The role of the apolipoprotein B gene in the pathogenesis of familial hypocholesterolaemia.

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Abstract

The role of the apolipoprotein B (apoB) gene in the pathogenesis of two familial hypocholesterolaemic disorders, recessive abetalipoproteinaemia and familial hypobetalipoproteinaemia has been investigated.

The structure of the apoB gene has been studied in four individuals with classical recessive abetalipoproteinaemia and four with familial hypobetalipoproteinaemia.

In two individuals from one family with familial hypobetalipoproteinaemia, a point mutation was detected in one apoB allele which is predicted to lead to premature termination of apoB messenger RNA translation and thereby accounts for the hypocholesterolaemic phenotype. Since the mutation predicts the synthesis of an abnormal, truncated form of apoB, the apolipoproteins produced by one of these individuals were examined, but no such protein was detected. Similar experiments on two individuals from another family with hypobetalipoproteinaemia however, demonstrated the presence of a different, larger, truncated form of apoB. Subsequent studies revealed that this family had a frameshift mutation in one apoB allele which could fully account for the phenotype of one of the individuals, and partly account for phenotype of the other. The latter individual was found to have, in addition to the truncated apoB species, an abnormally low level of full length apoB. In an attempt to elucidate the molecular defect responsible for this finding, the 5′ flanking region of the apoB gene was sequenced in this individual, but no abnormality was found.

In contrast to the hypobetalipoproteinaemic individuals, no abnormalities in the apoB gene were detectable in any of the individuals with abetalipoproteinaemia. The results of these studies however, did enable the transmission of the parental apoB alleles to the affected children to be followed. It is concluded that, within the families studied, the apoB gene is discordant with abetalipoproteinaemia and that therefore, the disorder must be caused by a defect in another gene which is involved in the synthesis or secretion of apoB-containing lipoproteins from both the liver and intestine.

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Chapter 1: Introduction

1.1 Objectives of the work described in this thesis.

The aim of this thesis has been to investigate the role of the apoB gene in the pathogenesis of familial hypocholesterolaemias. The particular disorders studied have been recessive abetalipoproteinaemia and familial hypobetalipoproteinaemia. The apoB gene and where appropriate, it's product have been studied in a series of individuals with these disorders.

In this chapter, the plasma lipoproteins, the apolipoproteins contained within them, the lipoprotein-processing enzymes and the lipoprotein receptors are reviewed. This is followed by a consideration of the major hypocholesterolaemic disorders, including the two under investigation.

1.2 The lipoproteins.

Lipid is transported in the circulation as water soluble macromolecular complexes known as lipoproteins. Each lipoprotein particle consists of a hydrophobic core composed mainly of triglyceride and cholesterol ester surrounded by a monomolecular layer of phospholipid and free cholesterol plus one or more specific proteins known as apolipoproteins.

The lipoproteins have traditionally been separated into various classes according to the density at which they float during ultracentrifugation (Havel *et al.*,1955). They can be further classified on the basis of particle size, electrophoretic mobility, or affinity chromatography.

Plasma lipoprotein fractions.

On the basis of flotation density and size, human plasma lipoproteins can be divided into five major classes; chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL).

Chylomicrons ($d<0.95$ g/ml), are the largest lipoproteins, with diameters ranging from 70 to 1200 nm. Their average composition by mass is 86% triglyceride, 7% phospholipid, 3% cholesteryl ester, 2% free cholesterol and 2% apolipoprotein. (Havel and Kane., 1989). They are synthesised by intestinal mucosal cells and secreted into lymph to transport dietary cholesterol and triglyceride from the site of absorption to various cells in the body. Their main apolipoprotein constituents are apoB48, apoAI and apoAIV. It is likely that there is only one molecule of apoB48 per chylomicron particle (Elovson *et al.*, 1988). Unlike the other apolipoproteins, apoB48 remains tightly bound to the chylomicron particle and does not transfer to any other lipoprotein particle. In the plasma, chylomicrons acquire apoE and C apolipoproteins from HDL.

In the periphery, 80-90% of the triglyceride is removed from the chylomicron by the action of the endothelial enzyme lipoprotein lipase. One of the C apolipoproteins, apoCII, is a crucial cofactor required for lipoprotein lipase action. The liberated fatty acids are used as an energy source by some cells and taken up and stored as triglyceride by adipocytes. At the same time as the removal of triglyceride, the chylomicron's surface compo-

nents, including phospholipid, free cholesterol and some of the exchangeable apolipoproteins are transferred to HDL.

Chylomicron remnants are the lipoprotein particles generated by the action of lipoprotein lipase on chylomicrons. They are enriched in cholesterol and are beta-migrating, very low density lipoprotein (ß-VLDL). Under normal circumstances they are rapidly cleared by a hepatic remnant receptor (which may be the LDL-receptor related protein; see below) which recognises apoE. They may however may accumulate in the plasma of patients with type III hyperlipoproteinaemia.

Very low density lipoprotein (VLDL d<1.006 g/ml) are 30-80 nm particles secreted by the liver for the transport of endogenously synthesised cholesterol and triglyceride. Their average composition by mass is 55% triglyceride, 18% phospholipid, 12% cholesteryl ester, 7% free cholesterol and 8% apolipoprotein (Havel and Kane., 1989). Nascent VLDL particles contain apoB100 and C apolipoproteins, with or without apoE (Fielding and Fielding., 1986). Like apoB48, apoB100 does not transfer to any other lipoprotein particle. As with chylomicrons, VLDL triglycerides are hydrolysed to free fatty acids by peripheral and hepatic lipoprotein lipases to generate smaller, cholesterol enriched lipoproteins. Similarly, as their triglyceride content is reduced, surface components, including phospholipid, free cholesterol and C apolipoproteins are transferred to HDL. Simultaneously the VLDL particles acquire cholesteryl ester from HDL together with addition-

al apoE.

Intermediate density lipoproteins (IDL 1.006<d<1.019 g/ml) and low density lipoprotein (lDL 1.019<d<1.063 g/ml) are the products VLDL metabolism. On average, IDL contains, by mass, 23% triglyceride, 19% phospholipid, 29% cholesteryl esters, 9% free cholesterol and 19% apolipoprotein (Havel and Kane., 1989). Some of the IDL particles, probably those richest in apoE, are taken up by the liver by interaction with the LDL (apoB,E receptor). The remainder are metabolised further. The LDL particle is the end product of VLDL metabolism, is small and is the major cholesterol carrying particle human plasma, containing about 2/3 of the plasma cholesterol. On average, the mass composition of LDL is 6% triglyceride, 22% phospholipid, 42% cholesteryl ester, 8% free cholesterol and 22% apolipoprotein, of which the sole component is apoB100.

ApoB100 on LDL binds to the LDL receptor of hepatic and peripheral cells, but with less affinity than apoE. LDL particles are accordingly removed from the plasma more slowly than IDL and at least 90% of the total plasma apoB100 is contained in LDL (Schonfield *et al.*, 1974). There is now accumulating evidence to suggest that the development of coronary atherosclerosis is causally related to elevated plasma levels of LDL cholesterol (Consensus conference., 1985).

High density lipoproteins (HDL 1.063<d<1.21 g/ml) are the smallest of the lipoproteins (40-60 Angstroms). They arise from a variety of sources. In addition to the liver and intestine, HDL precursors arise as a by-product of the

action of lipoprotein lipase on chylomicrons by the generation of phospholipid protein discs from the remnant surface (Tall *et al.*, 1978).

HDL is involved in the reverse cholesterol transport pathway, whereby precursor HDL acquires cholesterol from the periphery for subsequent transport to the liver (Eisenberg., 1984). In man, cholesterol acquisition by HDL precursors is brought about by the action of the enzyme lecithin:cholesterol acyltransferase (LCAT), which catalyses the transfer of fatty acids from the beta position of lecithin to the beta position of cholesterol. Cholesteryl ester is subsequently transferred to triglyceride-rich lipoproteins via cholesterol ester transfer protein (CETP). In addition, HDL accepts surface components of triglyceride-rich lipoproteins during lipolysis by lipoprotein lipase. These include phospholipid, free cholesterol and exchangeable apolipoproteins. In man, lipolysis is associated with an increase in the size of HDL particles, as a result of this transfer of surface components and the esterification of cholesterol. The smaller HDL are referred to as HDL_3 and the larger particles as HDL_2. On average, HDL_3 contain, by mass, 3% triglyceride, 35% phospholipid, 13% cholesteryl ester, 4% free cholesterol and 55% apolipoprotein, whilst HDL_2 contain 5% triglyceride, 33% phospholipid, 17% cholesteryl esters, 5% free cholesterol and 40% apolipoprotein (Havel and Kane., 1989). The main apolipoprotein components of HDL are apoAI and apoAII. The liver generated HDL also contain apoE.

The esterification of peripheral cholesterol by LCAT removes cholesterol from peripheral cells and other lipoproteins and the cholesteryl ester is delivered to the liver by a variety of routes. There is the receptor mediated uptake of the remnants of the triglyceride-rich lipoproteins and LDL discussed above. Cholesterol ester in HDL particles can also be taken up by the liver directly, following the hydrolysis of HDL phosphatidyl choline by hepatic lipase, a process which converts HDL_2 back into HDL_3. In addition, apoE-containing HDL particles can be taken up by hepatic apoE receptors (Havel and Kane., 1989).

The levels of the various lipoproteins are determined by a large variety of genes whose products control their synthesis, processing and catabolism. Amongst these genes, are those coding for the apolipoproteins AI, AII, AIV, B, CI, CII, CIII, D, E and (a), the lipoprotein-processing enzymes (peripheral and hepatic lipases and LCAT), the LDL receptor and the LDL receptor related protein (LRP). These components of lipoprotein metabolism will now be considered individually prior to a introduction to abetalipoproteinaemia and hypobetalipoproteinaemia and related disorders.

1.3 Apolipoproteins

The protein components of the plasma lipoproteins are known as apolipoproteins. Their function is primarily lipid transport. They maintain the integrity of the individual lipoprotein particles and control the flux of lipid between the gut, the liver and the periphery.

(a) Apolipoprotein B.

Apolipoprotein B (apoB) is the predominant apolipoprotein of the cholesterol and triglyceride rich plasma lipoproteins (Chylomicrons, VLDL, IDl and LDL) and is essential for their assembly and secretion.

The apoB gene lies on chromosome 2 (Mehrabian 1986). It extends over 43 kilobases and comprises 29 exons and 28 introns, with an extremely asymmetrical distribution of introns, most of them appearing in the 5' terminal one third of the gene. Exons 26 and 29 are particularly large (7572 and 1906 bp respectively), the former being by far the largest reported at the time for a vertebrate gene (Blackhart *et al.*, 1986).

Two different sized variants of apoB are present in normal human plasma. Because absolute estimates of molecular weights of these apoB species were found to differ substantially between laboratories, but because there relative molecular weights would be expected to remain constant in a given analytical system, Kane proposed a centile system for these and other apoB variants, such as those formed by proteolytic digestion (Kane *et al.*, 1980.; Kane., 1983). In this system, the liver derived and largest form, found in VLDL and LDL is designated apoBl00 and the relative mobilities of all other variants are expressed on a centile scale. The intestinal species, present in chylomicrons is smaller, displaying an apparent molecular weight that is 48% of that of apoBl00. It is thus known as apoB48 (Kane., 1980).

Two naturally occurring proteolytic fragments of apoBl00

are apoB26 and apoB74 (Kane., 1983). These are generated by kallikrein cleavage at a unique site in the amino terminal quarter of apoB100 (Cardin *et al.*, 1984).

ApoB100 is virtually the only protein component of LDL. The apoB100 precursor contains 4563 amino acids and gives rise to a mature protein of 4536 amino acids following cleavage of a 27 amino acid signal peptide (Knott *et al* ., 1986; Yang *et al.*, 1986; Chen *et al.*, 1986). It is one of the largest monomeric proteins known, with a calculated relative molecular weight of 512,937 (Yang *et al.*, 1986). ApoB48 corresponds to the amino terminal 2152 residues of apoB100 and arises by co- or post- translational editing of intestinal mRNA to introduce a U in place of a C at position 6666, causing codon 2153 (TAA, Glutamine) to change to a translational stop codon (CAA) (Powell *et al.*, 1987; Chen *et al.*, 1987; Hospattanker *et al.*, 1987; Higuchi *et al.*, 1988).

Unlike the other lipoproteins, apoB100 is insoluble in aqueous media after delipidation and is completely non-exchangeable between lipoprotein particles (Kane., 1983). Although the primary amino acid sequence is now known, the protein's tertiary structure and conformation in LDL have been only partially determined.

Within the apoB100 sequence are two regions rich in basic amino acids (residues 3147-3157 and 3359-3367), which have been reported to be linked by a disulphide bridge between cysteine residues 3167 and 3297, the second of these sequences having some homology to the receptor binding region of apoE (Knott *et al.*, 1986). Flanking these re-

gions, are epitopes for receptor blocking monoclonal antibodies and it may be that they together form the receptor binding domain of apoB100 (Knott *et al.*, 1986).

A cross-species comparison of the basic domains 3147-3157 and 3359-3367 has shown that the former is phylogenetically the primary region involved in interaction with the LDL receptor, but that the latter has become more basic from chicken to man and may have developed a more important role in this respect in humans (Law and Scott., 1990).

Based on differential susceptibility to tryptic digestion, a model for the structure and conformation of apoB100 in LDL has been proposed which divides the protein into five domains (Yang *et al.*,1986; Yang *et al.*, 1989); 1) residues 1-1000, largely trypsin releasable 2) residues 1001-1700 with alternating releasable and non-releasable regions 3) residues 1701-3070, largely non-releasable 4) residues 3071-4100 mainly releasable and containing the putative receptor binding domain and 5) residues 4101-4536 almost wholly non-releasable (Figure 1).

Studies by Deloof *et al* (1987) provide evidence for the existence of several internal repeats in apoB100. They used a specially developed computer programme to produce simultaneous alignment of several repeated sequences in an iterative way, thus generating consensus sequences. Two categories of internal repeat were identified; amphipathic helical regions and hydrophobic, proline rich domains. They suggested that both classes of repeat might contribute to the specific lipid binding ability of apoB.

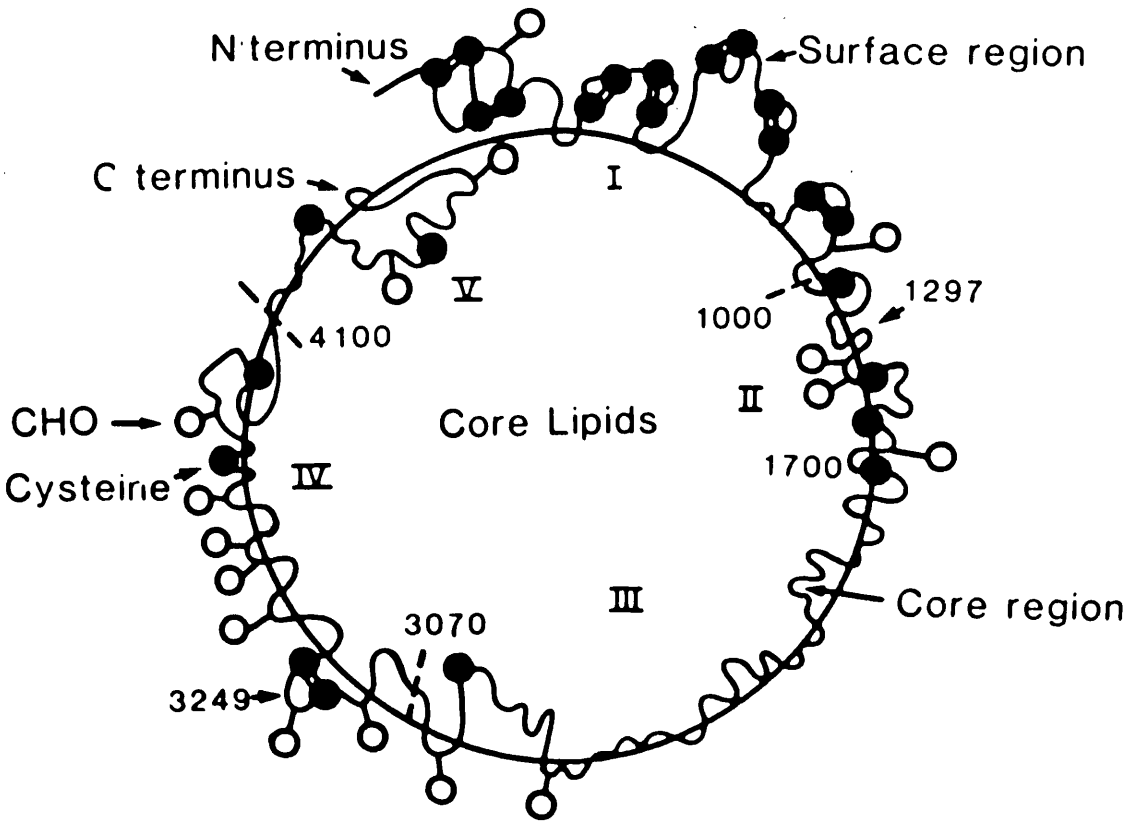


Figure 1.

Schematic representation of apoB100 structure on LDL. The trypsin releasable regions are shown on the outside and the trypsin non-releasable regions inside the core of the LDL particle, although this orientation is only hypothetical. The five domains proposed by Yang *et al* (1989) are separated by dashed lines. The thrombin cleavage sites at residues 1297 and 3249 are marked. The location of the 16 identified N-glycosylated carbohydrates are indicated by (O), cysteine residues by (●) and disulphide bridges by (=). (From Yang *et al* (1990)).

Amphipathic helices have been studied by other groups using a variety of methods (Segrest *et al.*, 1974; Sparrow and Gotto., 1982) and are considered to be involved in lipid binding. The fact that, on intact LDL particles, such regions are mostly inaccessible to trypsin (Yang *et al.*, 1986; 1989) provides further support to this view. The proline rich repeats on the other hand, consist predominantly of hydrophobic
residues which are predicted to form beta sheets and turns, also able to interact with lipid in the LDL particle (Deloof *et al.*, 1987).

The additive effect of the amphipathic and proline rich domains might account for the observation that unlike the smaller apolipoproteins, apoB is not exchangeable between the different classes of lipoprotein particle (Kane., 1983).

The apoB sequence contains 25 cysteine residues, 12 of which are present in the first 500 residues. Two free cysteine residues (sulphydryl form) have been localised to positions 3734 and 4190 (cysteine residues 22 and 24) of apoB100 on the LDL particle using a fluorescent sulphydryl probe (Kim *et al.*, 1989). It is likely that one of these is involved in forming the intermolecular link with apo(a) to give rise to lipoprotein (a).

Isolation of tryptic and peptic peptides by Yang *et al* (1990) have has shown that of the remaining 23 cysteines, sixteen exist in disulphide form and an five in sulphydryl form, with two residues (no's 19 and 25) not yet confirmed (Figure 1).

(b) Apolipoprotein E.

Apo E is synthesised primarily by the liver, but is also produced by various other organs not involved in lipoprotein synthesis (Blue *et al.*, 1983; Reue *et al.*, 1984). These include brain, heart, lung, spleen, adrenal and kidney, particularly the renal cortex. It acts as a ligand for both the LDL (apoB,E) receptor and the LDL-receptor related protein which is thought to be involved in the uptake by the liver of chylomicron remnants, IDL and apoE-containing HDL (see below). It is present in interstitial fluid, where it apparently participates in cholesterol redistribution between cells and seems to be involved in the repair response to tissue injury. Increased levels are found for instance, at the site of peripheral nerve injury and regeneration (Ignatius *et al.*,1986; Boyles *et al.*, 1986).

ApoE is a protein with a relative molecular mass of 34,000. It was initially termed the "arginine rich apolipoprotein" and was first identified as a constituent of VLDL by Shore and Shore (1973). Animals fed a high level of fat and cholesterol accumulate cholesterol enriched, apoE containing lipoproteins in their plasma. These include chylomicron remnants and VLDL remnants (IDL), referred to collectively as ß-VLDL and an HDL subclass referred to as HDL_1 or HDL_c (Mahley., 1981). ApoE enriched ß-VLDL accumulate in the plasma of individuals with type III hyperlipidaemia (Havel and Kane., 1973). This disorder is usually associated with the presence of homozygosity for E2 variant of apoE, which binds poorly to the

hepatic remnant receptors relative to the more common E3 and E4 variants (Weisgraber *et al* ., 1981).

The apoE gene is present on chromosome 19 (Olaisen *et al*., 1982; Das *et al*., 1985), together with apoCI (Tata *et al*., 1985) and apoCII (Jackson *et al*., 1984). The gene is 3.7 Kb in length with four exons (Das *et al*., 1985; Paik *et al*., 1985). The primary translation product consists of 317 amino acids, with an 18 residue amino terminal signal peptide. The mature protein contains 299 amino acids, with an Mr of 34,200.

As mentioned above, the liver is the most important source of apoE, where it is secreted by hepatocytes primarily as a component of VLDL. Mouse peritoneal macrophages have also been found to release apoE when loaded with cholesterol (Basu *et al*., 1981; Basu *et al*., 1983). The apoE is released as an apoE-phospholipid disc, which can subsequently combine with HDL and hence redistribute cholesterol to cells expressing the LDL receptor. ApoE is also a major apolipoprotein in cerebrospinal fluid (which lacks apoB and LDL) and is probably responsible for CSF lipid transport. Tissue culture experiments have demonstrated that it is the astrocyte which secretes apoE (Pitas *et al*., 1987).

The secondary structure of apoE has been predicted using the Chou-Fasman algorithm (Chou and Fasman., 1978). The alpha-helical content of human apoE has been determined experimentally to be about 65% (Roth *et al*., 1977). As with apoB, amphipathic alpha-helical domains, with polar residues on one face and apolar residues on the other,

have been postulated to be important in lipid binding (Segrest *et al.*, 1973). The protein has been shown by physicochemical studies to contain two distinct structural domains. Whilst the amino-terminal (192) residues have the features of a compact globular protein, the carboxy-terminal (216-219) residues, form mostly amphipathic helices like a typical lipoprotein. It appears that the ability of apoE to self associate into tetramers resides in this carboxy terminal domain (Aggerbeck *et al.*, 1988).

The receptor binding domain of apoE has been mapped by a variety of strategies to residues (140-160) in the globular domain (Innerarity *et al.*, 1983; Weisgraber *et al.*, 1983). The receptor binding domain is rich in basic amino acids, with doublets and triplets of arginine and lysine residues. Substitutions with neutral amino acids at certain of these residues reduces receptor binding and can cause type III hyperlipidaemia. The most common substitution of this kind is found in the apoE2 variant mentioned above, where arginine-158 is replaced by cysteine. The receptor binding of apoE may represent an ionic interaction between basic residues in this region and acidic residues in the ligand binding domain of the LDL receptor (Brown and Goldstein., 1986).

In addition to it's lipid transport role, other functions of apoE are emerging. ApoE may have an immunoregulatory role; apoE containing lipoproteins can lead to both stimulation and suppression of lymphocytes. ApoE is also known to bind to heparin like molecules and may hence modify the extracellular matrix, altering cell-matrix or growth

factor-matrix interactions and thereby modulating the mitogenic activity of neurons and smooth muscle cells (Mayley., 1988).

