to apo-B100 or, in the case of apo-B(Arg_{1306} → Term), the protein may not be secreted at all. Alternatively, they may be degraded extracellularly or be rapidly cleared from the circulation, particularly if present on an abnormal lipoprotein particle. However, consideration of the differences in size and behaviour of these variants compared to apo-B100 and apo-B48 suggests that the length of apo-B is critical for lipoprotein production. LDL and chylomicrons each contain about 500 kD of protein (30,31,32). This is consistent with there being one molecule of apo-B100 (Mr 512 kD before glycosylation) in LDL and two molecules of apo-B48 (Mr 241 kD before glycosylation) in chylomicrons.

Truncated apo-B variants may therefore be inadequate for the assembly of normal triglyceride-rich lipoproteins or for maintaining their structural integrity. Thus in heterozygotes, the amounts of apo-B37 and apo-B39 are much lower than apo-B100 and apoB(Arg_{1306} → Term) was not detectable at all in plasma.

No truncated apo-B was found in plasma lipoproteins from subject AD, only normal sized apo-B100 and apo-B48. Prolonged exposures of Western blots of VLDL from the homozygote DD revealed trace amounts of apo-B100 (not shown) suggesting that the defective paternal apo-B allele directs the synthesis of low levels of full length apo-B. DD therefore resembles the compound heterozygote HJB analysed by Young et al in whom one allele gives apo-B37 and the other makes traces of apo-B100. Greatly reduced hepatic apo-B mRNA levels have been observed in two patients with homozygous hypobetalipoproteinaemia (14), suggesting that mutations affecting gene expression, mRNA splicing or mRNA stability are responsible for the disease in these individuals. Such a mutation might be the cause of hypobetalipoproteinaemia in subject AD and explain the trace amount of apo-B100 in the compound heterozygote DD.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council. SEH and PJT receive support from the British Heart Foundation (grant RG5) and the Charing Cross Sunley Research Centre. We thank Lesley Sargeant for typing the manuscript.

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