(c) The A apolipoproteins.

(i) Apolipoprotein A1.

Human apoA1 circulates in the plasma as a component of HDL and to a lesser extent, chylomicrons. It is not found in significant amounts in chylomicron remnants, VLDL, IDL or LDL. The two major sources of apoA1 are the liver and the intestine. Hepatic apoA1 enters the circulation in association with nascent HDL particles with little cholesterol core. The intestinal form is associated with chylomicrons, but transfers to HDL following the action of lipoprotein lipase. It has a plasma concentration of 100-150 mg/dl (Assmann., 1982) and a half life of about 4 days (Fidge *et al.*, 1980).

ApoA1 has been sequenced by Brewer et al (1978). It is a single polypeptide containing 243 amino acids with a calculated Mr of 28,100. The ApoA1 mRNA codes for a translation product with an 18 amino acid signal peptide which is cleaved co-translationally and a 6 amino acid propeptide. Newly secreted apoA1 is a 249 amino acid proprotein which is processed slowly to the mature protein (Edelstein *et al.*, 1983).

A large percentage of the apoA1 protein is composed of tandemly repeated 22 amino acid segments, interrupted by proline residues and predicted to be amphipathic, alpha-helical in structure (Baker *et al.*,1974). As with apoB and apoE, such regions are implicated as crucial for lipid

binding. Nakagawa *et al.* (1985), proposed that 44 residue peptides, composed of two 22 residue peptides, punctuated by a helix-breaking proline, forms a crucial structural unit with a concave hydrophobic surface. The concavity of the proline containing segment matches closely the curvature of the surface of HDL3 (diameter, 40-50 Angstroms) and thus ideally suited for absorption to the HDL surface. Such 22 residue periodicity has since been found been found in other apolipoproteins, including apolipoproteins AII, AIV, CII, CIII and E; and an 11 residue repeat has been found in apoCI (Wen-Hsiung *et al.*, 1988).

In addition to it's structural role in maintaining HDL integrity, apoA1 serves as a co-factor for LCAT (Fielding *et al.*, 1972). The protein's amphipathic helical regions seem likely to be involved in this activation. Synthetic apoA1 peptides containing amphipathic repeats can activate LCAT (Sparrow and Gotto., 1982) and genetic variants unable to activate LCAT are predicted to perturb the structure of the amphipathic regions (Rall *et al.*, 1983). Homozygous apoAI deficiency occurs at a frequency of one per million in the population. Such individuals have very low HDL levels, premature vascular disease and corneal opacity. Obligate heterozygotes have half normal levels of HDL (Norum *et al.*, 1982; Schaefer *et al.*, 1981).

Three distinct classes of mutation have been described (Breslow., 1989). In type I, both apoAI and apoCIII are deficient (Karathanasis *et al.*, 1983 (a)). The defect results from gene rearrangement on chromosome 11, for instance a 6.5 Kb insertion (Karathanasis *et al.*, 1983(b))

or a 6.0Kb inversion (Karathanasis *et al.*, 1987).

In type II, there is deficiency of apoAI, apoCIII and apoAIV, due to deletion of the entire locus (Ordovas *et al.*, 1988). In type III, apoA1 only, is deficient, due to a small insertion in the apoA1 gene, which shifts the reading-frame and causes premature termination (Breslow., 1989).

ApoA1 Milano is due to an autosomal dominant mutation found in individuals from a small Italian village. Affected individuals have 33% of normal cholesterol levels and 60% of normal apoAI levels, but do not suffer from an increased risk of vascular disease (Franceschini *et al.*, 1980). The mutation responsible for the variant is an cysteine to arginine substitution at residue 173, leading to homo- and hetero-dimeric apoAI containing particles (Weisgraber *et al.*, 1980,1983; Roma *et al.*, 1988).

(ii) Apolipoprotein AII.

In man, apoAII is the second most abundant protein in HDL. It's major site of synthesis is the liver. It has a plasma concentration of 30-40 mg/dl (Assmann., 1982) and a half life of about 4 days (Fidge *et al.*, 1980).

In addition to a structural role in HDL, apoAII, either isolated, or when present in HDL, appears to activate hepatic lipase in a temperature and pH dependent manner, with maximal activation at pH 7.5 and 37 degrees centigrade (Jahn *et al.*, 1983).

In man, apoAII is a dimer of identical, 77 amino acid subunits linked by a disulphide bridge between the cysteine-6 residues (Brewer., *et al.*, 1972). Studies on

apoAII biosynthesis in the hepatocellullar carcinoma line, Hep G2 and intestinal epithelium shows that the protein is synthesised in a prepro form with an 18 amino acid signal peptide and a 5 amino acid pro segment which is partially cleaved on secretion into the medium (Gordon *et al.*, 1983). The apoAI gene consists of 4 exons and 3 introns and is 1337 base pairs in length (Tsao *et al.*, 1985).

Like other apolipoproteins, apoAII has a high degree of ordered secondary structure with amphipathic, alpha-helical domains (Sparrow and Gotto., 1982).

There is evidence that variations in the apoAII gene can influence serum apoAII levels and HDL structure. In particular, homozygosity for an *Msp* 1 polymorphism in the apoAII gene is associated with a significant increase in apoAII levels and a significantly lower apoAI/apoAII ratio (Scott *et al.*, 1985).

(iii) Apolipoprotein AIV.

In man, apoAIV is a major component of newly secreted chylomicrons, but only a minor component of HDL and is not found in significant amounts in chylomicron remnants, VLDL and LDL. Unlike most other apolipoproteins, apoAIV is also found in a lipoprotein free form in plasma. ApoAIV mRNA in the rat is abundant in both the liver and intestine, but is abundant only in the small intestine in man, with only trace expression in other tissues (Elshourbagy *et al.*, 1986). Its total serum concentration is about 15 mg/dl (Assmann., 1982).

The apoAIV gene is located on chromosome 11, about 14 Kb downstream of the apoAI gene in the same orientation,

with the apoCIII gene located between them in the opposite orientation (Elshourbagy *et al.*, 1986). The close linkage of the three genes raises the possibility of a common control mechanism over their expression.

The mature plasma apoAIV contains 376 residues, consisting throughout it's length of multiple tandem, 22 residue repeats with alpha-helical, amphipathic potential (Elshourbagy *et al.*, 1986). The overall metabolic role is unknown, although Steinmetz *et al* (1986) showed that like apoAI, apoAIV can activate LCAT in vitro. The two apolipoproteins however, appear to have differing activities in this respect, depending on the nature of the hydrocarbon side chains of the sn-α-phosphatidylcholine acyl donor (Steinmetz *et al.*, 1986).

(d) The C apolipoproteins.

Apolipoproteins CI, CII and CIII are low molecular weight (6,600-8,800), exchangeable and have diverse functions. In the fasting state, the C apolipoproteins are mostly HDL associated, but following the absorption of dietary fat or the synthesis of VLDL by the liver, they redistribute to the surface of chylomicrons and VLDL. When the triglyceride core of these lipoprotein particles is hydrolysed by lipoprotein lipase, the C apolipoproteins, along with other excess surface components (phospholipid, unesterified cholesterol and other exchangeable apolipoproteins) transfer back to HDL.

(i) Apolipoprotein CI.

The smallest of the C apolipoproteins is apoCI, which contains 57 amino acids, has a calculated Mr of 6,605 and

a plasma concentration of 60 mg/L (Curry *et al.*, 1982). The protein sequence has been obtained directly (Schulman *et al.*, 1975; Jackson *et al.*, 1974) and confirmed by sequencing a cDNA clone (Knott *et al.*, 1984). A 26 residue pre segment is co-translationally cleaved but there is no propeptide (Knott *et al.*, 1984). The Chou-Fasman algorithm (Chou and Fasman., 1978) predicts that the protein has a high helical content and a significant proportion of potentially lipid binding, amphipathic alpha-helical regions (Segrest *et al.*, 1974).

ApoCI has been found to activate LCAT (Soutar *et al.*, 1975) and may thus be involved in the esterification of free cholesterol transferred from chylomicrons and VLDL to HDL following lipolysis.

(ii)Apolipoprotein CII.

Apolipoprotein CII is a 79 amino acid peptide with a molecular weight of 8916. The primary amino acid sequence of apoCII has been determined (Jackson *et al.*, 1977; Hospattankar *et al.*, 1983). The apoCII gene has been mapped to chromosome 19 (Jackson *et al.*, 1983). The protein is synthesised with a co-translationally cleaved 22 amino acid prepeptide, but does not appear to have a propeptide (Sharpe *et al.*, 1984). Chou-Fasman analysis (Chou and Fasman., 1978) predicts the presence of three sequential alpha-helical regions which are potentially lipid binding (Mantulin *et al.*, 1980). Some beta-sheet and beta-turn structure is also predicted.

Apolipoprotein CII is present in the plasma at a concentration of 3-5 mg/dl (Nestel and Fidge., 1982). It plays

an important role in lipoprotein metabolism as an activator of lipoprotein lipase (LaRosa *et al.*, 1970; Havel *et al.*, 1970). Patients with apoCII deficiency develop severe hypertriglyceridaemia and have impaired plasma clearance of chylomicrons and VLDL. The clinical picture resembles that of lipoprotein lipase deficiency, but is corrected transiently by the infusion of normal plasma or purified apoCII (Breckenridge *et al.*, 1978). The protein has also been shown to activate LCAT, though less effectively than apolipoproteins AI, CI or CIII (Jonas *et al.*, 1984).

(iii) Apolipoprotein CIII.

Apolipoprotein CIII is present in the plasma at a concentration of 10-15 mg/dl (Nestel and Fidge., 1982). It consists of a 79 amino acid peptide with a calculated Mr of 8751. Residue 74 is a threonine, to which a carbohydrate side chain is attached, containing 1 mole each of galactosamine and galactose and 0, 1 or 2 moles of sialic acid per mole of protein (Brewer *et al.*, 1974). This gives rise to three forms of the protein referred to as CIII-0, CIII-1 and CIII-2, to indicate the number of sialic acid present.

Isoelectric focusing in the presence of 6M urea gives Pi values for these subtypes ranging from 4.7 to 5.1 (Marcel *et al.*, 1979). The sequence of a full length cDNA clone indicates that the protein is synthesised with a 20 amino acid prepeptide, but no propeptide (Sharpe *et al.*, 1984). Chou-Fasman analysis (Chou and Fasman., 1978) suggest the presence of amphipathic helical regions which serve as

lipid binding domains (Sparrow and Gotto., 1982). ApoCIII has been shown to activate LCAT, more effectively than apoCII and apoAII, but less so than apoA1 and apoCI with apoCIII-1 being more active than apoCIII-2 (Jonas *et al.*, 1984).

Excess apoCIII will inhibit apoCII activation of lipoprotein lipase (Brown and Baginsky., 1972) but the specificity of this action is not clear. ApoCIII will also inhibit the uptake by rat liver of synthetic triglyceride emulsions and rat lymph chylomicrons (Shelburne *et al.*, 1980). Jahn *et al* (1983) showed that apoCIII, either isolated or when combined with HDL, inhibits hepatic lipase, in contrast to apoAII which has a stimulatory effect.

As mentioned above in discussion of apoAI, a combined deficiency of apoAI and apoCIII occurs due to a gene rearrangement on chromosome 11, that causes premature development of atherosclerosis (Noram *et al.*, 1982; Karathansis *et al.*, 1983 (a); 1983 (b); 1987).

(e) Apolipoprotein D.

ApoD is a glycoprotein with an apparent Mr of 33,000 present in HDL. It appears to form a cholesterol ester transfer complex with LCAT in whole plasma that synthesises and distributes cholesterol ester between lipoprotein particles (Fielding and Fielding., 1980), although there conflicting evidence about the need for it's presence for this activity (Albers *et al.*, 1981).

ApoD is 169 amino acid protein, predicted by Chou-Fasman analysis (Chou and Fasman., 1978) to have an alpha-helical content of less than 5%. This is in contrast to

the substantial amounts of alpha-helix present in the other apolipoproteins.

The nucleic acid sequence of apoD cDNA and it's predicted amino acid sequence has been compared to other known gene and protein sequences in the GenBank-TM and Dayhoff sequence data bases (Drayna *et al.*, 1986). No significant amino acid homology with apolipoproteins of the A, B, C and E group is apparent. The protein does however display a high degree of homology with members of the α-2-μ-globulin superfamily, including human retinol-binding protein, human α-1-microglobulin, ungulate ß-microglobulin, rodent α-2-μ-globulin and tobacco hornworm insecticyanin.

The tissue distribution of apoD mRNA is also distinct from most other apolipoproteins, being more abundant in adrenal, kidney, pancreas, small intestine and placenta than the liver (Drayna *et al.*, 1986).

(f) Apolipoprotein(a).

Apo(a) is a glycoprotein with a molecular weight ranging from 300 kDa to 700 kDa. It is found in an LDL-like particle known as lipoprotein(a) (Lp(a)) where it is linked to apoB100 via a disulphide bridge.

Lp(a) was first described by Berg (1963), as an antigen present in about 30% of a Northern European population. Further studies have shown that all plasma contains Lp(a), but that the concentration range is very wide, from less than 1, to greater than 100 mg/dl (Groener and Kostner., 1987). It is also clear that there is a correlation between high Lp(a) levels and atherosclerosis (Albers *et*

al., 1977). In a study of 307 white patients undergoing coronary angiography, Dahlen *et al* (1986), found an apparent threshold for coronary risk occurring at 30-40 mg/dl. Because in other respects, Lp(a) resembles LDL, it is probable that it's postulated atherogenicity is due to the apo(a) component.

Partial amino acid sequencing of apo(a) (Eaton *et al.*, 1987), followed by cloning and sequencing of apo(a) cDNA (McLean *et al.*, 1987), shows that the protein has an extreme similarity to plasminogen and a high degree of internal repetition.

At the 5'ends of the apo(a) and plasminogen cDNA's, the first 93 base pairs, comprising 45 base pairs of 5' untranslated sequence and 16 codons of signal peptide, show near identity. Thereafter, the sequences abruptly diverge. Plasminogen has an amino terminal tail and 5 "kringle" domains, followed by a serine protease domain. This contains an arginine which is cleaved in the mature protein by tissue-type and urokinase plasminogen activators, to produce active plasmin. In contrast, the apo(a) sequence continues with 3 final residues of signal peptide and a mature amino terminus that is similar to kringle 4 of plasminogen, followed by a 37-fold multiplication of the exons encoding a kringle 4-like domain, a non repeated kringle 5-like domain and a serine protease domain. However, the arginine which is cleaved in plasminogen to produce plasmin activity is substituted by serine in apo(a). Attempts to activate Lp(a) with tissue plasminogen activator (t-PA), urokinase or streptokinase, have not

been successful (McLean *et al.*, 1987). Another potential similarity to plasminogen, that of fibrin binding, has been shown to be only a very weak property of apo(a) (McLean *et al.*, 1987). The linkage between apoB100 and apo(a) is likely to be via a free cysteine residue in kringle repeat number 36 (McLean *et al.*,1987).

A significant effect of apo(a) may be to interfere with lipoprotein uptake by one or more of the receptors which bind LDL, modified LDL or other lipoprotein particles. Armstrong *et al* (1985), have shown that removal of apo(a) from Lp(a) particles, greatly enhances their affinity for the LDL receptor of cultured human fibroblasts.

Recently, Miles *et al* (1989) demonstrated that Lp(a) inhibits the binding of ^{125}I-labelled plasminogen to human umbilical vein cells (HUVEC) and human monocyte-like U937 cells. Hajar *et al* (1989) showed that, in addition to inhibiting plasminogen binding to HUVEC, Lp(a) can inhibit plasmin generation by tissue plasminogen activator (t-PA) at the cell surface. They also demonstrated by immunohistochemical studies, the presence of Lp(a) in the endothelium and intimal subendothelium of atherosclerotic human coronary arteries derived from autopsy specimens. It is suggested that physiological levels of Lp(a) may serve to control the otherwise unopposed generation of plasmin at the endothelial surface resulting from the action of t-PA on bound plasminogen. In contrast however, elevated Lp(a) levels would over-suppress the cell's fibrinolytic mechanism, resulting in a prothrombotic tendency. The presence of large amounts of Lp(a) within

coronary lesions may thus lead to impaired fibrinolysis and hence progressive atherosclerosis.

1.4 The lipoprotein-processing enzymes.

Three enzymes play a key role in lipoprotein metabolism, lipoprotein lipase, hepatic lipase and lecithin-cholesterol acyltransferase (LCAT).

(a) Lipoprotein lipase and hepatic lipase.

Lipoprotein and hepatic lipases are members of a multigene family that also includes pancreatic lipase, although there are more homologies between lipoprotein and hepatic lipases than between either of them and pancreatic lipase. Both lipoprotein lipase and hepatic lipase are inhibited by apoCI and apoCIII and lipoprotein lipase, but not hepatic lipase, requires apoCII as a cofactor (Augustin *et al.*, 1978). In man, the lipoprotein lipase gene is about 30 kb in length with 10 exons, whilst the hepatic lipase gene spans over 60 kb with 9 exons (Olivecrona and Bengtsson-Olivecrona., 1990).

Lipoprotein lipase is synthesised by a variety of tissues, but predominately in adipose tissue and striated muscle, where, following secretion, it is bound to heparin sulphate on the endothelial surface of capillaries. It is required for the efficient hydrolysis of triglycerides in chylomicrons and VLDL. In the postprandial state, it's activity in adipose tissue is induced by insulin and in skeletal and cardiac muscle, it's activity remains increased under catabolic conditions (Havel and Kane., 1989).

The human cDNA for lipoprotein lipase codes for a mature

protein of 448 amino acids, with a molecular weight of 50,394 before and about 60,000 after glycosylation. (Wion *et al.*, 1987). Homozygous lipoprotein lipase deficiency occurs at an incidence of about 1 per million, and is characterised by marked fasting chylomicronaemia, eruptive xanthomata, hepatosplenomegaly and recurrent pancreatitis (Olivecrona and Bengtsson-Olivecrona., 1990). Heterozygous deficiency has been estimated at 1 in 500 (Brunzell, J.D., 1989). It can give rise to a hyperlipidaemic state, or may have no detectable effect, suggesting that some individuals can handle triglyceride transport adequately with only half-normal amounts of the enzyme (Olivecrona and Bengtsson-Olivecrona).

Hepatic lipase is synthesised by hepatocytes and has an aminoacyl chain molecular weight of 53,394 (Havel and Kane., 1989). It is located primarily on the hepatic sinusoidal surfaces, but it's activity has also been detected in other tissues, including the adrenal and ovary (Komaromy and Schotz., 1987). The presumed function of the enzyme is the hydrolysis of triglyceride in IDL and triglyceride and phospholipid in HDL_2 to produce HDL_3 and it may also have some action on VLDL and chylomicron remnants (Komaromy and Cooper., 1987). The activity of the enzyme is increased by androgens and decreased by oestrogens (Havel and Kane., 1989).

Hepatic lipase deficiency in man has been reported (Breckenridge *et al.*, 1982; Carlson *et al.*, 1986) but is very rare. It gives rise to a syndrome whereby triglyceride accumulates in HDL (hyperalphatriglyceridaemia). As a

result, the HDL is almost exclusively of a size corresponding to the HDL_2 density fraction, supporting the hypothesis that a primary function of hepatic lipase is associated with the degradation of plasma HDL_2 (Carlson *et al.*, 1986). Other abnormalities include triglyceride enrichment of LDL and an abnormally cholesterol-rich VLDL with retarded electrophoretic mobility (ß-VLDL). A kindred has been recently reported by Auwerx *et al* (1990) in which a deficiency hepatic lipase is coexistent with mild lipoprotein lipase deficiency. The result of this compound heterozygosity is a syndrome with the lipoprotein characteristics of hepatic lipase deficiency superimposed on a variable degree of hypertriglyceridaemia characteristic of heterozygous lipoprotein lipase deficiency.

(b) Lecithin:cholesterol acyltransferase.

Lecithin:cholesterol acyltransferase (LCAT) is secreted by hepatocytes into the blood where it acts on species of HDL which contain apoD, to esterify cholesterol with a fatty acyl residue of lecithin. The esters produced are transferred, via cholesteryl ester transfer protein (CETP) to LDL and to a certain extent, VLDL and HDL, and in this way, cholesterol is returned from the extrahepatic tissues to the liver (Havel and Kane., 1989).

1.5 The Lipoprotein Receptors.

(a) The LDL Receptor.

The LDL receptor is a cell surface glycoprotein, capable of binding two different apolipoproteins, apoB100 and apoE (Goldstein and Brown., 1986). It's affinity for lipoproteins containing multiple copies of apoE (IDL) is actually

up to 20 fold higher than that for LDL itself, which contains only one copy of apoB (Innerarity and Mayley., 1978).

Sequence data has been obtained from a full length cDNA clone of the human LDL receptor (Russell *et al.*, 1983; Yamamoto *et al.*, 1984). The amino terminus of the LDL receptor consists of a 21 amino acid hydrophobic signal sequence that directs the protein to the endoplasmic reticulum and is cleaved immediately following translation. The resultant 839 amino acid protein is subsequently combined with sugar chains by both N and O linkages and can be divided into five distinct domains.

The first domain of the LDL receptor consists of the amino terminal 292 amino acids containing seven 40 amino acid repeat units, each containing 6 cysteine residues involved in disulphide bridge formation. Each repeat has a number of negatively charged amino acids near it's carboxy terminal, which are complementary to positively charged residues on the putative receptor binding domains of apoE (Mayley and Innerarity., 1983; Innerarity *et al.*, 1984) and of apoB (Knott *et al.*, 1986; Yang *et al.*, 1986). Studies by van Driel *et al* (1987), suggest that the first of these repeats binds calcium and that calcium binding to the other repeats is involved in the lipoprotein receptor function.

The second domain consists of approximately 400 amino acids, with 35% homology to part of the extracellular domain of the EGF precursor, a carboxy terminal part of which gives rise to the 53 amino acid peptide EGF.

The third domain of the LDL receptor is immediately external to the cell membrane and contains 58 amino acids, 18 of which are serine or threonine residues which bear the O-linked sugar chains. The forth domain is a stretch of 22 membrane spanning hydrophobic amino acids and the fifth, a cytoplasmic tail, containing 50 amino acids which are highly conserved among species (Goldstein and Brown., 1986).

The LDL Receptor pathway.

Following their synthesis, LDL receptors appear on the cell surface after about 45 minutes where they interact, via an adaptor protein, with a protein known as clathrin and cluster together to form clathrin coated pits. Within 3-5 minutes, the coated pits invaginate, forming coated vesicles which rapidly loose their clathrin coat and fuse to form larger sacs known as endosomes. The internal pH of the endosome subsequently falls to below 6.5 as a result of the operation of an ATP driven proton pump and at this pH, any bound LDL dissociates from it's receptor (Brown and Goldstein., 1986). The free receptors then apparently cluster and the sections of membrane containing them pinch off to form recycling vesicles which return to the cell surface. The freed LDL is delivered to lysosomes by membrane fusion with the remainder of the endosome. Here the apoB of the LDL is hydrolysed into amino acids and the cholesterol esters are hydrolysed to cholesterol by an acid lipase. Each LDL receptor cycles around in this fashion once every 10 minutes, performing several hundred trips in it's 20 hour lifespan. The cholesterol

which is delivered to the cell interior as a result, has several effects on cholesterol metabolism. It suppresses transcription of the rate limiting enzyme 3-hydroxy,3-methyl, glutaryl coenzyme A reductase. (HMGCoA reductase). (Luskey *et al.*, 1983) and accelerates the enzyme's degradation (Gil *et al.*, 1985). It also activates a cholesterol esterifying enzyme, acylCoA-cholesterol acyltransferase (ACAT), which serves to store excess cholesterol in the cytoplasm as cholesterol ester droplets (Goldstein *et al.*, 1974). In addition, it suppresses further LDL receptor synthesis, by reducing receptor mRNA concentrations (Brown and Goldstein., 1975; Russell *et al.*, 1983). This latter action acts as a negative feedback mechanism, preventing excessive cholesterol accumulation within cells (Goldstein and Brown., 1977).

One in 500 persons has a mutation within one of their LDL receptor genes which gives rise to a defective receptor and the clinical syndrome of heterozygous familial hypercholesterolaemia. Because of the reduction in their LDL receptor function, such individuals have an accumulation of LDL particles in their plasma. The result is an acceleration of the atherosclerotic process, with myocardial infarction at 30-40 years of age. Homozygotes, or compound heterozygotes, for the disorder, number about 1 per million persons and have considerably accelerated atherosclerosis, often having myocardial infarcts in childhood.

(b) <u>LDL-Receptor related protein (LRP).</u>

Although the LDL receptor quite clearly plays a central role in lipoprotein metabolism, it cannot be the only

receptor with specificity for apoE, since the levels of apoE containing lipoproteins (chylomicron remnants, IDL and HDL_c) are not elevated in animals or patients with defective LDL receptors (Kita, T. *et al.*, 1982; Brown and Goldstein., 1983). Recently Herz *et al* (1988) have reported the isolation and sequencing of another receptor with apoE affinity. It is an abundant liver protein of 503 kd that has close structural and biochemical similarities to the LDL receptor. The molecule contains 4544 amino acids and apparently has four functional regions: i) an extracellular domain resembling four copies of the ligand binding and the EGF-precursor homologous region of the LDL-receptor ii) a region of growth factor repeats, similar to that found in EGF iii) a membrane-spanning segment and iv) a 100 amino acid cytoplasmic tail with two copies of possible signal for clustering into coated pits. The extracellular domain contains cysteine motifs with a net negative charge of 158, strongly suggestive of an ability to bind positively-charged ligands such as apoB or apoE. A further similarity to the LDL receptor is the finding that like the latter, LRP also binds calcium, indicating that it may interact with apoE in a similar fashion. Herz *et al* (1988) suggest that the LRP may be responsible for binding apoE-containing chylomicron remnant and HDL_c particles and that certain conditions in which apoE-containing lipoproteins are elevated might be caused by defects in protein.

1.6 Abetalipoproteinaemia

Abetalipoproteinaemia is a rare, autosomal recessive disorder, first described by Bassen and Kornzweig (1950). The patient that they described was an 18 year old Jewish girl with atypical retinitis pigmentosa, malformed erythrocytes and a form of ataxic neuropathy, with loss of deep tendon reflexes. During childhood, the patient was thought to have coeliac disease, based on the presence of chronic diarrhoea. The patient's brother also had malformed erythrocytes. The parents were consanguineous with no abnormal findings, suggestive of recessive inheritance of a rare allele. Singer *et al* (1952) subsequently reported a similar case, but without retinitis pigmentosa. Jampel and Falls (1958) reported a follow-up examination of the same patient, 6 years later, noting a progression of ataxia and the appearance of retinitis pigmentosa. They also noted that the patient had a very low total cholesterol value. Later, patients with similar clinical history were found to have an almost complete absence of ß-lipoprotein (Salt *et al.*, 1960; Mabry *et al.*, 1960).

(a) Plasma Lipoproteins.

The deficiency in abetalipoproteinaemia is in those plasma lipoproteins that contain apoB. There does appear to be some lipoprotein material in the ultracentrifugation density interval in which chylomicrons, VLDL, IDL and LDL are found. Attempts to detect circulating apoB by sensitive immunochemical methods have been without success (Kostner *et al.*, 1974; Scanu *et al.*, 1974), but immunoblotting studies with monoclonal antibodies has revealed

the presence of trace amounts of apoB100 in some patients (Gregg *et al.*, 1988; Scanu *et al.*, 1990). The defect is presumed to be one of the synthesis of apoB, or the assembly of apoB containing lipoproteins. Plasma triglycerides fail to rise after a fatty meal. Some exogenous polyunsaturated fatty acids are apparently absorbed, but the concentrations present in both plasma and adipose tissue are decreased (Barnard *et al.*, 1966). A large proportion of cases are said to result from consanguine matings. Obligate heterozygotes for classic abetalipoproteinaemia are both clinically and biochemically normal.

Examination of the lipoproteins of the $d<1.006$ g/ml and $1.006<d<1.063$ g/ml ultracentrifugation density fractions, shows that apoAI apoAII and apoE are all present (Scanu *et al.*, 1974). In the LDL density fraction ($1.019<d<1.063$ g/ml), there is a decreased ratio of lecithin to sphingomyelin (almost 1, compared to a normal ratio of 2) and the amount of unesterified cholesterol is increased from 1/3 to 1/2 of the total (Scanu *et al.*, 1974).

Almost all the plasma cholesterol in abetalipoproteinaemia is carried in HDL. As with the lower density lipoproteins, the HDL has an abnormally low ratio of lecithin to sphingomyelin (5:4, compared to a normal ratio of 8:1) and there is an increase in the ratio of unesterified to esterified cholesterol (0.7, compared to 0.3).

Decklebaum *et al* (1982), has investigated the HDL subfractions in abetalipoproteinaemia and found that HDL_2 is increased from the 10% normally found, to 65% of the total HDL mass. Both the HDL_2 and the HDL_3 particles

contained 2-2.5 fold more cholesterol ester than normal. Abetalipoproteinaemic HDL could be modified towards normal HDL by allowing VLDL triglyceride to exchange with the cholesterol ester contained within it, in the presence of lipoprotein lipase. It is suggested that it is the lack of cholesterol ester transfer to such triglyceride rich lipoproteins in abetalipoproteinaemia, which results in the accumulation of large, cholesterol ester rich HDL.

Apolipoproteins AI and AII within abetalipoproteinaemic HDL, are immunochemically and electrophoretically normal. (Kostner *et al.*, 1974; Scanu *et al.*, 1974). The C apolipoproteins in abetalipoproteinaemia have the usual amino acid composition, but the monosialated apoCIII$_1$, usually associated with VLDL, is absent (Gotto *et al.*, 1971; Scanu *et al.*, 1974). There is apparently an oversialylation process favouring the formation of apoCIII$_1$, containing 2 mol of sialic acid per mol of protein (Scanu *et al.*, 1974).

ApoE is present in greater than normal amounts in abetalipoproteinaemia, much of it in mixed disulphide form with apoAII (Herbert *et al.*, 1980). It may be that this reflects a role for HDL in substituting to some extent for LDL in cholesterol delivery to peripheral tissues, since apoE binds with high affinity to the LDL receptor. Blum *et al* (1982) have performed detailed studies of apoE containing lipoproteins in abetalipoproteinaemia, in an attempt to resolve the apparent paradox of absent LDL in the absence of a derepressed LDL receptor pathway. According to the Brown and Goldstein principle of feedback

regulation of cholesterol biosynthesis by the LDL receptor pathway, abetalipoproteinaemia should be accompanied by rapid rates of cholesterol biosynthesis, high concentrations of LDL receptors on cell surfaces, high levels of HMGCoA reductase and low levels of ACAT. Several laboratories have presented data however, to indicate that the LDL receptor pathway is not derepressed and cholesterol biosynthesis not excessive (Myant *et al.*, 1978; Reichl *et al.*, 1978; DeWitt *et al.*, 1983). Where increased total body cholesterol biosynthesis has been reported, by sterol balance studies (Illingworth *et al.*, 1980 (a)), or urinary mevalonic acid excretion (Illingworth *et al.*, 1989), they can largely be explained on the basis of reduced dietary cholesterol absorption.

Blum *et al* (1982), studied HDL subfractions from nine patients with abetalipoproteinaemia, with respect to their ability to compete with ^{125}I-LDL for to specific binding to human fibroblast LDL receptors. They showed that an apoE rich subfraction of HDL, present in the patients, had 10-25 times the binding potency per mg of protein than normal LDL. The apoE rich, HDL_2 was found to be a potent inhibitor of HMGCoA reductase in the fibroblasts. It is suggested that apoE rich HDL in abetalipoproteinaemia has the ability to deliver cholesterol to tissues via the LDL receptor pathway, producing the normal cholesterol biosynthesis and LDL receptor activity, previously reported. These findings are supported by Alam *et al* (1983), who demonstrated inhibition of cholesterol biosynthesis in cultured human fibroblasts by abetalipoproteinaemic HDL_2.

In addition, Illingworth *et al* (1983 (a)), showed that abetalipoproteinaemic HDL_2 will down regulate human fibroblast LDL receptor activity. Further, Hagemenas and Illingworth (1987), demonstrated that abetalipoproteinaemic HDL_2 can provide sufficient cholesterol to cells so that cholesterol esterification is stimulated.

A further abnormality of lipoprotein metabolism observed in abetalipoproteinaemia, is a consistently reduced activity of hepatic lipase and lipoprotein lipase (Illingworth *et al.*, 1983 (b)). It may be that the intravascular presence of triglyceride rich lipoproteins is required to up-regulate these enzymes on capillary endothelial cells and hepatocytes. The inability of subjects with abetalipoproteinaemia to form chylomicrons, appears to result in reduced levels of gastric inhibitory peptide (GIP), which is known to increase lipoprotein lipase activity. This reduction in GIP levels may partly contribute to the low lipoprotein lipase levels seen in abetalipoproteinaemia (Illingworth *et al.*, 1983 (b)).

LCAT activity has also been shown to be reduced by half in abetalipoproteinaemia (Cooper and Gulbrandsen., 1971). LCAT can also acylate lysolecithin (a by-product of the LCAT reaction), but this activity is greatly reduced in abetalipoproteinaemia. However, the addition of normal LDL will activate this reaction up to 22 fold (Subbaiah., 1982). Holmquist *et al* (1988), confirmed that LCAT activity is reduced by half in abetalipoproteinaemia and that this reduction involves α-LCAT(esterification of free cholesterol of HDL) and ß-LCAT (esterification of free

cholesterol of VLDL and LDL) activities. The authors point out however, that although LCAT activity is reduced in abetalipoproteinaemia, the secretion of triglyceride rich lipoproteins cannot be a prerequisite for LCAT secretion, since VLDL, LDL and chylomicrons are completely absent in the disorder.

Recent studies by several workers have shown that apo(a) is detectable in patients with abetalipoproteinaemia either as free apo(a) or as a complex with apoB (Gregg *et al.*, 1988; Holmquist *et al* ., 1989; Menzel *et al.*, 1990). The studies of Gregg *et al* (1988) and Holmquist *et al* (1989) indicate that apo(a) can be secreted independently of apoB containing lipoproteins, suggesting that it's appearance in LDL like particles of normal individuals may be secondary to it's secretion.

(b) Clinical and Pathophysiological Features of Abetalipoproteinaemia

(i) Gastrointestinal Effects.

Fat malabsorption in abetalipoproteinaemia is present from birth, with poor appetite, vomiting, abdominal distension and steathorroea. This has lead to erroneous diagnoses of coeliac disease. Dietary restriction of gluten however, fails to correct these clinical abnormalities. Restriction of dietary fat, combined with diminished intolerance to fatty food can lead to the disappearance of steathorroea in later life. The pathway for fat absorption however, does not involve chylomicron formation, with the passage of resynthesised triglyceride into the lymph. Indeed, chylomicrons are absent from the circulation, and

from jejunal biopsy specimens taken at any time after a fatty meal (Ways *et al.*, 1967). The histological appearance of intestinal villi is characteristic, mucosal cells, especially those near the villous tip, being extensively vacuolated (Dobbins., 1966; Weinstein *et al.*, 1973). Fat staining with oil red O, reveals that this vacuolation is due to lipid droplets, the lipid content being 1.5-3.5 times normal (Ways *et al.*, 1967). There is an increased number of lysosomes within the mucosal cells however (Dobbins., 1966) and it may be that some of the accumulating triglycerides are hydrolysed and subsequently transported in the portal venous system bound to albumin. This would account for the relative mildness of steathorreoa in the disease.

The failure of chylomicron formation in abetalipoproteinaemia leads to defective absorption of the fat soluble vitamins. Dietary vitamin A is hydrolysed to retinol before absorption, then reesterified, usually with palmitic acid in the mucosal cell, before being secreted in chylomicrons in intestinal lymph. Vitamin A levels in abetalipoproteinaemia are low and absorption is flat, but supplementation can produce levels within the normal range by as yet, unknown mechanisms (Havel and Kane., 1989).

Vitamin K malabsorption also occurs in abetalipoproteinaemia and hypoprothrominaemia, with abnormal haemostasis can result (Caballero and Buchanan., 1980).

The most significant vitamin deficiency is that of vitamin E. Abetalipoproteinaemia in fact, provides an ideal model to study the consequences of vitamin E deficiency in man,

since untreated, patients have undetectable serum concentrations (Muller *et al.*, 1983). Tocopherol absorption is greatest when fat is present, but will also occur in the absence of dietary lipid (McCormick *et al.*, 1960). Small chylomicron formation occurring in the absence of fat, probably accounts for this. Large oral loads of water miscible vitamin E in abetalipoproteinaemic individuals do not induce detectable levels in the following 24 hours (Muller *et al.*, 1974). Following absorption, it is likely that LDL is important in vitamin E transport, since intramuscular injection results in only relatively low blood levels. Administration of very large quantities of fat or water miscible preparations of the vitamin over prolonged periods however, will produce significant blood levels (Muller *et al.*, 1974)

The beneficial effects of vitamin E therapy occur despite an inability to achieve normal plasma vitamin E levels however (Muller and Lloyd., 1982). Bieri *et al* (1984), demonstrated normal hepatic stores in a treated patient, in spite of subnormal plasma levels. They suggest that HDL may provide sufficient tissue delivery of the vitamin. In addition Kayden *et al* (1983), showed that some abetalipoproteinaemic patients on massive supplementation with vitamin E, can achieve normal concentrations in their adipose tissue.

The liver can also become lipid laden and vacuolated and this has lead, in some instances to elevated transaminase levels and micronodular cirrhosis, although the latter may be a direct result medium chain triglyceride substitu-

tion in the diet (Partin *et al.*, 1974).

(ii) Neuromuscular Effects.

The major neurological manifestation of abetalipoproteinaemia is the appearance of a spinocerebellar disorder. Heavily myelinated sensory neurones, with cell bodies in the dorsal root ganglia appear to be selectively involved. As a result, the earliest neurological finding is the loss of stretch reflexes. Position and vibration sense are lost early and positive Romberg sign, characteristic of a sensory ataxia, can be elicited.

Other neurological pathways are less severely affected in abetalipoproteinaemia. Pyrimidal tract involvement is evidenced by the appearance of extensor plantar responses in some patients and the loss of anterior horn cells at post mortem (Sobrevilla *et al.*, 1964). The cerebellum may show some loss of nuclei (Sobrevilla *et al.*, 1964) or may be normal (Dische and Porro., 1970) and it is likely that cerebellar signs in abetalipoproteinaemia result more from an interruption of the spinocerebellar pathways, rather than significant intrinsic cerebellar degeneration. It is now generally considered that the neurological degenerative changes in abetalipoproteinaemia are secondary to the severe deficiency of vitamin E which occurs in the disorder. Several lines of evidence suggest that vitamin E is required for normal neurological function in man. Vitamin E deficiency has been linked with neurological pathology in other malabsorption states, including congenital biliary atresia (Rosenblum *et al.*, 1981),cystic fibrosis (Sung *et al.*, 1980) and short bowel syndrome (Howard *et*

al., 1982). The resemblance to the changes in abetalipoproteinaemia is striking. It is now clear however, that early therapy with vitamin E in abetalipoproteinaemia can prevent the development of neurological complications and in patients with established complications, may arrest or reverse the neuropathy (Muller *et al.*, 1970; Muller *et al.*, 1977; Illingworth *et al.*, 1980 (b); Muller and Lloyd., 1982).

The mode of action of vitamin E in the nervous system is not clear. It is clearly important in maintaining the integrity and stability of cell membranes. The vitamin interacts via it's phytyl side chains with polyunsaturated fatty acids, especially arachidonic acid, present in membrane phospholipids (Diplock and Lucy., 1972). With the vitamin E molecule anchored in this way, the reactive hydroxyl group on it's chromane ring may serve to scavenge highly reactive free radical groups and thus protect unsaturated membrane lipids from peroxidation. In addition, other molecules, such as sulphur or selenium containing non-haem-iron proteins, may be held in their correct oxidation state by the vitamin (Muller *et al.*, 1983).

Following on from the spinocerebellar degeneration and disuse in abetalipoproteinaemia, weakness and muscle atrophy inevitably occurs. Primary skeletal muscle disease may also occur. Kott *et al* (1977), reported the case of a 26 year male with abetalipoproteinaemia, who had, in addition to CNS lesions, a myopathy. Biopsy revealed the accumulation of ceroid pigment in striated muscle, with

myofibril degeneration in some muscle fibres. Myopathy in man can certainly result from vitamin E deficiency and be reversed by supplementation (Tomasi., 1979). Cardiac lesions have also been noted in abetalipoproteinaemia, with interstitial myocardial fibrosis and excessive deposition of lipochrome pigment in cardiac muscle (Dische and Porro., 1970)

(iii) Ocular Effects.

The other major complication of abetalipoproteinaemia is the development of retinitis pigmentosa. This is atypical, in that cones in addition to rods are destroyed, but is otherwise indistinguishable from commoner types of retinal pigmentary degeneration.

Vitamin A deficiency was originally considered to be an important factor in retinal degeneration in abetalipoproteinaemia. Sperling *et al* (1972), treated two abetalipoproteinaemic brothers with retinal complications, with intramuscular water soluble vitamin A and found that the electoretinogram response returned in the younger brother, but not the older, more severely affected brother. Others have found that vitamin A will not prevent the development of retinal degeneration (Wolf *et al.*, 1964; Muller *et al.*, 1977).

It is now considered that vitamin E deficiency is primarily responsible for the retinal, as well as the neurological degenerative changes in abetalipoproteinaemia. Vitamin E deficient rats show significant loss of photoreceptor cells and the accumulation of lipofuschin deposits in the retinal pigment epithelium and vitamin A deficiency

appears to accelerate the retinal degeneration. It may be that vitamin E deficiency results in the oxidation of both photoreceptor membranes and retinal vitamin A stores (Robinson *et al.*, 1979; Robinson *et al.*, 1980).

Vitamin E deficiency has also been linked to retinitis pigmentosa occurring in the short bowel syndrome, with improvement in visual fields following supplementation (Howard *et al.*, 1982). There is accumulating evidence to suggest that vitamin E supplementation with or without vitamin A and K can prevent the progression of retinal degeneration in abetalipoproteinaemia (Muller *et al.*,1977; Bishara *et al.*, 1982; Muller *et al.*, 1983; Runge *et al* ., 1986).

(iv) Haematological Effects.

Abetalipoproteinaemia is accompanied by the presence of a large population of abnormal erythrocytes in the peripheral blood. It is this finding that prompted the first case report and the first description of acanthocytes, derived from the Greek word for spine or thorn, akantha.

The acanthocytes in abetalipoproteinaemia are seen well in wet preparations, and best of all by scanning electron microscopy. The shape of the erythrocytes prevents rouleaux formation and results in a very low erythrocyte sedimentation rate.

The lipid composition of the red cell membranes reflects the abnormal composition of the plasma lipoproteins (Phillips., 1962; Ways *et al.*, 1963; Iida *et al.*, 1984). The cholesterol and phospholipid content are greater than normal, the ratio of sphingomyelin to lecithin is in-

creased from 0.9 to more than 1.4 and there are more saturated fatty acids contained within the sphingomyelins. The erythrocytes apparently acquire their shape as a result of an abnormal distribution of lipid between the two bilayer leaflets (Lange and Steck., 1984). Acanthocytes are not found in the bone marrow however, the membrane distortion being acquired in the peripheral circulation. Transfused normal cells will apparently undergo similar changes in shape in patients with abetalipoproteinaemia (Kayden., 1972).

Untreated, patients with abetalipoproteinaemia have red cells with increased autohaemolysis and increased sensitivity to peroxide induced haemolysis. Addition of tocopherol to in vitro incubation mixtures, corrects autohaemolysis (Kayden *et al.*, 1965) and dietary supplementation with water miscible preparations of vitamin E protects patient's acanthocytes from peroxide induced haemolysis (Dodge *et al.*, 1967). Most adult patients with abetalipoproteinaemia do not have significant anaemia however and that seen in children is probably secondary to other nutritional deficiencies.

(c) Diagnosis and Treatment of Abetalipoproteinaemia.

A presentation of malabsorption with retinitis pigmentosa, neuromuscular deficits, especially involving the posterior spinocerebellar pathways, is highly suggestive of abetalipoproteinaemia. The simultaneous presence of very low cholesterol and triglyceride levels and the absence of readily detectable apoB in the serum, establishes the diagnosis as highly likely.

Further support for the diagnosis is provided by the finding of lipid-laden enterocytes in a jejunal biopsy specimen. The establishment of clinically and biochemically normal obligate heterozygotes within the patient's family is required to confirm the diagnosis of classical abetalipoproteinaemia, in contrast to homozygous hypobetalipoproteinaemia (see below).

Early diagnosis of abetalipoproteinaemia may allow the prevention of early death and reduction of morbid consequences in later life. Restriction of triglyceride and long-chain fatty acids will relieve the gastrointestinal effects of the disorder in infancy and childhood.

It is now clear that the most important deficiency to correct is that of vitamin E, in order to limit retinal and neurological degeneration (Muller *et al.*, 1970; Azizi *et al.*, 1978; Illingworth *et al.*, 1980; Runge *et al.*, 1986). In addition vitamins A and K should be given, sufficient to maintain normal serum levels of retinol and a normal prothrombin time.

Although the beneficial effects of vitamin A and E replacement from an early age are clear, the effects on adult cases of abetalipoproteinaemia is less convincing. MacGilchrist *et al* (1988), for example, have treated two adult female cases for 8 and 10 years with vitamins A and E and linoleic acid. In one case, visual function improved initially, but deteriorated thereafter, whilst neuropathy progressed slowly. In the second case, visual function was unaffected and neuropathy slowed, but did not halt. These cases suggest that, once the disease is

established, vitamin supplementation does not prevent eventual progression of retinal and neurological damage, reinforcing the need for early implementation of therapy.

1.7 Hypobetalipoproteinaemia

In a homozygous form, hypobetalipoproteinaemia gives rise to clinical and biochemical features which may make it indistinguishable from abetalipoproteinaemia (Malloy and Kane., 1982). The distinction with abetalipoproteinaemia lies in the characteristics of the obligate heterozygote carriers. In heterozygous abetalipoproteinaemia, the clinical and biochemical features are essentially normal, whilst in heterozygous hypobetalipoproteinaemia, LDL cholesterol and apoB levels are usually well below the fifth percentile and individuals may show some of the clinical characteristics of homozygotes.

It is likely that one of the earliest reports of an individual with abetalipoproteinaemia, in fact had homozygous hypobetalipoproteinaemia (Salt *et al.*, 1960), as both her parents and her paternal grandfather had abnormally low levels of beta-lipoprotein (mother, 216 mg/dl; father, 149 mg/dl; grandfather, 289 mg/dl; NR 300-580 mg/dl). Non of these relatives had any neurological, ocular or haematological pathology however and in the parents, chylomicron absorption after a fatty meal was normal.

In 1966 van Buchem *et al* described a patient with ß-lipoprotein (LDL) deficiency but normal α-lipoprotein (HDL) levels, but without any manifestation of steatorrhoea or neuropathy. His fundi did show changes, with scattered fine, shiny dots on both sides, but these did not appear to progress with time and although he had a lowered red cell lecithin to sphingomyelin ratio, there were no acanthocytes. Two of his brothers showed mild ß-

lipoprotein deficiency, but his third brother and children showed no abnormalities. It is likely that the three brothers described represent cases of heterozygous hypobetalipoproteinaemia.

Mars *et al* (1969), described a three generation family with hypobetalipoproteinaemia, with 13 affected individuals. Serum levels of cholesterol and phospholipid were low, but triglyceride levels were low-normal and α-lipoprotein levels, normal. Nine of the affected individuals (those with serum cholesterol levels less than 100 mg/dl) had acanthocytosis, but this could be reversed by the *in vitro* addition of hyperlipaemic serum. Their phenotypic characteristics were highly variable, the proband having the most severe abnormalities, with a progressive demyelinating disorder and the lowest levels of beta-lipoprotein and cholesterol. Vitamin E levels were found to be low in five of the eleven persons tested, with the proband having the lowest value (0.3 mg/dl). The pattern of inheritance in this family was clearly dominant. Since this report there have been many others of familial hypobetalipoproteinaemia, showing dominant inheritance and occurring in both heterozygous and homozygous form (Glueck., 1977).

(a) Plasma Lipoproteins.

Individuals with homozygous hypobetalipoproteinaemia have severe hypolipidaemia. VLDL levels are very low and almost their cholesterol is present in HDL, with very little LDL cholesterol. Total apoB levels are very low and can only be detected by sensitive assays such as

enzyme linked immunosorbant assay (ELISA) (Thrift *et al.*, 1986). Triglyceride may be very low, or in some cases normal, as in that described by Steinberg *et al* (1979). Although sometimes lower than normal, the HDL fraction contains the usual A and C apolipoproteins. As with abetalipoproteinaemia, cholesterol biosynthesis in, homozygous hypobetalipoproteinaemia, measured by sterol balance studies (Illingworth *et al.*, 1979), or urinary mevalonic acid excretion (Illingworth *et al.*, 1989), is not excessive. Any increases may be explained on the basis of enhanced faecal losses, rather than induction resulting from the absence of circulating LDL, since, as mentioned under abetalipoproteinaemia, apoE rich HDL can effectively substitute for the latter. Unlike patients with abetalipoproteinaemia, the activities of hepatic lipase and lipoprotein lipase are normal (Illingworth *et al.*, 1983).

In contrast, individuals with heterozygous hypobetalipoproteinaemia have only moderate hypolipidaemia, with lipid levels sometimes overlapping into the normal range. VLDL levels are usually low and levels of total cholesterol usually below the fifth percentile however, with the bulk of the deficiency residing the LDL fraction. HDL concentrations are essentially normal. As a result of their low LDL cholesterol levels, heterozygotes appear to be immune to the development of atherosclerosis and coronary artery disease (Kahn and Glueck., 1978).

(b) Clinical and pathophysiological features of hypobetalipoproteinaemia.

(i) Gastrointestinal Effects

In homozygous hypobetalipoproteinaemia, the gastrointestinal manifestations are similar to those of abetalipoproteinaemia. Fat ingestion does not usually result in chylomicronaemia (Salt *et al.*, 1960; Cottrill *et al.*, 1974), and there is fat malabsorption. Examination of jejunal biopsy specimens demonstrates excessive accumulation of neutral fat in the jejunal mucosal cells (Cottrill *et al.*, 1974). In addition, hepatic steatosis may also be present. The two homozygous individuals examined by Cottrill *et al* had liver biopsies demonstrating large amounts of triglyceride and foam cells in portal spaces on frozen section. It is presumed that this accumulation of triglyceride in intestinal mucosa and liver is due to failure to elaborate chylomicrons and VLDL respectively. Plasma levels of the fat soluble vitamins are accordingly low.

In heterozygotes, gastrointestinal symptoms are either mild or absent (Mars *et al.*, 1969), but mild steatorrhoea may occur. The appearance of jejunal biopsies in heterozygotes is usually normal however (Mars *et al.*, 1969). It has been suggested by Granot and Deckelbaum (1989) however, that both plasma and tissue levels of vitamin E should be measured in heterozygotes and that supplementation with the vitamin and a diet low in long chain fatty acids initiated in appropriate cases.

(ii) Neuromuscular Effects

The appearance of neuromuscular complications in hypobetalipoproteinaemia is highly variable, giving support to the view that the disorder represents a heterologous group of molecular defects. There are reports of homozygotes both with (Ross *et al.*, 1988) and without (Steinberg *et al.*, 1979) neuromuscular complications. On the other hand, although heterozygotes are nearly always free from neuromuscular effects, there are reports of clear heterozygotes with obvious neuromuscular complications. A patient described by Scott *et al* (1979), with a totally normal brother and son, born in 1922, did not walk until age 4 years and was then clumsy with slurred speech. In 1974, he required a stick for walking and a wheelchair for long journeys. Neurological examination suggested a posterior column lesion, cerebellar disease, peripheral neuropathy and proximal myopathy. Vitamin E levels measured in 1976 were found to be undetectable. In general however, the neuromuscular effects seen in hypobetalipoproteinaemia are not as severe as those occurring in abetalipoproteinaemia and in general are usually seen only in homozygotes.

(iii) Ocular Effects

Retinitis pigmentosa has not been demonstrated in heterozygous hypobetalipoproteinaemia, but some homozygotes have shown changes. As with the neuromuscular effects however, retinal changes, if they occur, are of later onset and follow a more benign course than in abetalipoproteinaemia.

(iv) Haematological Effects

Patients with homozygous hypobetalipoproteinaemia have typical acanthocytes in their peripheral blood, with the same red cell membrane lipid changes seen in abetalipoproteinaemia. Some heterozygotes show a small proportion of acanthocytes, but their peripheral blood film is more often than not normal. Homozygotes occasionally demonstrate prolongation of their prothrombin time, presumably as a result of vitamin K malabsorption. A homozygous female individual described by Biemer and McCammon (1975), for example, was discovered as a result of a bleeding diathesis which developed after the birth of her daughter.

(c) Diagnosis and Treatment of Hypobetalipoproteinaemia

The clinical, biochemical and haematological features of homozygous hypobetalipoproteinaemia are indistinguishable from abetalipoproteinaemia. Thus, malabsorption, with retinitis pigmentosa and neuromuscular deficits involving the spinocerebellar pathways are found. Examination of the blood will likewise reveal acanthocytosis, with very low cholesterol and triglyceride levels and a virtual absence of apoB. Jejunal biopsy will demonstrate lipid laden enterocytes as in abetalipoproteinaemia. The differentiation from abetalipoproteinaemia is provided by examination of the parents and other first degree relatives. Obligate heterozygote relatives will show abnormally low cholesterol, and apoB levels and may also have a small proportion of acanthocytes. Some mild clinical symptoms, such as fat malabsorption may also be present. It is important however to exclude secondary causes of

hypobetalipoproteinaemia in such individuals, such as malnutrition, gastrointestinal disease causing malabsorption, hypothyroidism and others (Malloy and Kane., 1982).

1.8 Normotriglyceridaemic Abetalipoproteinaemia

Normotriglyceridaemic abetalipoproteinaemia is variant of abetalipoproteinaemia first described by Malloy *et al* (1981). In this form of the disease, there is a selective deficiency of normal VLDL and LDL, but normal fat absorption and chylomicron formation. In contrast to patients with classical abetalipoproteinaemia, the patient described by Malloy *et al* was obese, with a normal plasma triglyceride level, normal small intestinal histology and visible chylomicronaemia following an oral fat load. A further difference was found in the HDL fractions. Whilst in classical abetalipoproteinaemia, there is an increased proportion of HDL_2 relative to HDL_3, largely due to a marked decrease in the latter, Malloy *et al* (1981) found not only low levels of HDL_3, but in addition, a virtual absence of HDL_2.

Also unlike classical abetalipoproteinaemia, she had only a very small percentage of acanthocytes (no more than 1%). As with the usual form of abetalipoproteinaemia however, the patient had developed an ataxia and her serum had no normal LDL and essentially undetectable levels of vitamin E. The levels did however increase in to the normal range on supplementation and the patient's ataxia then showed a striking improvement, suggesting that the vitamin E deficiency was not as severe as with normal abetalipoproteinaemia. Unlike classical abetalipoproteinaemia, the serum vitamin A level and the prothrombin time were normal, indicating normal transport of vitamins A and K. In common with classical abetalipoproteinaemia, the ratio of

sphingomyelin to lecithin was increased and there was a deficiency of the monosialated form of apoCIII. Low levels of apoD were observed and were considered to be a consequence of the HDL abnormalities.

No apoB100 was detectable in the patient's serum, but apoB48 was easily demonstrated, suggesting that the normotriglyceridaemic phenotype results from a selective failure of apoB100 secretion with intact apoB48 production. Unfortunately, because the affected child had been adopted and no relatives were available for testing, although Malloy *et al* referred to her condition as normotriglyceridaemic abetalipoproteinaemia, there was no formal proof that she did not have an equivalent form of hypobetalipoproteinaemia. Subsequent studies by Hardman *et al*(1989), viewed in conjunction with the findings of this thesis and of several other workers confirm that this is indeed the case (see Discussion p.172).

A second case, also referred to as normotriglyceridaemic abetalipoproteinaemia, was reported by Takashima *et al* (1985). Their patient was a 1-year old Japanese infant who presented with malnutrition and was found to have acanthocytosis, with LDL deficiency, but significant amounts of triglyceride-rich lipoproteins in her plasma. Although at 6 months, in addition to apoB48, a faint band corresponding to apoB100 was found in the d<1.006 lipoproteins on SDS polyacrylamide gel electrophoresis, by 18 months, this was no longer seen and the apoB48 band was more pronounced. Intestinal fat absorption was not normal, but both plasma triglyceride and apoB48 were found to

increase following an oral fat load. By 20 months, there was no obvious malnutrition, retinopathy, or neurological complications. Unlike the patient of Malloy *et al* (1981), this individual did not have a deficiency of HDL_2, rather HDL_2 predominated over HDL_3.

Unlike the previous case, some relatives were available for investigation. The mother was hyperlipidaemic in the third trimester of pregnancy, but had been normolipidaemic before pregnancy; the father, although his total cholesterol was low (117 mg/dl), had normal LDL-cholesterol and apoB levels and the older brother had a normal lipoprotein profile. The recessive pattern of inheritance confirms the diagnosis of normotriglyceridaemic, abetalipoproteinaemia in contrast to hypobetalipoproteinaemia.

A similar patient has also been described by Herbert *et al* (1985). Their patient was hospitalised at 10 months because of persistent diarrhoea and failure to thrive. At 1 year, hepatomegaly and the presence of 5-10% acanthocytosis was noted. Both liver and jejunal biopsies were performed and demonstrated the presence of both intestinal mucosal fat accumulation and hepatic steatosis. No retinal or neuromuscular abnormalities were observed, although, as the authors pointed out, this would be unusual at such a young age, even in classical abetalipoproteinaemia. Cholesterol and triglyceride levels in the plasma were 38 and 63 mg/dl respectively, normal LDL was absent, but lipoproteins in the $1.006<d<1.063$ density interval were large, triglyceride rich, chylomicron and chylomicron remnant-like particles. These particles were present

in the patients fasting plasma, indicating prolonged dietary fat absorption. The serum vitamin E level was low at 1.8 mg/dl (normal, 5-20 μg/ml). Unlike the patient of Malloy *et al* (1981), but like that of Takashima *et al* (1985), both HDL_2 and HDL_3 were present. The deficiency of the monosialated form of apoCIII ($ApoCIII_1$) was more severe than that seen in the Malloy patient, with a complete absence of the protein. As in typical abetalipoproteinaemia, the ratio of lecithin to sphingomyelin was reduced.

Examination of the patient's triglyceride-rich lipoproteins showed that they contained apoB48, but no apoB100. Both parents were phenotypically normal with normal plasma cholesterol and triglyceride levels, indicating a recessive pattern of inheritance characteristic of a form of abetalipoproteinaemia, in contrast to hypobetalipoproteinaemia. The authors conclude that their patient's phenotype results from a recessive genetic defect which abolishes apoB100 production and perhaps, in addition, in view of the impaired fat absorption, may also limit apoB48 production.

Treatment of normotriglyceridaemic abetalipoproteinaemia should be as for classical abetalipoproteinaemia, with restriction of dietary fat, particularly during infancy and supplementation with vitamins E and A.

1.9 Normotriglyceridaemic Hypobetalipoproteinaemia

As with abetalipoproteinaemia, forms of hypobetalipoproteinaemia exist where the plasma triglyceride level is essentially normal. As mentioned above, although the parents are not available for study, the case of normotriglyceridaemic abetalipoproteinaemia reported by Malloy *et al* (1981) can, in the light of recent studies on the same individual by Hardman *et al* (1989), taking into account the findings of this thesis and of many other workers, be more accurately referred to as normotriglyceridaemic hypobetalipoproteinaemia (see Discussion p.172).

The hypobetalipoproteinaemic individual reported by Steinberg *et al* (1979) had a similar phenotype, with very low levels of LDL cholesterol, but only mild fat malabsorption and normal triglyceride levels. Chylomicrons persisted in the fasting state, the chylomicron half-life being 29 minutes, five times the normal value. There was a complete deficiency of the monosialated form of apoCIII ($apoCIII_1$).

In fact, normotriglyceridaemia is generally much more likely in hypobetalipoproteinaemia than in abetalipoproteinaemia. Certainly, many of the heterozygotes for the disorder have a normal plasma triglyceride level. Amongst homozygotes, in addition to the two cases referred to above, one of the individuals investigated in this study was also normotriglyceridaemic. In the patients studied by Malloy *et al* (1981) and perhaps Steinberg, this normotriglyceridaemia is due to a selective preservation of apoB48 production (Hardman *et al.*, 1989; Linton and

Young., Unpublished observations)

1.10 Chylomicron Retention Disease (Anderson's Disease)

This disorder was first described by Anderson *et al* (1961). They described the case of a 7 month old girl who presented with steatorrhoea and failure to thrive. On investigation, the plasma levels of cholesterol, triglyceride, phospholipid and vitamins A and E were found to be low and no chylomicrons were observed in the circulation following a fatty meal.

Although the case resembled that reported by Salt *et al* (1960), referred to as having abetalipoproteinaemia, but in fact more probably having homozygous hypobetalipoproteinaemia, there were important differences. Unlike the Salt case, Anderson's patient had no acanthocytosis, retinitis pigmentosa, or neurological abnormalities and did have approximately half normal levels of beta- (as well as alpha-) lipoprotein in her plasma. Anderson pro posed that the primary defect in the disorder lay in the synthesis of chylomicrons by intestinal cells.

Bouma *et al* (1986) studied a series of seven individuals from five kindreds with Anderson's disease. The patients all exhibited the typical features of the disorder; diarrhoea, steatorrhoea, decreased cholesterol, triglyceride, phospholipid, alpha- and -beta lipoproteins, absent post-prandial chylomicronaemia and lipid droplets in intestinal cells. As well as LDL levels, HDL levels were decreased in all of the subjects and increased amounts of apoE and E-AII complex was seen in the HDL. There was no acanthocytosis, retinitis pigmentosa, or ataxia. Exami-

nation of enterocytes obtained from intestinal biopsies following an overnight fast, showed an accumulation of numerous fat droplets. Using antibodies which recognise both apoB100 and B48 and apoB100 only, the presence of large amounts of apoB48, but not apoB100 was demonstrated. In the plasma, apoB100 was found, but no apoB48, the exact opposite what is seen in normotriglyceridaemic abetalipoproteinaemia.

It is likely that the relatively low levels of LDL are a secondary consequence of decreased VLDL secretion by the liver as a result of the interrupted supply of alimentary fatty acids. The reduction in HDL can be predicted as a result of the deficient production of triglyceride-rich lipoproteins from the intestine, which are known to contribute to HDL formation (Eisenberg *et al.*, 1984). The preponderance of apoE in the HDL can similarly be explained by it's predominantly liver origin.

Roy *et al* (1987) studied a further eight individuals with the disorder. They demonstrated a recessive inheritance of a malabsorption syndrome, with low plasma LDL, apoB and apoAI, and normal fasting triglycerides but no increase after a fatty meal, and no identifiable chylomicronaemia. Some of the individuals had undetectable vitamin E levels and showed signs of sensory polyneuropathy, but none had any evidence of retinitis pigmentosa. Lipid laden enterocytes were demonstrated, with increased immunoperoxidase localisation of apoB. Following a fat load, the enterocytes contained large numbers of fat particles, morphologically similar to chylomicrons, accumulating in the endo-

plasmic reticulum and Golgi zone, especially the latter. No such particles were observed in the intercellular spaces or lacteals. Potential defects leading to this chylomicron retention are suggested by Roy *et al* (1987), including a defect in the glycosylation process normally operative in the Golgi, in the lipid transporting microtubules, or in the plasma membrane recognition sites for chylomicrons-like particles.

Levy *et al* (1987 (a)) studied the biosynthesis of chylomicrons in cultured jejunal explants from six patients with chylomicron retention disease. Incubation of the explants with ^{14}C-palmitate showed that they were capable of normal biosynthesis of triglyceride, phospholipid and cholesteryl ester, but that, with the exception of phospholipid these lipids were retained in the tissue and did not appear in the culture medium. When incubated with ^{3}H-leucine and ^{14}C-mannose, normal protein synthesis, but with reduced glycosylation, was observed. Immunoblotting demonstrated the presence of intestinal apoB48. The authors note that the inhibition of glycosylation in rat jejunal enterocytes has been found to block lipoprotein transport (Hoffman *et al.*, 1981). They suggest that the defect in the disorder lies in the final assembly of chylomicron particles, perhaps related to a defect in the glycosylation of chylomicron protein.

Chapter 2: Methods

Experimental strategies utilised in this thesis to investigate the molecular defects in abetalipoproteinaemia and hypobetalipoproteinaemia

To begin with, the gross structure of the apoB gene was examined by Southern blotting experiments on genomic DNA extracted from peripheral blood, of individuals with abetalipoproteinaemia and hypobetalipoproteinaemia. Patients with abnormal Southern blots were then studied further using specific mapping probes and the polymerase chain reaction to amplify specific regions of the apoB gene, followed by sequencing by the dideoxy chain termination method. In addition, protein studies were conducted on certain individuals, in order to characterise their apoB, followed by appropriate polymerase chain reaction amplification and sequencing. These strategies have helped to define the role of the apoB gene in the pathogenesis of these two disorders and in some instances revealed specific apoB mutations.

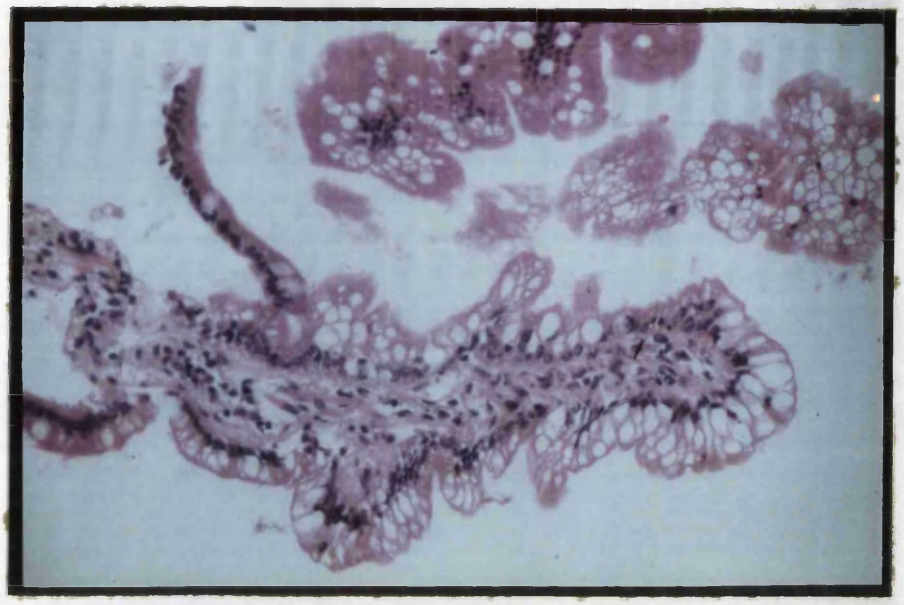
2.1 Subjects

(a) Hypobetalipoproteinaemic Individuals.

The D Family.

The individual investigated in this study with homozygous hypobetalipoproteinaemia, DD, was a patient who presented initially with fat malabsorption. On investigation, she was found to have greater than 50% acanthocytosis and plasma apoB levels less than 2% of normal, with a very low total cholesterol and low-normal triglyceride level.

(a)



(b)

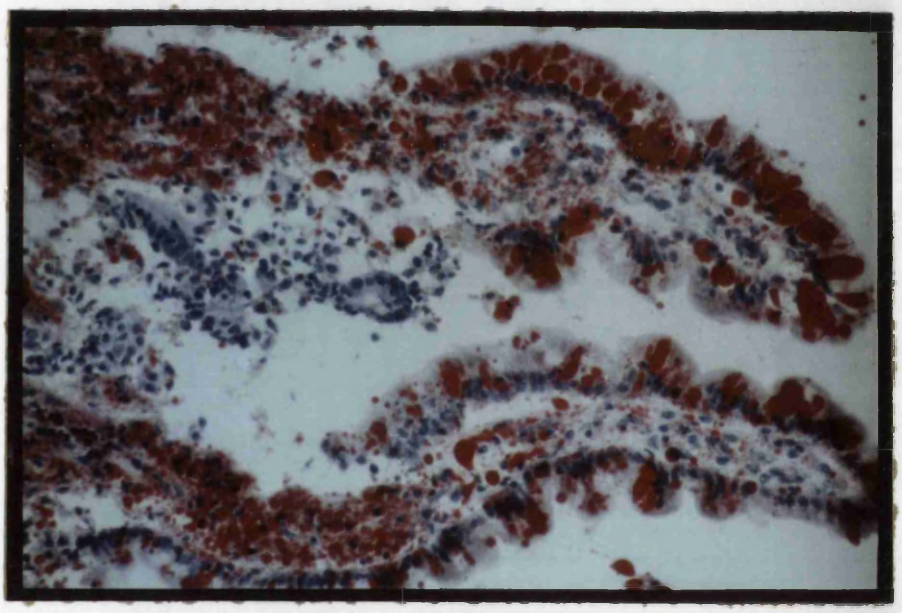


Figure 2.

Jejunal biopsy from subject DD with homozygous hypobetalipoproteinaemia (a) H&E section showing vacuolated enterocytes (b) Oil Red-O stain showing that this is due to an excessive accumulation of lipid within the cells. (Photomicrographs courtesy of Dr. Ashley Price, Consultant in Histopathology, Northwick Park Hospital).

A jejunal biopsy demonstrated the presence of lipid-loaded enterocytes (Figure 2). She had no neurological or retinal disease. Investigation of her parents, AD and PD, revealed that they both had haematological and lipid abnormalities of intermediate severity, with 10% acanthocytosis and apoB and total plasma cholesterol values below the fifth percentile for their age and sex, on each of several occasions on which they were measured. The clear co-dominant pattern of inheritance of abnormalities from the parents, AD and PD, to DD, confirmed the diagnosis of homozygous hypobetalipoproteinaemia, in contrast to abetalipoproteinaemia, in DD and heterozygous hypobetalipoproteinaemia in AD and PD.

The P Family.

Patient CP was the mother of a young child, ChP, who presented with fat malabsorption. On investigation, both mother CP and daughter, ChP, were found to have moderately low apoB and total cholesterol and low-normal triglyceride levels, but no acanthocytosis. The father, MP and second daughter, LP, were both clinically and biochemically normal. The dominance of inheritance of lipid abnormalities from CP to ChP confirmed the diagnosis of heterozygous hypobetalipoproteinaemia in both patients.

(b) Abetalipoproteinaemic Individuals.

The M Family.

Patient CM, presented in infancy, with fat malabsorption and was found on investigation to have acanthocytosis and very low plasma concentrations of cholesterol, triglyceride and apoB.

Patient PM was the brother of CM, diagnosed at birth, with the same haematological and biochemical profile.

Both parents were clinically entirely normal, with no haematological or biochemical abnormalities. The clear recessive pattern of inheritance established the diagnosis of abetalipoproteinaemia in their children, in contrast to homozygous hypobetalipoproteinaemia.

The J Family.

Patient MJ presented in infancy with failure to thrive and fat malabsorption and was subsequently found to have very low plasma concentrations of cholesterol, triglyceride and apoB.

Patient SJ was the sister of MJ, diagnosed at birth, with the same biochemical abnormalities. Another sib, NJ was unaffected.

As with the M family, both parents were clinically and biochemically normal, likewise establishing the diagnosis of abetalipoproteinaemia in their two affected children.

2.2 Reagents, chemicals, materials and equipment.

(a) Preprepared solutions

Lysis buffer

per litre:

109.5 g Sucrose:-	0.32 M Sucrose
10 ml 1M Tris:-	1 mM Tris-HCL, pH 7.5
5 ml 1M $MgCl_2$:-	5 mM $MgCl_2$
10 ml autoclaved Triton X100:-	1% Triton X100

Aliquot and autoclave.

75 mM NaCl, 25 mM EDTA

per litre:

75 ml 1 M NaCl

50 ml 500mM EDTA

Aliquot and autoclave.

Tris-saturated phenol

equilibration buffer:

per 500 ml:

74.4 g EDTA:-	0.4 M EDTA
24.2 g Tris:-	0.4 M Tris
80 g NaOH:-	4 M NaOH

Aliquot and autoclave. To 1 litre of water-equilibrated phenol add 1 g hydroxyquinoline and 2 ml ß-mercaptoethanol and shake thoroughly. Add 10 ml of the equilibration buffer, mix thoroughly and check that pH of the aqueous phase is approximately 8.0.

3M Sodium acetate

per 100 ml:

40.8 g sodium acetate

Adjust pH to 5.2 with glacial acetic acid, aliquot and autoclave.

Tris-Borate-EDTA Buffer (TBE) X 10

per 5 litres:

540 g Tris

275 g Boric acid

40 g EDTA

Ethidium bromide

per 10 ml water:

0.1 g ethidium bromide

Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved, wrap the container in aluminium foil and store at 4 °C.

Orange G sample-buffer

per 10 ml:

4 g Ficoll:-	40% Ficoll
0.5 ml 500 mM EDTA:-	25 mM EDTA
1 mg Orange G:-	Trace Orange G

Labelling solution

25 µl 1M HEPES pH 6.6

25 µl DTM

7 µl OL

Aliquot and store at -20°C.

1M HEPES pH 6.6

per 10 ml:

283 g HEPES

Adjust pH with 1M NaOH. Filter through 0.2µm Gelman acrodisc.

DTM

2 μM 25 mM dATP:-	100 μM dATP
2 μM 25 mM dGTP:-	100 μM dGTP
2 μM 25 mM dTTP:-	100 μM dTTP

494 μM TM buffer Aliquot and store at -20°C.

TM buffer

per 10 ml:

2.5 ml 1M Tris:-	250 mM Tris, pH 8.0
250 μl 1M $MgCl_2$:-	25mM $MgCl_2$
500 μl ß-mercaptoethanol:-	50 mM ß-mercaptoethanol

6.75 ml distilled water

Aliquot and store at -20°C.

OL

250 μl random primers at 90 U/ml

1 μl 250 mM EDTA

1 μl 250 mM Tris-HCL, pH 7.5

Store at -20°C.

TENS

per 500 ml:

5 ml 1M Tris:-	10 mM Tris-HCL, pH 8.0
5 ml 500 mM EDTA:-	5 mM EDTA, pH 8.0
50 ml 1M NaCL:-	100 mM NaCl
5 ml 20% SDS:-	0.2% SDS

Make up with autoclaved reagents.

P60-TENS

5 g P60

200 ml TENS

Allow P60 to swell overnight at room temperature.

Prehybridisation solution

12 ml distilled water

5 ml 20X SSC:- 5X SSC

1 ml 20% SDS:- 1% SDS

1 ml 10% BLOTTO:- 0.5% BLOTTO

1ml 10 mg/ml salmon sperm DNA:- 0.5 mg/ml carrier DNA

The carrier DNA is denatured immediately before adding it to the hybridisation solution by heating at 100°C for 5 minutes then cooling rapidly in ice-water.

20X SSC

per litre:

175.3 g NaCL

88.2 g sodium citrate

Adjust pH to 7.0 with 10N NaOH and sterilize by autoclaving.

10% BLOTTO

per 100 ml autoclaved distilled water:

10 g dried milk-powder

0.2 g sodium azide

Store at 4°C.

Salmon sperm DNA

per 100 ml autoclaved distilled water:

1 g salmon sperm

Sonicate in 50 ml Falcon tubes for 15-20 min. and store at -20°C.

SOB medium

per 100 ml:

2.0 g Bactotryptone:- 2.0%

0.5 g Bacto yeast extract:- 0.5%

1.0 ml 1M NaCl:- 10 mM

0.25 ml 1M KCl:- 2.5 mM

Make up to volume with glass distilled water and autoclave for 20-30 minutes.

Magnesium buffer

per 50 ml:

10.16 g $MgCl_2$:- 10 mM

12.3 g $MgSO_4$:- 10 mM

Autoclave for 20-30 minutes.

RFI

per 100 ml:

1.2 g RbCl:- 100 mM

0.99 g $MnCL_2$:- 50 mM

3 ml 1M Potassium acetate:- 30 mM

0.15 g $CaCL_2$:- 10 mM

15 g Glycerol:- 15% w/v

Adjust pH to 5.8 with dilute acetic acid (0.2M), sterilize with 0.22 μm pore filter and store at 4°C.

RF2

per 100 ml:

0.12 g RbCl:- 10 mM

1.1 g $CaCl_2$:- 75 mM

0.21 g MOPS:- 10 mM

15 g Glycerol:- 15% w/v

Adjust pH to 6.8 with dilute NaOH, sterilize with 0.22 μm pore filter and store at 4°C.

(b) Chemicals, enzymes and cloning vectors and suppliers

Absolute ethanol	B.D.H.
Acrylamide	B.D.H.
Alkaline phosphatase	Anglian Biotech.
Ammonium persulphate	B.D.H.
ApoB assay kit	Orion Diagnostics.
Aprotinin (Traselol)	Bayer Pharmaceuticals.
Bacto yeast extract	Difco Labs.
Bactotryptone	Difco Labs.
Benzamidine	Sigma.
Boric acid	B.D.H.
Bovine serum albumen	Sigma.
Bromophenol blue	Sigma.
Calcium chloride	B.D.H.
Chloroform	Rathburn Chemicals.
Cholesterol assay kit	Boehringer Manheim.
Citric acid	B.D.H.
^{32}P-CTP	Amersham International.
Coomassie Brilliant Blue	Sigma.
dATP, dGTP dTTP	Pharmacia.
Dimethyldichlorosilane	B.D.H.
Dithiothreitol	Sigma.
E. coli DH5αF'	Life technologies.
*Eco*RI	Anglian Biotech.
*Eco*RV	Anglian Biotech.
EDTA	B.D.H.
Ethidium bromide	Sigma.
Ficoll	Pharmacia.
Formaldehyde	B.D.H.

Gelatin	B.D.H.
Glacial acetic acid	B.D.H.
Glycerol	B.D.H.
Glycine	B.D.H.
HEPES	Sigma.
*Hinc*II	Anglian Biotech.
Hydrochloric acid	B.D.H.
IPTG	Sigma.
Iodine 125-protein A	Amersham International.
Isoamyl alcohol	Rathburn Chemicals.
M13tg131	Amersham International.
Magnesium chloride	B.D.H.
Methanol	B.D.H.
ß-Mercaptoethanol	Sigma.
N,N,-bis-Methylacrylamide	B.D.H
Milk powder (Marvel)	Cadbury's.
Mineral oil	B.D.H.
*Msp*I	Anglian Biotech.
Orange G	Sigma.
PEG	Sigma.
Phenol	Rathburn Chemicals.
Phenylmethylchloroketone	Sigma.
Phenylmethylsulphonyl fluoride	Sigma.
Polybrene	B.D.H.
Potassium bromide	B.D.H.
Potassium chloride	B.D.H.
Proteinase K	B.D.H.
*Pvu*II	Anglian Biotech.
Random primers	Pharmacia.

Rubidium chloride	Sigma.
Salmon sperm	Sigma.
Scintillation fluid	National Diagnostics.
Sequenase USB sequencing kits	Cambridge Bioscience.
Silver nitrate	B.D.H.
Sodium acetate	B.D.H.
Sodium azide	Sigma.
Sodium carbonate	B.D.H.
Sodium chloride	B.D.H.
Sodium citrate	B.D.H.
Sodium dodecylsulphate (SDS)	B.D.H.
Sodium hydroxide	B.D.H.
Soybean trypsin inhibitor	Sigma.
TY agar	Difco Labs.
T4 DNA polymerase	Anglian Biotech.
T4 polynucleotide kinase	Anglian Biotech.
*Taq*1	Anglian Biotech.
Taq polymerase	Cetus.
Tetramethylethylenediamine	B.D.H.
Triglyceride assay kit	Metachem Diagnostics.
Tris-HCL	B.D.H.
Triton X100	B.D.H
Ultra-pure urea	B.D.H.
X-gal	Sigma.
*Xba*I	Anglian Biotech.

(c) Other materials and suppliers.

Clinwrap	Perfowrap.
DE81 paper	Whatman.
Electrical tape No. 56	3M.

Emitpen	Dupont.
Eppendorf tubes	B.D.H.
Falcon tubes	Becton Dickinson.
Hybond-N membranes	Amersham International.
Kodak XAR-5 film	Kodak.
3MM filter paper	Whatman.
Nalgene Type S filters	B.D.H.
Nitrocellulose sheets	BioRad.
P20, P200, P1000 Gilson Pipetman	Anachem Ltd.
P60	BioRad.
Pasteur pipettes	B.D.H.
Polythene roll for hybridisation bags	Transadlantic Plastics.
Protein electrophoresis plates	Hoeffer Scientific.
Plastic pipettes, 10 ml	Sterilin.
Saran wrap	B.D.H.
Scotties tissues	Scott.
Syringes	B.D.H.
Zeta Probe membrane	BioRad.

(d) Equipment and suppliers.

380 A oligonucleotide synthesiser	Applied Biosystems.
Bioscan counter	V.A. Howe.
DACOS	Coulter Electronics.
Hamilton 1000 diluter	V.A. Howe.
Heat sealer	Hybaid.
BT3 heating block	Grant Instruments.
MSE Microcentaur Microcentrifuge	Kontron instruments.
Mini-Moniter Type 510	Mini Instruments.
Model 583 gel drier	BioRad.

MSE Europa 65M	Kontron Instuments.
Multistat III centrifugal analyser	IL.
Sorvall RT 6000 centrifuge	Dupont.
Sorvall AH650 rotor	Dupont.
Ultra-violet light boxes	U.V. Products.
Waterbaths; Plain	Grant Instruments.
Waterbaths; Shaking	Kotterman.
Western Blot electrophoresis tank	Hoeffer.
X-ray cassettes	Fuji X-Ray.

(e) Addresses of suppliers

Amersham International, Aylesbury, England.

Anachem Ltd., Luton, Bedfordshire, England.

Anglian Biotech Ltd., Colchester, England.

Applied Biosystems, Warrington, Cheshire, England.

B.D.H. Chemicals, Poole, Dorset, England.

Bayer Pharmaceuticals UK Ltd., Newbury, Berkshire, England.

Becton Dickinson, Cowley, Oxford, England.

Biorad Labs Ltd., Watford, England.

Boeringer Manheim UK, Lewes, East Sussex, England.

Cadbury's, Bourneville, England.

Cambridge Bioscience, Cambridge, England.

Cetus-Perkin Elmer Ltd., Beaconsfield, England.

Coulter Electronics Inc., Luton, Bedfordshire, England.

Difco Labs., East Molesley, Surrey, England.

Dupont UK Ltd., Stevenage, Herts, England

Fuji X-Ray Ltd., London, England.

Grant Instruments, Cambridge, England.

Hoeffer Scientific, Newcastle Under Lyme, England.

Hybaid Ltd., Teddington, Middlesex, England.

IL (UK) Ltd, Warrington, Cheshire, England.

Kodak Ltd., Hemel Hempstead, Hertfordshire, England.

Kontron Instruments Ltd., Watford, England.

Life Technologies, Paisley, Scotland.

Metachem diagnostics, Rugby, England.

Mini Instruments, Burnham on Croach, Essex.

3M, Bracknell, Berkshire, England.

National Diagnostics, Aylesbury, England.

Orion Diagnostica, Espoo, Finland.

Perfowrap Ltd., High Wycombe, England.

Rathburn Chemicals Ltd., Walkerburn, Scotland.

Scott Ltd., East Grinstead, Sussex, England.

Sigma Chemical Co. Ltd., Poole, Dorset, England.

Sterilin Ltd., Teddigton, Middlesex, England.

Transadlantic Plastics, Surbiton, surrey, England.

USB, Cleveland, Ohio, U.S.A.

U.V. Products Ltd., Cambridge, England.

V.A. Howe & Co. Ltd., London, England.

Whatman Labsales Ltd., Maidstone, Kent, England.

2.3 Methods used in the investigation of both hypobetalipoproteinaemic and abetalipoproteinaemic patients.

(a) Specimen collection.

Whole venous blood was collected into EDTA and plasma stored at 20° C, prior to estimation of cholesterol, triglyceride and apoB concentrations.

(b) Cholesterol Assay.

Cholesterol was estimated by the cholesterol oxidase-phenol-aminophenazone (CHOD-POP) method, using a commercial kit supplied by Boehringer-Manheim Corp.

Test Principle.

cholesterol ester + H_2O ----cholesterol esterase----> cholesterol + RCOOH

cholesterol + O_2 ----chol. oxidase----> δ^4-cholestenone + H_2O_2

2 H_2O_2 + 4-aminophenazone + phenol ----peroxidase----> 4-(p-benzoquinone-mono-imino)-phenazone + 4 H_2O

The absorbance of the coloured end-product is measured at 546 nm against a reagent blank.

The analyses was performed on an automated chemistry analyser known as a DACOS (Discrete Analyser with Continuous Optical Scanning).

(c) Triglyceride Assay.

Triglyceride was estimated by a modification of Fossati and Prencipe (1982) method, using a commercial kit supplied by Metachem Diagnostics.

Test Principle.

triglycerides ----lipase----> glycerol + fatty acids

glycerol + ATP ----glycerol kinase----> glycerol-3-phosphate + ADP

glycerol-3-phosphate + O_2 ----glycerophosphate oxidase----> H_2O_2 + dihydroxyacetone phosphate

H_2O_2 + 4-aminoantipyrine + 3,5-dichloro-2-hydroxybenzene sulphonic acid ----peroxidase----> quinoneimine chromophore + HCL + $2H_2O$

The absorbance of the coloured end-product is measured at 520 nm against a reagent blank. As with the cholesterol assay, analyses were performed on a Coulter DACOS analyser.

(d) Apolipoprotein B Assay.

ApoB estimations were carried out by an immunochemical method, based on the measurement of immunoprecipitation by determination of absorbance at 340 nm. ApoB buffer, apoB antiserum and reference material for the assay were supplied in kit form by Orion Diagnostica. The kit was used to perform a kinetic immunoturbimetric assay on an IL Multistat III centrifugal analyser. Samples were diluted 1 in 15 on a Hamilton 1000 diluter. In the Multistat III analyser, diluted sample is mixed with antibody and the formation of anti-apoB/apoB complex is monitored by measuring the increase in absorbance at 340 nm between 3 and 360 seconds.

For samples with apoB levels < 50 mg/dl, apoB concentrations were determined by a competitive ELISA method, similar to that of Fruchart *et al* (1978).

2.4 Methods used in the investigation of hypobetalipoproteinaemic patients.

(a) Lipoprotein Preparation.

Venous blood samples were taken from patient and control subjects in a non-fasted state and mixed immediately with a protease inhibitor mixture, containing EDTA (7.5 mg/ml), polybrene (25 μg/ml), benzamidine (2 mM), aprotinin (100 Kallikrein-Inhibitory U/ml), soybean trypsin inhibitor (20 μg/ml), phenylmethylchloroketone (20 μg/ml) and sodium azide (175 μg/ml) (Cardin *et al.*, 1984). Plasma was collected after centrifugation in Falcon tubes at 3000 rpm for 10 minutes in a Sorvall RT6000 centrifuge and phenylmethylsulphonyl fluoride was added to a concentration of 75 μg/ml (Cardin *et al.*, 1985)

Plasma density was adjusted with an aqueous solution containing 153 g/L sodium chloride and 354 g/L potassium bromide, according to the formula below. If the density of this solution is termed D and the volume to be added, V, then;

$$\text{New density} = \frac{(\text{Old density} \times \text{Old volume}) + (D \times V)}{\text{Old volume} + V}$$

from which V can be calculated.

The plasma density was adjusted initially from 1.006 to 1.019. Ultracentrifugation was then performed at 100,000 g for 16 hours at 10°C, to yield a combined chylomicron, VLDL and IDL fraction ($d<1.019$).

The infranatant density was adjusted from 1.019 to 1.063. Subsequent ultracentrifugation at 100,000 g for a further 16 hours at 10°C, yielded an LDL fraction ($1.019<d<1.063$).

Finally, the infranatant from the second spin was adjusted from 1.063 to 1.21, followed by further ultracentrifugation at 100,000 g for 48 hours at 10°C, to give an HDL fraction (1.063<d<1.21) and an infranatant fraction (d>1.21) (Havel *et al.*, 1955). Centrifugation was carried out in an MSE Europa 65M ultracentrifuge using a Kontron TFT 65:38 rotor.

The isolated fractions were centrifuged a second time at 100,000 g for 16 hours at 10°C, in order to concentrate and purify them. Prior to the respins, the fraction densities were adjusted up as shown below to allow for the fall in density which occurs in top-fractions during the ultracentrifugation process.

1.019 --> 1.025

1.063 --> 1.085

1.021 --> 1.25

Recentrifugation was performed in an MSE Europa 65M ultracentrifuge using a Sorvall AH650 rotor. Following collection of the recentrifuged lipoprotein fractions, they were dialysed for 24 hours against 1000-fold excess of phosphate buffered saline, with two changes of buffer, to remove potassium bromide.

(b) Preparation of Apolipoprotein Fractions for SDS Polyacrylamide Gel Electrophoresis.

Lipoprotein fractions, obtained as above, were subsequently delipidated by dropwise addition to 20 volumes of a mixture of ethanol:diethyl ether (3:1). The precipitates so obtained were pelleted by centrifugation in Falcon tubes at 3,000 rpm for 10 minutes, then washed with ether

and dried under nitrogen. They were finally dissolved to a concentration of 2-3 mg/ml in 10% SDS, 1% ß-mercaptoethanol, prior to SDS polyacrylamide gel electrophoresis.

(c) SDS-Polyacrylamide Gel Electrophoresis (SDS PAGE).

Patient and control apolipoprotein fractions, prepared as above, were separated on 5-10% and 8-17% SDS PAGE gradient gels, using the system of Laemmli (1970).

Gels were prepared with a 4% stacking gel, from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N,N-bis-methylacrylamide. The separation gel contained in addition 0.375M tris-HCL (pH 8.8) and 0.1% SDS. Polymerisation was achieved chemically by the addition of 0.05% by volume of tetramethylethylenediamine (TEMED) and 0.033% ammonium persulphate. Volumes were measured and delivered using 10 ml plastic pipettes and P20 (20 μL), P200 (200 μL) and P1000 (1000 μL) Gilson Pipetman pipettes. The separation gel was poured to a height of 12 cm between two 15X15 glass plates, with 1.5 mm thick spacers, sealed with plastic tape. A 3 cm stacking gel, containing sample wells was layered on top of the separation gel containing 4% acrylamide, 0.125 M tris-HCL (pH 6.8) and 0.1% SDS and was polymerised chemically in the same way as the separating gel.

Gels were run in electrophoresis tanks containing 0.025 M tris and 0.192 M glycine and 0.1% SDS as an electrode buffer (pH 8.3). To the apolipoprotein samples, prepared as above, were added equal volumes of a sample loading buffer, containing 0.0625 M tris-HCL (pH 6.8), 2% SDS, 10% glycerol, 5% ß-mercaptoethanol and 0.001% bromophenol blue

as a tracking dye, using a P20 Gilson Pipetman. Prior to loading, samples were denatured completely by heating at 90° C for 5 minutes on a heating block. Loading was carried out using a P200 Gilson Pipetman. Gels were run at 200 volts until the bromophenol blue marker reached the end (approximately 4 hours).

Following electrophoresis, gels were stained with Coomassie brilliant blue or used to prepare Western blots.

(d) Coomassie Brilliant Blue Staining of gels.

Gels were simultaneously fixed and stained by immersion in a solution containing 1.25 gm of Coomassie brilliant blue dissolved in 250 ml of methanol, 250 ml of distilled water and 46 ml of glacial acetic acid. Following 1 hour in the fix/stain solution, gels were destained for 18 hours in 20% methanol, 10% acetic acid.

(e) Western Blotting.

Following electrophoresis, some gels were used in Western blotting (immunoblotting) experiments. In this technique, proteins are transferred electrophoretically to nitrocellulose paper (Burnette., 1981). Firstly, a sandwich was prepared with the following successive layers; 1) porous polyethylene sheet 2) two thicknesses of 3 MM filter paper 3)the SDS polyacrylamide gel slab 4) a nitrocellulose sheet cut to the size of the gel 5) two further thickness of 3 MM filter paper and 6) another porous polyethylene sheet. The whole was pre-wetted with buffer and enclosed between two perforated perspex sheets and inserted into a specially designed electrophoresis tank, containing a transfer buffer consisting of 20 mM tris base, 150 mM

glycine and 20% methanol. Transfer was accomplished by the application of a current of 0.5-1 amp, 17 volts, for 18 hours, overnight in a cold room (4°C). Following electrophoresis, the gel was removed and stained with coomassie brilliant blue as above, in order to check the completeness of protein transfer.

The nitrocellulose sheet was immersed in a buffer containing 0.15 M NaCl, 0.005 M ethylenediamine tetraacetic acid (EDTA), 0.05M tris-HCL (pH 7.4), 0.05% triton X100 and 0.25% gelatin, for 30 minutes at room temperature, in order to saturate the blot with protein and prevent non-specific binding at subsequent stages.

The blots were next probed with monoclonal antibody Sol-9 (Milne *et al.*, 1986) directed against an epitope which lies between residues 454 and 586 of human apoB (Pease *et al.*, 1990), by incubation in 10 ml of the same buffer containing 10 μL of antibody, for 2 hours at room temperature. Being directed to an amino terminal epitope, Sol-9 has the capacity to probe truncated forms of apoB in addition to apoB100. Excess antibody was removed by shaking the nitrocellulose filters in three changes of buffer for a total of 1 hour, also at room temperature.

The antibody treated filters were next incubated in a solution of Iodine 125-protein A in buffer (1-2 μC in 10 ml buffer), again for 1 hour at room temperature, rinsed in three changes of buffer as before, blotted dry between 3 MM filter paper, wrapped in Saran Wrap and exposed overnight in an X-ray cassette at -70°C.

(f) Preparation of DNA.

Genomic DNA was isolated from 10 ml of peripheral blood that had been collected in EDTA, by a method based on that of Kunkel *et al* (1977). To 5 ml aliquots of blood in Falcon tubes, were added 45 ml of lysis buffer, followed by mixing by inversion several times. Tubes were then allowed to stand on ice for 10 minutes, before pelleting nuclei by spinning in a Sorvall RT6000 centrifuge at 2200 rpm for 10 minutes. The two pellets so produced per patient sample were then combined by suspension in 4.5 ml of 75 mM NaCl, 25 mM EDTA, pH 8.0. This was followed by the addition of 0.5 ml of 5% SDS containing 2 mg/ml of proteinase K. The samples were mixed well and incubated at 37°C overnight. Samples were then extracted with 5 ml tris-saturated phenol by rotating on an aliquot mixer for 30 minutes at room temperature, followed by 5 ml of chloroform:isoamyl alcohol mixture (24:1), with a further 30 minute mix. The two phases were separated by centrifugation for 10 minutes in an RT6000 centrifuge at 2200 rpm and the top layer removed, taking care not to remove the protein debris at the interface. This procedure was repeated and the residual phenol then removed by a third extraction with chloroform:isoamyl alcohol alone.

DNA was precipitated by the addition of 0.5 ml (1/10th vol.) of 3M sodium acetate, pH 5.0 and 11 ml of absolute ethanol, mixing by inversion, followed by standing at room temperature for 10 minutes. The precipitated DNA was spooled out using a hooked Pasteur pipette, then washed briefly in 70% ethanol and squeezed dry on the side of the

tube. The DNA pellets placed in 1 ml of 10 mM tris-HCL, 1 mM EDTA (pH 7.4) and allowed to dissolve at 4°C for 2 days or more in screw cap Eppendorf tubes.

(g) Southern Blotting.

Genomic DNA (2 μg), was digested overnight to completion with 10 U of *Taq*1 restriction enzyme in the presence of bovine serum albumen and restriction buffer, as recommended by the manufacturer. The digests contained 3.0 μL of x10 buffer, 3.0 μL of 1 mg/ml bovine serum albumen, 10 U of restriction enzyme (1-2 μL) and sterile, glass distilled water to a total volume of 30 μL. Incubation was carried out at 65°C and evaporation was limited by overlaying 2 drops of mineral oil. The μL volumes were measured and delivered using a P20 Gilson Pipetman.

Digests were electrophoresed in 10x10x1cm 0.8% agarose slabs containing tris-borate-EDTA buffer and 2 μg/ml of ethidium bromide as a DNA stain. Samples were loaded in the presence of 1/10th volume of sample-buffer containing Orange G as a marker using a P200 Gilson Pipetman and gels run at 30 volts until the dye reached the end.

The genomic restriction fragments were depurinated by soaking the gel in 0.25 M HCL for 15 minutes, in order to reduce the size of the larger fragments and accelerate the subsequent blotting process. After a brief wash in distilled water, restriction fragments were transferred overnight to Zeta-Probe nylon membranes in a Southern blot assembly.

Approximately 600 ml 0.4 M NaOH was placed in a plastic tray with a glass plate positioned across, bearing a

double thickness 3 MM paper wick. The gels were placed upside down on the wicks and covered with a piece of Zeta-Probe membrane, cut to the same size and soaked in 0.4 M NaOH. The edges of the membrane were then sealed with clingfilm and two further layers of 3 MM paper of the same dimensions, soaked in 0.4 m NaOH, applied. Care was taken to remove air bubbles at each stage. Finally, two boxes of tissues were applied and covered with a glass plate bearing lead weights.

After overnight blotting, the completeness of DNA transfer was checked by examining the gel slabs for residual DNA under ultra violet light. The membranes were rinsed briefly in 2x SSC prior to hybridisation with ^{32}P probes.

(i) Preparation of ^{32}P-labelled probes.

cDNA inserts from plasmids pABF, pSB9 and pAB1, which together cover the coding sequences within the apoB gene, were oligolabelled with ^{32}P, prior to subsequent hybridisation to the Southern blots. In some instances, 1.6 kb *Hind*III/*Xba*I and 0.75 Kb *Xba*I genomic fragments were similarly labelled and used to probe specific Southern blots.

To oligolabel the probes, 60 ng of cDNA or genomic DNA fragments were first denatured by boiling for 90 seconds, followed by cooling for 2 minutes to 0°C on ice. To the denatured DNA was added 11.5 μL of cold labelling solution, 1.0 μL of 10 mg/ml bovine serum albumen, 5 μL of ^{32}P dCTP and 2.5 U (0.5 μL) of Klenow DNA polymerase to a final volume of 25μL. The μL volumes were measured and delivered using a P20 Gilson Pipetman.

Following overnight incubation, an equal volume of bromophenol blue TENS, was added to the oligolabelling mix and the probe isolated on a P60 gel filtration column. This was prepared by plugging a Pasteur pipette with glass wool and filling it to within 0.5 cm of the top with P60 in TENS. The probe was then loaded onto the column and eluted with TENS. Labelled DNA was collected into an Eppendorf tube whilst monitoring with a Gieger counter, with it's probe positioned at the bottom of the column. Collection was commenced when counts reached 50 cpm and stopped when they had fallen to 100 cpm.

A 2 μL aliquot of the collected fraction was added to 10 ml of scintillation fluid for subsequent counting in a Bioscan counter. The counts obtained were used to calculate the total counts of the probe preparation and it's specific activity (cpm/μg DNA).

(ii) Hybridisation of probes to Southern blots.

In order to limit non-specific binding of probe to the Zeta-Probe membranes and thus reduce background, a prehybridisation procedure was first carried out. Blots were sealed in polythene hybridisation bags with 10 ml of a prehybridisation mix containing sonicated, heat denatured salmon sperm, taking care to exclude air bubbles. The bags were placed in a 65°C shaking water bath and prehybridisation allowed to proceed for 1/2-24 hours.

Following incubation, the prehybridisation mix was replaced with 10 ml of fresh mix containing ^{32}P probe, with an activity of 10^6 cpm/ml, that had been denatured by boiling for 5 minutes. The bag was then resealed, taking

care to exclude air bubbles and incubated in a 65°C shaking water bath overnight.

Following hybridisation the membranes were removed from the bags and rinsed briefly in 2x SSC. They were then washed successively by vigorous agitation for 15 minutes at room temperature in 2x SSC/0.1% SDS, 0.5x SSC/0.1% SDS and 0.1x SSC/0.1%SDS. A final wash was carried out in 0.1x SSC/1% SDS at 60°C for 30 minutes.

(iii) Autoradiography

Following the final wash, membranes were rinsed briefly in 0.1% SSC, the blotted lightly between 3 MM filter paper and wrapped immediately in Saran Wrap. The bottom left hand corner of the blot was marked with ^{35}S radioactive ink to enable subsequent orientation of the autoradiograph. The blot, in Saran Wrap, was taped to the base of an X-ray cassette and a similar sized piece of fast film, followed by an intensifying screen, applied in a darkroom, under red light. The sealed cassette was placed in a -70°C freezer for 1-4 days exposure.

(h) Oligonucleotide Preparation.

The oligonucleotides for use in the polymerase chain reaction amplification of patient genomic DNA, were synthesised on an Applied Biosystems 380A synthesiser. They were subsequently purified on Applied Biosystems OPC columns according to manufacturers instructions and dissolved at 1 mg/ml in water. The oligonucleotides synthesised and their position within the apoB coding sequence are shown below.

For determination of the P family apoB gene mutation.

CP1 21'mer CTGTTAGGACACCAGCCCTCC 4072-4092

CP2 23'mer GCCACCACTGTAGGAGGCGGACC 4256-4234

For determination of the D family mutation in the maternal apoB allele.

DD1 23'mer CCCTCACCTCCACCTCTGATCTG 4756-4778

DD2 22'mer CTTAAGTCCTTCTTGACTGACC 5339-5318

DD3 24'mer CAGGCCATGATTCTGGGTGTCGAC 5271-5294

DD4 24'mer CCCATTGCCATTTGTATGTGCATC 5873-5852

DD5 24'mer GTAATGGCCCCGTTTACCATGACC 5823-5846

DD6 23'mer GGCATGTGAAACTTGTCTCTCCC 6542-6520

For determination of the 5' flanking region sequence of the D family paternal apoB allele.

D7 24'mer GAGGTTGCTCTTCCCCAGAGGCCT -660 - -636.

D8 24'mer GGTGCCTTCTTCAGCTGACCCACC -301 - -324.

D9 24'mer GGTGGGTCAGCTGAAGAAGGCACC -324 - -301.

D10 25'mer GGGCTCCTCAGCTGCAGCAACCGAG +59 - +35.

D7 contained a *Stu*I restriction enzyme site (underlined). D8, D9 and D10 were modified from the original sequence by single base changes (in bold) to include *Pvu*II sites (underlined). The presence of these sites enabled the resultant PCR products to be blunt-ended prior to sequencing, by *Stu*II/*Pvu*II digestion (see below).

(i) Polymerase Chain Reaction

The polymerase chain reaction is a technique that allows specific DNA sequences to be amplified, producing a selective enrichment of sequences up to 2000 base pairs long by a factor of a million or more (Saiki *et al.*, 1987). The target sequence is defined by two oligonucleotides, one

complementary to the sense strand at the 5′ end of the sequence and one to the antisense strand at the 3′ end. Genomic DNA is mixed with the oligonucleotides in a buffer containing dATP, dCTP, dGTP and dTTP and after a denaturation step, the thermostable *Taq* DNA polymerase (isolated from *Thermus aquaticus*) is added and the temperature is reduced to allow the mixture to anneal. The mixture is then raised to an intermediate temperature to promote the synthesis of new complementary strands between the oligonucleotides, thereby amplifying the target sequence by two. Repeated application of this triple temperature cycle results in successive doubling of the target sequence until a million-fold amplification is achieved.

Patient genomic DNA samples (5 μg) were subjected to 30 cycles of polymerase chain reaction amplification. The reactions were carried out in 10 mM tris-HCL pH 8.5, 50 mM KCL, 2.5 mM $MgCl_2$, 200 mM each of dTTP, dCTP, dGTP and dATP and 1 μg each of 5′ and 3′ oligonucleotide. Incubations were performed in three water baths set at the three reaction temperatures.

Following a prolonged initial denaturation step at 95°C for 10 minutes, 2 units of *Taq* polymerase (Cetus) was added. The reaction mixes were then held at 55°C for 1.5 minutes to allow the first annealing step to take place, then 2 minutes at 70°C for the first extension reaction. The subsequent 28 cycles consisted of 1.25 minutes at 95°C for denaturation, 1.5 minutes at 55°C for annealing and 2 minutes at 70°C for extension. The 30th cycle was completed with a 10 minute extension step.

(j) Cloning into phage M13.

Following the polymerase chain reaction, fragments of amplified DNA were purified from the reaction mixtures. This was accomplished by running the mixtures at 100 volts in 1% agarose gels containing tris-borate-EDTA (TBE) buffer and 2 μg/ml ethidium bromide as a DNA stain. The amplified DNA was identified by examining the minigels under long-wave ultra violet light in a darkroom. Small pieces (1x0.5 cm) of DE81 paper pre-prepared for electroelution were then inserted into cuts made in front of and behind the amplified bands. The DNA was then electroeluted onto the anodic DE81 pieces by electrophoresis at 100 volts for a further 10 minutes. The cathodic DE81 pieces served to prevent contamination from higher molecular weight species of genomic DNA. After elution from the DE81 paper, the amplified DNA was blunt-ended. In the case of the products from oligonucleotides PD1-2 and DD1-6, this was accomplished using T4 DNA polymerase (Maniatis *et al.*, 1982). In the case of the product of D7 and D8, which contained respectively *Stu*I and *Pvu*II restriction enzyme sites, this was achieved by performing a double digest with *Stu*II and *Pvu*II. For the product of D9 and D10, a digest with PvuII alone was used.

After blunt-ending, the products were phoshorylated with T4 polynucleotide kinase (Maniatis *et al.*, 1982). Approximately 50 ng of DNA was ligated to 10 ng of M13tg131 vector that had been digested with *Eco*RV and dephosphorylated with alkaline phosphatase (Maniatus *et al.*, 1982).

(k) Preparation of Competent Cells.

E. Coli DH5αF' cells were inoculated into two tubes each containing 2 ml SOB medium and 20 μL magnesium buffer and grown up at 37°C overnight.

The 4 ml of medium was transferred into 40 ml SOB to which had been added 400 μL magnesium buffer in a sterile 300 ml conical flask. Cells were grown up in a shaking incubator at 37°C. The optical density (OD) of a culture aliquot was measured at 600 nm at 20 minute intervals. When the OD reached approximately 0.5, corresponding to about $6x10^7$ cells, the culture was collected into a Falcon tube and left on ice for 15 minutes.

The cells were pelleted by centrifugation in an RT6000 centrifuge at 2500 rpm for 15 minutes. The supernatant was removed by Pasteur pipette, followed by draining the pellets by inverting the tubes over tissues. The pellets were resuspended in 1/3 of the culture volume of RF1 and left on ice for 15 minutes. After repelleting by a further 15 minutes centrifugation at 2500 rpm in an RT6000 centrifuge, the cells were resuspended in 1/12.5 of the culture volume of RF2 and left on ice for 30 minutes.

Finally, 50μL aliquots of the cells were flash-frozen in an ethanol/dry ice mix, then stored at -70°C.

(l) Sequencing

One tenth of the amplified DNA/M13tg131 ligation mixes were used to transform 100 μL of competent E.coli DH5αF' cells by incubation for 30 minutes on ice, followed by 2 minutes of heat shock at 42°C. To 2.5 ml of top agar containing 50 μl of a 2% solution of X-gal and 50 μl of a

2% solution of IPTG were added 200 µl of a mid-log phase culture of plating cells together with the transformed cells and the mixture was then spread over the surface of pre-poured TY agar plates. Following overnight incubation at 37°C, single colourless recombinant phage plaques were picked and inoculated into 1.5 ml of a 1:100 dilution in warm TY medium of an overnight culture of plating cells. After shaking the cells for 7 hours at 37°C, cells were removed from the resultant phage suspensions by centrifugation. 250 µl of 20% PEG in 2.5 mMol NaCl were then added to the supernatant and the mixtures allowed to stand at room temperature for 30 minutes. Precipitated phage was then pelleted by centrifugation in a MSE Microcentaur microcentrifuge for 10 minutes, the supernatant removed and the pellets resuspended in 100 µl TE pH 8.0. DNA was then prepared from the pellets by vortexing with 50 µl phenol followed by 50 µl chloroform. After recentrifugation the supernatants were removed and a further phenol-chloroform extraction performed. DNA was then precipitated with an equal volume of 5 mMol ammonium acetate and 2.5 volumes of absolute ethanol. Finally, the template DNA was dissolved in 30 µl TE pH 8.0 and 2 µl run on a mini-gel to check it's concentration.

Sequencing reactions were performed using Sequenase and [^{35}S]dATPαS. Sequenase is a chemically or more recently genetically modified variant of bacteriophage T7 DNA polymerase (Tabor and Richardson., 1987) which lacks the 3'-5' exonuclease activity of the wild-type enzyme and has high processivity (average number of nucleotides incorpo-

rated before dissociation), high speed and is able to utilise a range of nucleotide analogues. Sequenase USB sequencing kits were used to perform the sequencing reactions according to the manufacturer's instructions.

(i) Preparation of Sequencing gels.

Two glass sequencing plates, one whole and one lugged, were first washed with detergent, rinsed with deionised water and wiped dry with an absorbent paper towel. The plates were then wiped with ethanol and left to dry for 5 minutes, before application of a little dimethyldichlorosilane spread over their surfaces with a paper towel.

Plastic plate spacers were positioned at the edges of the whole glass plate, siliconised face up and the plate with lugs positioned on top, siliconised face down. The plates were held together by two pieces of tape on each side, before applying tape to the full length of both sides with 5 mm overlaps at each end which were folded over. The full length of the bottom of the gel was sealed with a further piece of tape. Finally, the edges of the tape along the two sides and bottom of the plate were sealed further with a second application of tape.

To prepare the gel, 10 ml of a solution of 30% by weight of acrylamide and 0.8% by weight of N,N'-bis-methylacrylamide was mixed with 5 ml of 10X tris-borate-EDTA buffer, 50 ml glass distilled water and 350μL ammonium persulphate in 250 ml conical flask. To the mixture was then added 25 g of ultra-pure urea, which was dissolved by running hot water over the flask. The solution was vacuum filtered

and placed in a 150 ml beaker before addition of 30 μL tetramethylethylenediamine (TEMED). It was then taken up in a 50 ml syringe and mixed by drawing up and down on the syringe plunger. Finally the mixture was dispensed with the syringe between the gel plates, lying with the lugged end positioned on two coins to create a slight incline, taking care to avoid bubble formation. The plates were left undisturbed for 30 minutes to allow complete polymerisation.

(ii) Loading and Running Sequencing Gels.

Gels, prepared within the previous day were pre-run for 30 minutes and the sequencing reaction mixes were loaded 8 adjacent lanes at a time in the base order TCGA. For smaller amplified sequences (200-400) bases, gels were run for 6 hours; for longer sequences (400-800), 12 hours. After running, gel plates were separated and leaving the gel on one of the plates, then soaked in 10% acetic acid, 12% methanol for 30 minutes to remove the urea. The upper surface of the gel was then covered with a slightly larger piece of filter paper, inverted and the glass plate removed. A piece of Saran wrap was then layered over the gel before drying at 80°C in a gel drier.

(iii) Autoradiography.

After drying down, the Saran wrap was removed and the gels taped to the base of autoradiography cassettes before applying a cassette-sized sheet of Kodak XAR-5 film directly to the dried gel surface. Cassettes were kept at room temperature and the films developed after 24-36 hours.

2.5 Methods used in the investigation of abetalipoproteinaemic individuals.

(a) DNA preparation.

Genomic DNA was extracted from 10 ml of peripheral blood by a method based on that of Kunkel *et al* (1977), as for the hypobetalipoproteinaemic individuals.

(b) Southern Blotting.

Genomic DNA was digested with the restriction enzymes *Hinc*II, *Pvu*II, *Xba*I, *Eco*RI and *Msp*1 (2-10 U/μg DNA) under conditions recommended by the manufacturers. The digested DNA was electrophoresed in 1% agarose slabs and Southern blotted as for the hypobetalipoproteinaemic samples, with transfer to either Zeta-Probe or Hybond-N membranes.

(c) DNA Probes.

Southern blots were hybridised with four different apoB probes. The *Xba*1 and *Eco*R1 blots were probed with a mixture of two cDNA probes from the 3' end of the apoB gene, AB1 and AB7 (Knott *et al.*, 1985). These probes detected the presence or absence of a polymorphic *Xba*1 site at codon 2488 and a polymorphic *Eco*RI site at codon 4154 (Talmud *et al.*, 1987). The *Msp*1 blots were probed with a unique 2.0 Kb, *Hind*III, genomic apoB fragment, known as BH2. This detected variations in two *Msp*I sites in the hypervariable region in the 3' untranslated region of the gene (Knott *et al.*, 1986). The *Pvu*II and *Hinc*III blots were probed with a cDNA segment of 959 bp, containing the 5' coding region of apoB and 86 bp upstream of the initiation codon. This enabled detection of the *Pvu*II and *Hinc*II polymorphisms positioned towards the 5' end of the

gene (Darnfors *et al.*, 1986). A map of the apoB gene showing the position of the polymorphic sites and the fragment sizes of the *Xba*I, *Eco*RI, *Pvu*II and *Hinc*II polymorphisms together with the probes used is shown in Figure 3. Probes were labelled and hybridised to blots using techniques similar to those used for the hypobetalipoproteinaemic individuals.

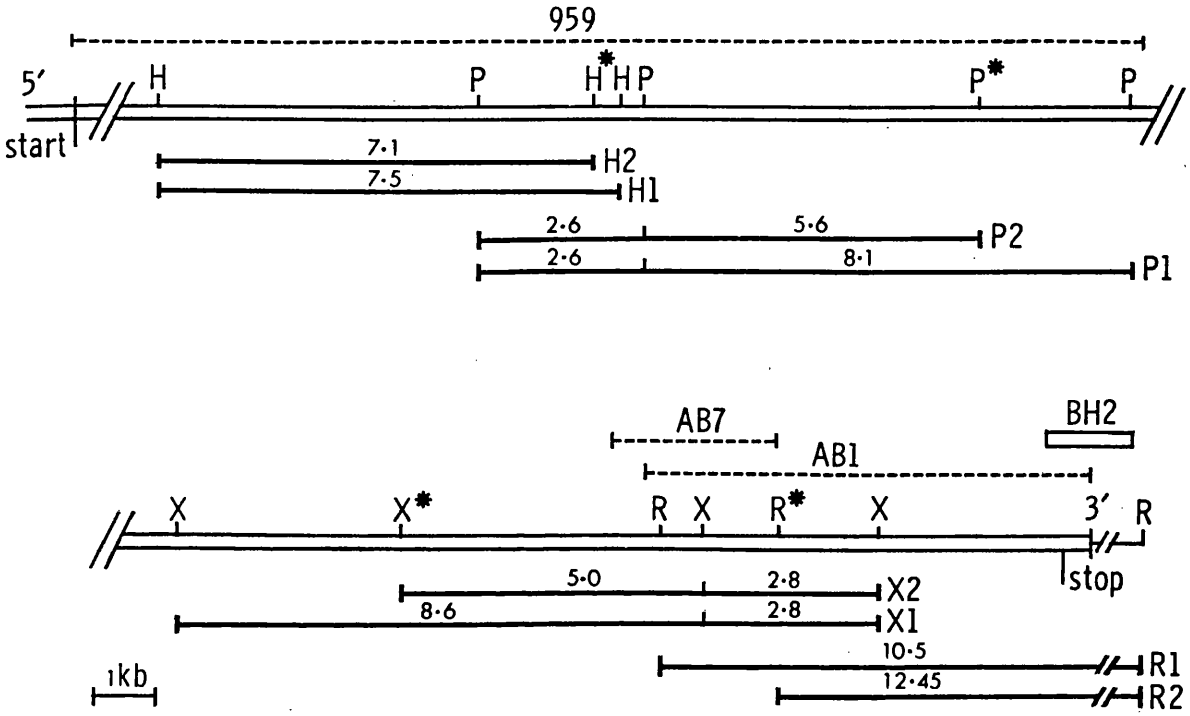


Figure 3.

A map of the 5' and 3' ends of the apoB gene showing the apoB RFLP's obtained using restriction enzymes *Hinc*II (H), *Pvu*II(P), *Eco*R1(R) and *Xba*1(X). The polymorphic restriction sites are indicated by (*). The cDNA probes used to detect these polymorphisms, 959, AB1 and AB7, are shown above in dashed lines and the fragment sizes obtained are shown below in solid lines. The genomic probe BH2, used to detect the 3' hypervariable *Msp*1 polymorphism is shown above as a bar.

Chapter 3: Results.

3.1 Hypobetalipoproteinaemic Individuals.

Two families with familial hypobetalipoproteinaemia were studied. The D family had an individual DD, with homozygous hypobetalipoproteinaemia with parents PD (mother) and AD (father) with heterozygous hypobetalipoproteinaemia. The P family had an individual CP, with heterozygous hypobetalipoproteinaemia with a normal spouse, MP, one normal daughter, LP and one daughter ChP, with heterozygous hypobetalipoproteinaemia. The plasma lipid and apoB concentrations of the D and P family members are shown in Table 1.

(a) Southern Blotting.

To begin with, genomic DNA from these individuals was digested with the restriction enzyme *Taq*1, then Southern blotted and probed with ^{32}P-labelled cDNA's spanning the apoB coding sequence.

Restriction patterns obtained from the members of the D family, DD, AD and PD, were identical to those obtained from normal controls. In the P family however, individual CP was heterozygous for the loss of a *Taq*1 restriction site when probed with a cDNA insert from plasmid pABF (Figure 4). Control genomic DNA gave a pattern of 5 bands of 0.8, 2.0, 2.8, 3.2 and 3.4 Kb. DNA from individual CP however, showed the same basic pattern, but with a novel band of 3.6 Kb and fainter than normal bands of 0.8 and 2.8 Kb. This pattern was compatible with the heterozygous loss of a Taq1 site situated between the 0.8 and 2.8 Kb fragments, which thus combine to give the novel 3.6 Kb fragment.

Subject	Age	Total Cholesterol (4.0-6.5 mmol/l)*	Triglyceride (0.3-1.8 mmol/l)*	ApoB (60-140 mg/dl)*
PD (Mother)	44	2.57	0.59	17
AD (Father)	48	2.82	0.32	28
DD (Daughter)	21	1.27	0.30	1.5
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 ♦	83

* Normal ranges are 5-95 percentile ♦ Non-fasting level

Table 1:- Plasma lipid and apoB concentrations of hypobetalipoproteinaemic individuals.

DD is a homozygote for the disorder; PD, AD, CP and ChP are heterozygotes and MP and LP are unaffected.

Study of the *Taq*1 restriction map of the genomic apoB sequence, indicated that the mutated *Taq*1 site was most likely to be in exon 25. To examine this hypothesis, *Taq*1 Southern blots of patient and control DNA were hybridised separately with two genomic probes produced by a double digest of plasmid pSB6 (which runs from intron 20 to exon 26), with restriction enzymes *Hind*III and *Xba*1. The *Hind*III/*Xba*1 fragment was 1.6 Kb and ran from the *Hind*III site in intron 23 to the *Xba*1 site just 3′ to the postulated mutated *Taq*1 site in exon 25. The *Xba*1/*Xba*1 fragment was 0.75 Kb and ran from the exon 25 *Xba*1 site to the *Xba*1 site at the 5′ end of exon 26, just 5′ to the next *Taq*1 site (Figure 5). The probes were thus complementary to the 2.8 and 0.8 Kb *Taq*1 restriction fragments respectively, predicted to be derived from this region.

Use of then probes demonstrated that the 1.6 Kb *Hind*III/*Xba*1 probe hybridised to the 2.8 Kb *Taq*1 fragment, whilst the 0.75 Kb *Xba*1/*Xba*1 fragment hybridised to the 0.8 Kb fragment on both patient and control Southern blots. This confirmed that the 2.8 and 0.8 Kb bands seen with probe ABF were derived from the intron 21-exon 26 region. On patient Southern blots, both probes also hybridised to the novel 3.6 Kb fragment, indicating that it resulted from the loss in one allele, of the *Taq*1 site in exon 25 as predicted.

The spouse, MP and daughter, LP both gave a normal pattern with probe pABF and the 0.75 and 1.6 Kb probes. The daughter ChP however, who had been undergoing investigation for fat malabsorption and been diagnosed as having

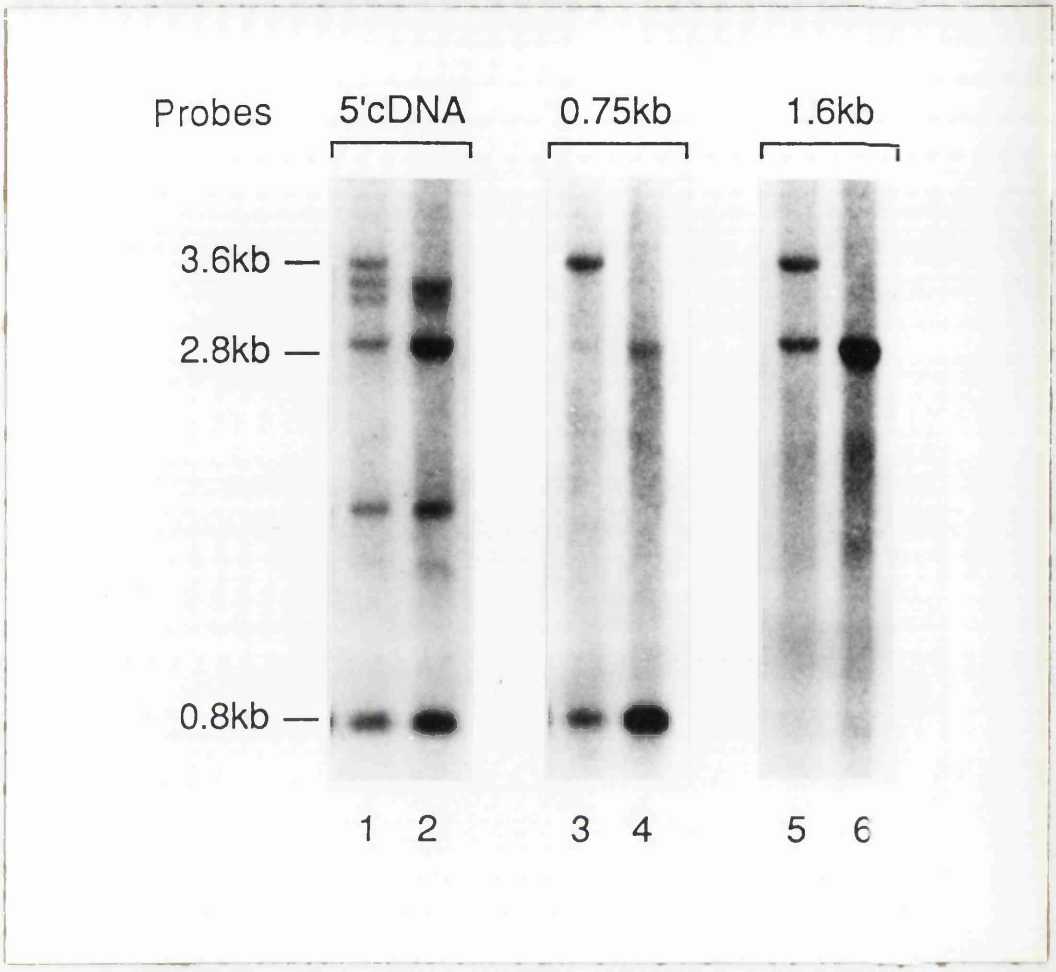


Figure 4

Genomic Southern blots of *Taq*1 digested DNA from subject CP. Lanes 1, 3 and 5, DNA from patient CP. Lanes 2, 4 and 6, control DNA. Lanes 1 and 2 were hybridised with pABF (Knott *et* ., 1986), a 6.5 Kb cDNA from the 5' end of the apoB message. Lanes 3 and 4 were hybridised with a 0.75 *Xba*1 genomic fragment and lanes 5 and 6 with a 1.6 Kb *Hind*III/*Xba*1 genomic fragment (Figure 5).

heterozygous hypobetalipoproteinaemia, gave Southern blots with the same novel band pattern as her mother with these probes, showing that she too was heterozygous for the loss of the exon 25 *Taq*1 site (Figure 6).

The restriction enzyme *Taq*1 recognises and cuts at the sequence TCGA, contained within which, is a CpG dinucleotide. In vertebrate genomes, the majority of CpG dinucleotides are thought to be methylated on the cytosine (Cooper., 1983). Such methylation apparently renders the cytosine susceptible to deamination to form thymidine (Coulandre *et al.*, 1978; Wang *et al.*, 1982). As a result, *Taq*1 site mutations commonly occur through spontaneous deaminations of the 5-methyl cytosines in methylated CpG dinucleotides within the TCGA recognition sequence, to give a C to T transition and a sequence no longer recognised by *Taq*1. The *Taq*1 site in exon 25 of the apoB sequence contains the arginine codon 1306 (CGA). If a C to T transition occurs in the coding strand at this point, the result is a transformation of the *Taq*1 sequence TCGA to TTGA. The sequence TTGA then contains the codon TGA, which in the mRNA is represented by UGA, a translation termination codon. If the transition occurs in the non-coding strand, the result is a transformation of sequence AGCT to AGTT, which, in the coding sequence, is represented by a transition of the sequence TCGA to TCAA. In this case the arginine codon CGA becomes a glutamine codon CAA. Thus, only if the exon 25 *Taq*1 site is lost by a C to T transition on the coding strand, is there likely to be a significant effect on apoB production compatible with the

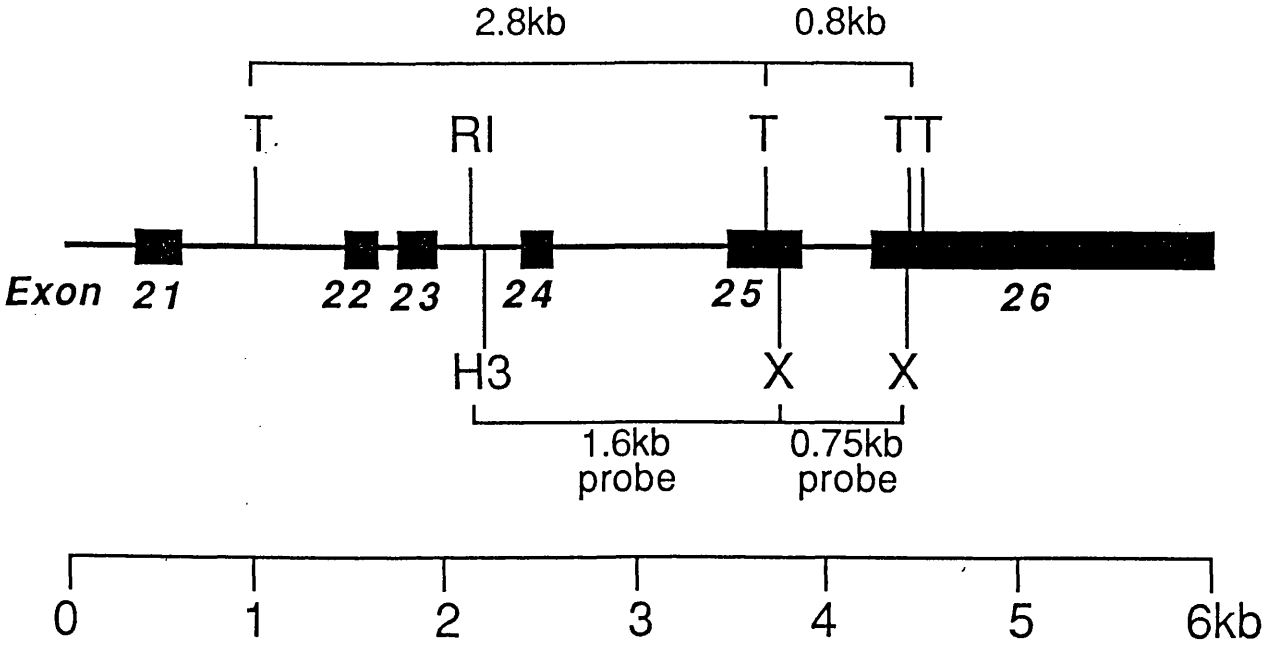


Figure 5.

*Taq*1 mutation in hypobetalipoproteinaemia. Restriction map of the apoB gene around the *Taq*1 mutation in patient CP. The positions of the two genomic probes and the *Taq*1 fragments which they detect are shown. Loss of the *Taq*1 site in exon 25 joins the two *Taq*1 fragments to give a 3.6 Kb band (Figure 4). Restriction enzyme sites are shown as T = *Taq*1, R1 = *Eco*R1, H3 = *Hind*III and X = *Xba*1.

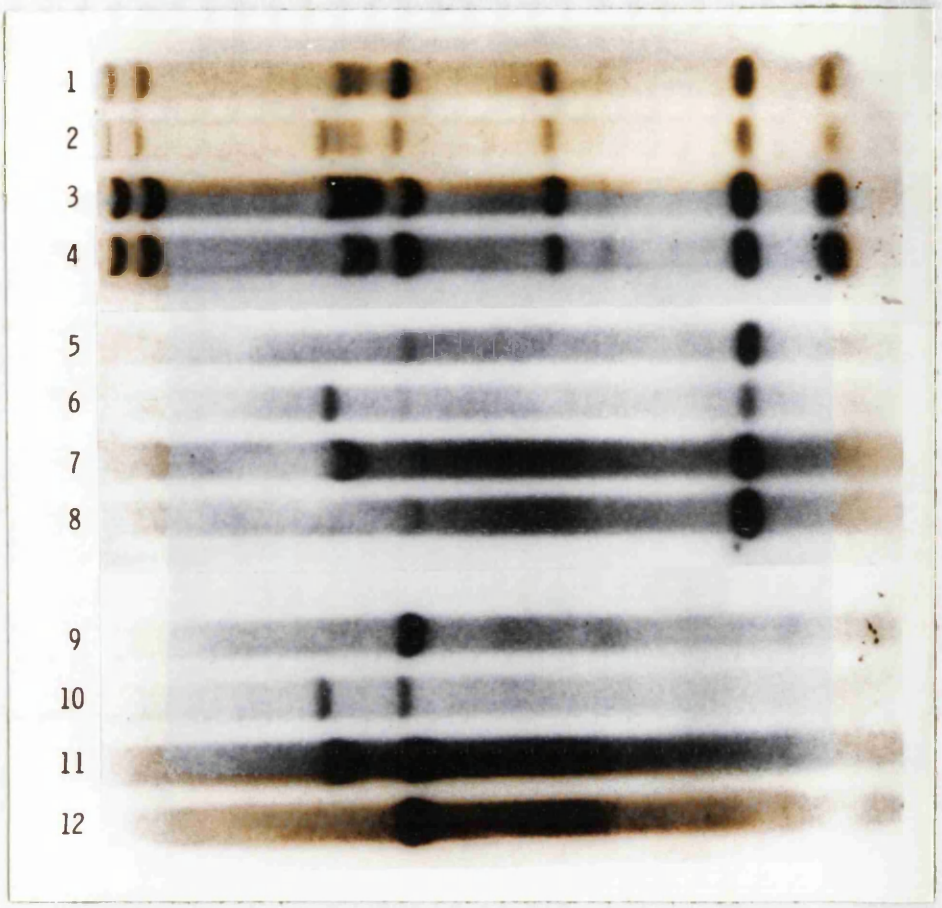


Figure 6.

Genomic Southern blots of *Taq*1 digested DNA from first degree relatives of subject CP. Lanes 1, 5 and 9, DNA from the husband MP; Lanes 2, 6 and 10, subject CP; Lanes 3, 7 and 11, the daughter ChP and Lanes 4, 8 and 12, the daughter LP.

Lanes 1-4 were probed with pABF (Knott *et al.*, 1986), a 6.5 Kb cDNA from the 5′ end of the apoB message; Lanes 5-8 with a 0.75 Kb genomic *Xba*1 fragment and Lanes 9-12, with a 1.6 Kb *Hind*III/*Xba*1 genomic fragment (Figure 5).

hypobetalipoproteinaemic phenotype. It was thus essential to obtain that part of the apoB sequence of subject CP's genomic DNA that contained the exon 25 *Taq*1 site, in order to demonstrate that arginine codon 1306 (CGA) had mutated to a stop codon (TGA), rather than a glutamine codon (CAA).

(b) Identification of truncated apoB species by SDS polyacrylamide gel electrophoresis and immunoblotting.

Plasma lipoproteins from members of the D and P families (DD, PD and CP) were fractionated into three density intervals by ultracentrifugation, $d<1.0019$ g/ml, containing a mixture of chylomicron, VLDL and IDL, $1.019<d<1.063$ g/ml, containing LDL and $1.063<d<1.21$ g/ml, containing HDL. The lipoprotein fractions were delipidated and their proteins separated, along with those of the infranatant fraction, $d<1.21$ g/ml, by gradient SDS polyacrylamide gel electrophoresis and gels were stained with Coomassie Brilliant Blue. Immunoblots of the lipoprotein gels were performed with an apoB monoclonal antibody Sol 9, directed to an epitope near the amino terminus of apoB (Pease *et al.*, 1990).

In the individual CP, the truncated apoB species predicted on the basis of termination at amino acid 1305 as a result of a stop signal at codon 1306 and calculated to be apoB29 on the centile system (Kane *et al.*, 1980; Kane., 1983), could not be demonstrated. Using both Coomassie Brilliant Blue staining and Western blotting, no apoB29 species could be detected in any of the lipoprotein fractions (Figures 7 and 8). Similarly, in the infranatant frac-

tion, although Western blotting could not be applied due to the high background signal produced by serum immunoglobulins, Coomassie Brilliant Blue staining failed to demonstrate any abnormal protein band of the size of apoB29 (Figure 7).

There were two possible explanations for the complete absence of apoB29 in patient CP. Firstly, the *Taq*1 site loss present in CP was due to an arginine (CGA) to glutamine (CAA) change at codon 1306. Secondly, the *Taq*1 site loss was due to an arginine (CGA) to stop (TGA) transition, but, for some reason, the short, apoB29 protein was not secreted, or was secreted, but was metabolised extremely rapidly. This finding reinforced the need to sequence the relevant region of the apoB alleles in CP.

In the individual DD, SDS polyacrylamide gel electrophoresis demonstrated the presence of an abnormal species of apoB in the chylomicron/VLDL/IDL fraction. The patient's LDL fraction was almost completely devoid of lipoprotein material. The abnormal apoB migrated to a position just behind the myosine standard (Mr 200 kDa) and was estimated to have a molecular weight of approximately 205 kDa. It was absent from the HDL and infranatant fractions. The truncated apoB species was readily demonstrable by Coomassie Brilliant Blue staining as well as Western blotting (Figures 9 and 10). In addition to the truncated apoB, DD showed the presence of a very small amount of apparently full length apoB100 in her chylomicron/VLDL/IDL fraction only. This was only just discernible by Coomassie staining (Figure 9), but more easily seen by Western blotting

(Figure 10).

The individual PD, who was the mother of DD, also showed the same truncated apoB species in her chylomicron/VLDL/IDL fraction and in her LDL fraction. In her case however, it was found in the presence of a much larger quantity of full length apoB100 and in the chylomicron/VLDL/IDL fraction, some apoB48 (Figures 9 and 10). As with her daughter, the short apoB was absent from the HDL and infranatant fractions.

The truncated apoB species present in DD and PD suggested that there might be an abnormality in their apoB coding sequence. Approximate sizing of the protein on SDS polyacrylamide gel electrophoresis suggested that it represented an apoB of between B37 and B40 on the centile system (Kane *et al.*, 1980; Kane., 1983). This could result from a mutation present in the 5' end of exon 26. As with individual CP, it was clearly essential to sequence the relevant region of the apoB alleles in DD and PD.

(c) <u>Characterisation of the apoB gene mutations by amplification of genomic DNA, cloning and sequencing.</u>

In order to elucidate thee precise nature of the apoB mutations in the P and D families, genomic DNA from the relevant individuals was amplified using the polymerase chain reaction (PCR) (Saiki *et al.*, 1987) and sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977). In the individual CP, in whom the truncated apoB species, B29, predicted by the presence of an arginine (CGA) to stop (TGA) transition at codon 1306, could not be

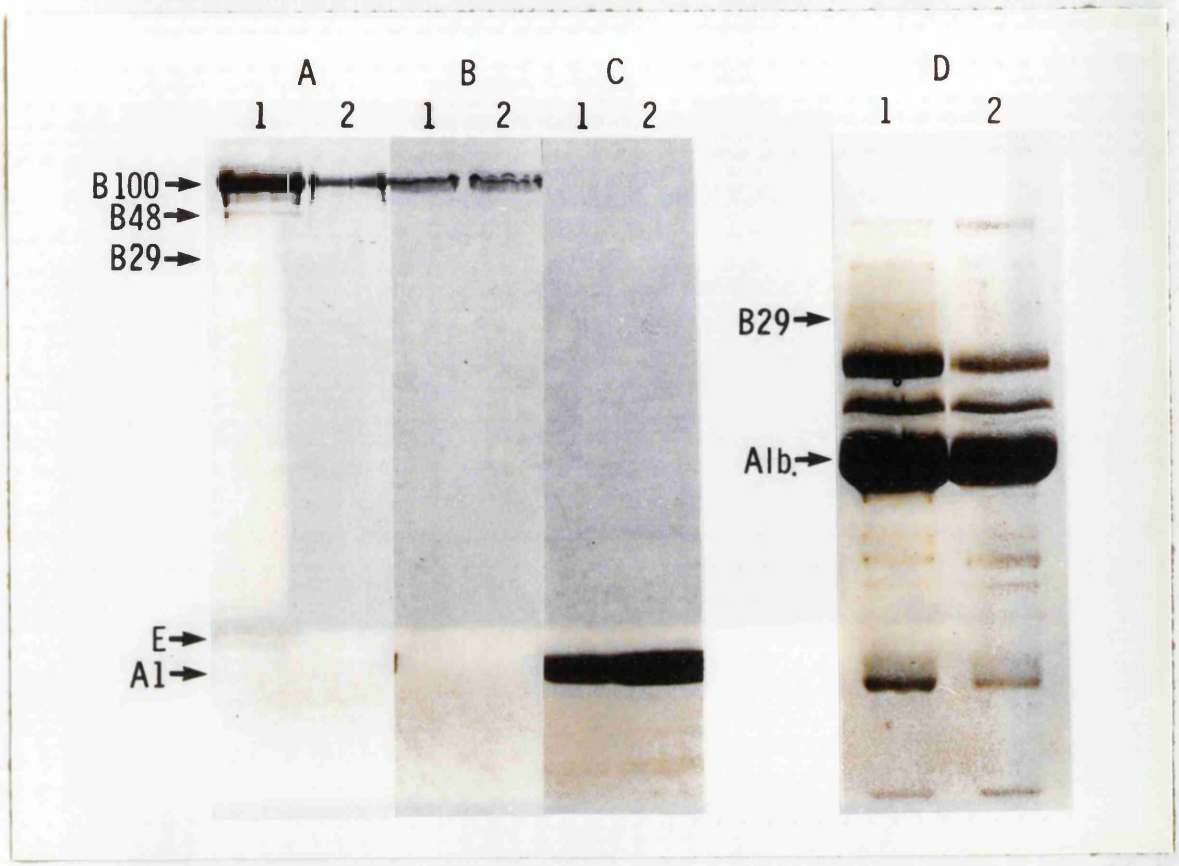


Figure 7.

Polyacrylamide gel electrophoresis of delipidated lipoprotein fractions of subject CP. 1 = Apolipoproteins from control subject. 2 = Apolipoproteins from subject CP. A = VLDL B = LDL C = HDL D = Infranatant fractions. The positions of apoB100, apoB48, apoE, apoA1 and albumen (Alb.) are marked, together with the expected position of the predicted truncated form of apoB, apoB29. No such protein is detectable however.

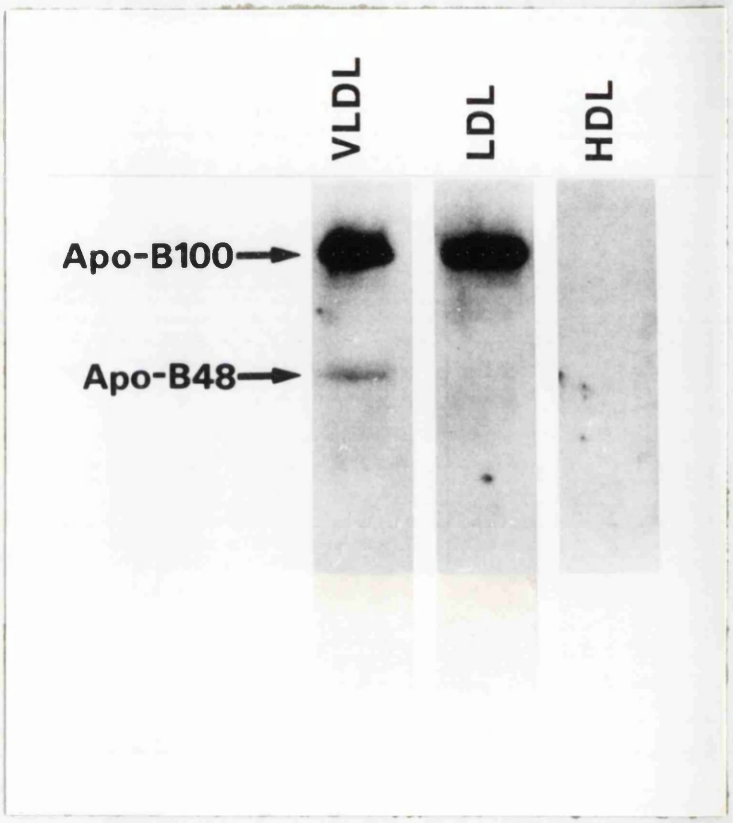


Figure 8.

Western blots of delipidated lipoprotein fractions from subject CP. 50 μL of protein from each fraction were loaded and blots were prepared and probed with anti-human apoB antibody, Sol 9 (see Methods). No truncated form of apoB (predicted to be 29% of the size of apoB100, apoB29) was detectable.

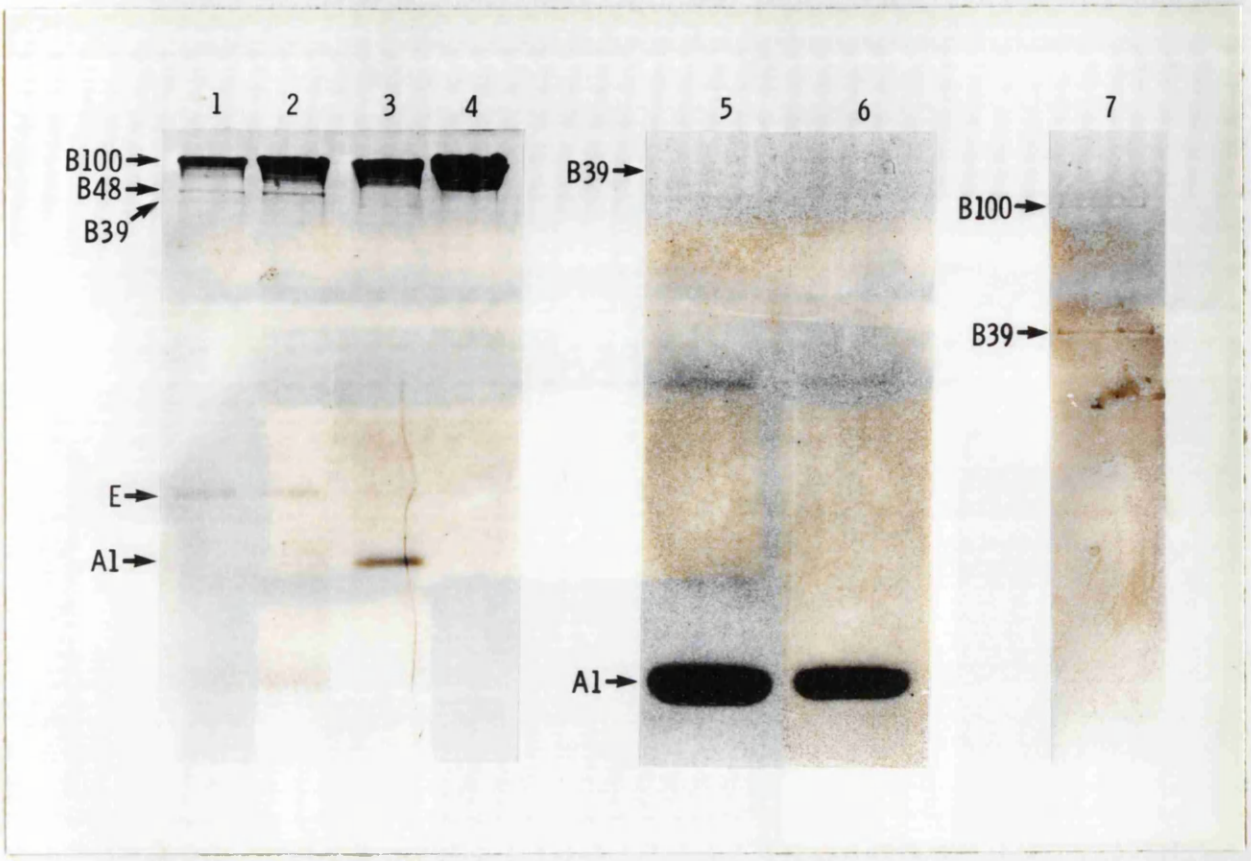


Figure 9.

Polyacrylamide gel electrophoresis of delipidated lipoprotein fractions from subjects PD and DD. 1 = VLDL from PD with 2 = Control VLDL 3 = LDL from PD 4 = Control LDL 5 = HDL from PD 6 = Control HDL 7 = VLDL from DD. The positions of apoB100, apoB48, apoB39, apoE and apoA1 are shown. ApoB39 is present in both the VLDL and LDL, but not the HDL of PD (predicted position indicated) and in the VLDL of DD. There was no protein the size of apoB39 in the LDL, HDL and infranatant fractions of DD nor the infranatant fraction of PD (not shown).

ApoB100 is present in abundance in the VLDL and LDL of PD, but is absent from the LDL and present at considerably reduced concentrations in the VLDL of DD.

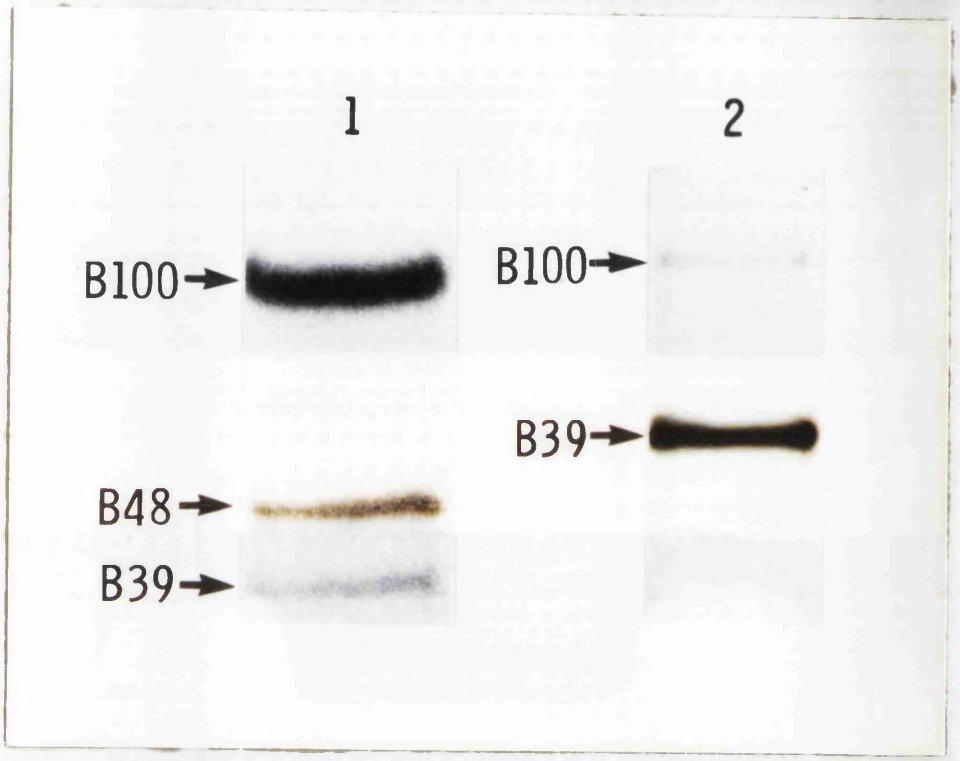


Figure 10.

Western blot of delipidated VLDL from 1. PD and 2. DD. 50 μg of protein were run and blots were prepared and probed with anti-human apoB antibody Sol 9 (see Methods).

In PD, three species of apoB, apoB100, apoB48 and the mutant, truncated apoB species, apoB39 are present. In DD, the major form of apoB present is apoB39, but there is also a small amount of apoB100 present.

demonstrated in the plasma, a section of exon 25, 185 bp long, running from nucleotides 4072 to 4256 of the apoB mRNA, was amplified. This was accomplished using specially synthesised oligonucleotides CP1 and CP2 (see methods). The amplified region spanned the *Taq*1 site which had been shown to be lost in one allele of CP (nucleotides 4124-4127). The PCR fragment was cloned into M13 and a total of 12 clones of the were sequenced in order to be reasonably sure that both alleles were represented. The *Taq*1 site, TCGA, was present in approximately half of the clones, but replaced by TTGA in the remainder (Figure 11). This confirmed that the arginine codon 1306, CGA, was indeed replaced by a stop codon, TGA, in one allele of CP, through mutation of the CpG dinucleotide.

The mutation predicts that the hypobetalipoproteinaemic phenotype in patient CP and her daughter ChP, is due to an in-frame termination codon resulting from a C to T transition at nucleotide 4125 in the coding sequence of one apoB allele. The truncated variant of apoB predicted by the mutation can be referred to as apoB(Arg_{1306}-->Term). It has a calculated pre-glycosylation molecular weight of 146.2 kDa. On the centile system (Kane *et al.*, 1980; Kane., 1983), it can be referred as apoB29 (Figure 13).

For the patients DD and PD, predicted from the size of their truncated apoB species seen on polyacrylamide gel electrophoresis, to have a mutation in one apoB allele near the 5' end of exon 26, a rather larger stretch of apoB coding sequence was amplified. This was necessary in

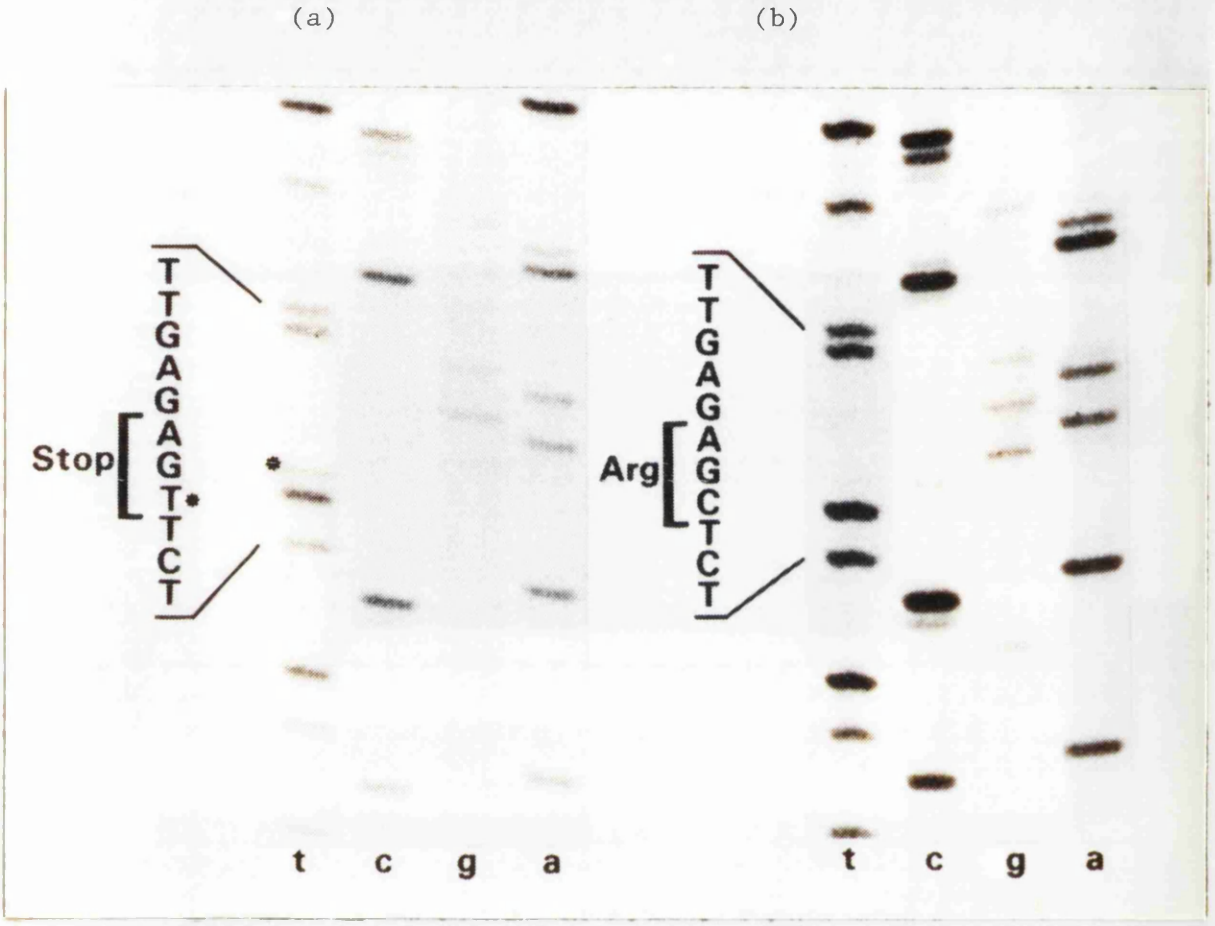


Figure 11.

DNA sequence ladder of apoB gene mutation in the P family. (a) mutant and (b) normal sequences from patient CP showing C to T transition (asterisk) converting arginine codon 1306 to a stop codon.

view of the relative imprecision of sizing high molecular weight proteins by electrophoresis. A section of exon 26, of approximately 1.7 Kb was amplified in three overlapping segments, using oligonucleotides DD1 to DD6 (see methods). The PCR products obtained were cloned and sequenced as for patient CP.

In both DD and PD, approximately half of the clones examined had the apoB sequence usually seen in this region. The remainder of the DD and PD clones however showed deletion of nucleotide G 5591 (Figure 12). The effect of this mutation is to remove the third base of leucine codon 1794 to produce a frame shift in which histidine codon 1795 becomes a methionine codon. Translation then continues in +1 frameshift for a further four amino acids, Tryp-Leu-Val-Thr, before termination at codon 1800.

The predicted apoB variant can be referred to as apoB(His_{1795}-->Met-Tryp-Leu-Val-Thr-Term) and has a calculated pre-glycosylation molecular weight of 201.6 kDa. This is consistent with the molecular weight of around 205 kDa of the mature truncated protein, estimated by migration on SDS-polyacrylamide gel electrophoresis. On the centile system (Kane *et al.*, 1980; Kane., 1983), the protein can be designated apoB39 (Figure 13).

Although both PD and DD were both heterozygotes for the apoB39 mutation, only PD was a clinical/biochemical heterozygote for hypobetalipoproteinaemia. DD had the clinical and biochemical features of homozygous hypobetalipoproteinaemia and on examination of her lipoproteins showed, in addition to apoB39, a very large reduction in

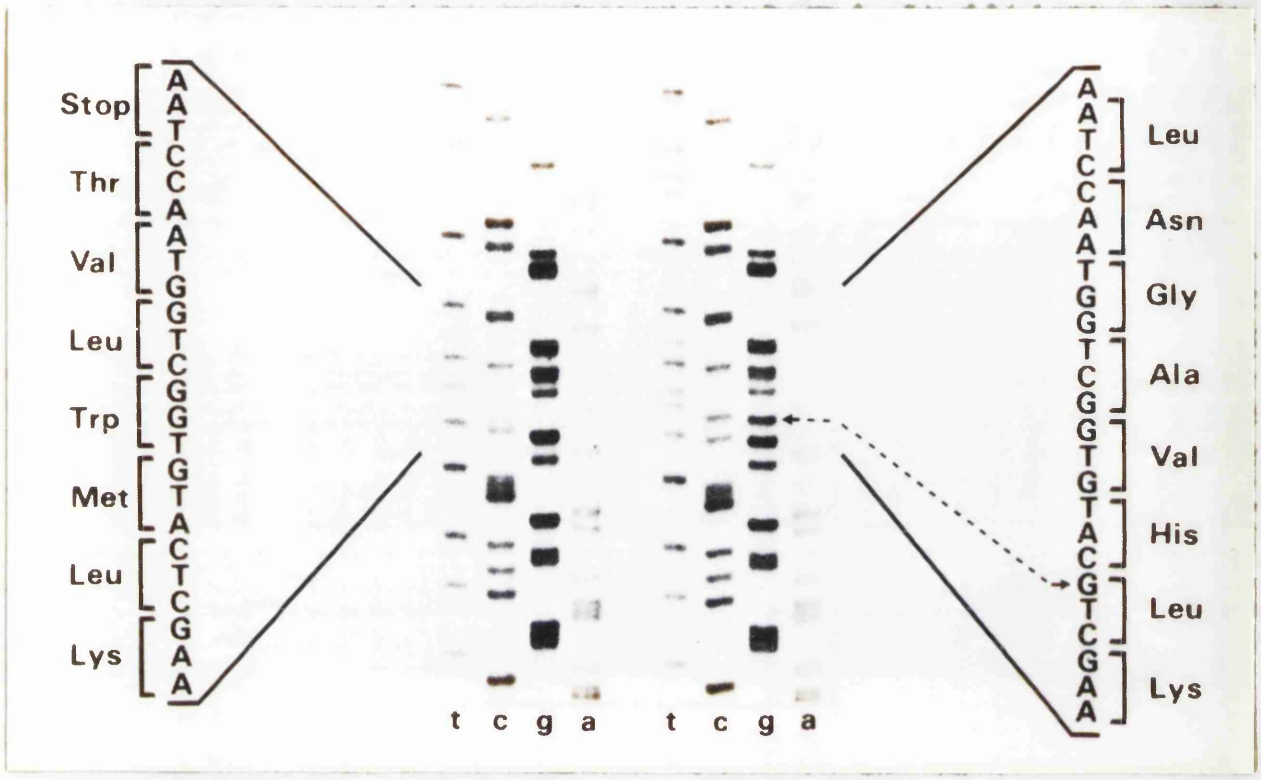


Figure 12

DNA sequence ladder of apoB gene mutation in D family. (a) mutant and (b) normal sequences from patient DD showing deletion of a G nucleotide from leucine codon 1794 (arrowed). The frame-shifted and normal translations are displayed alongside the ladders.

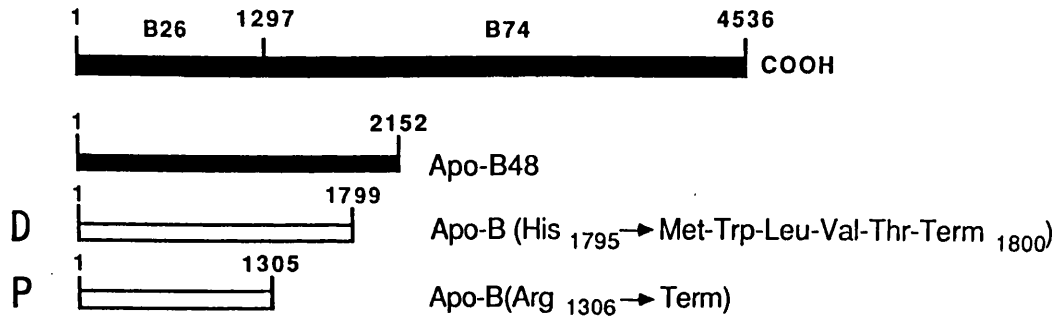


Figure 13.

Schematic representation of truncated apoB species predicted by the P family (P) and D family (D) apoB gene mutations. The normal forms, apoB100 and apoB48 are also shown. The kallikrein cleavage site at residue 1297 (Hardman *et al.*, 1986), which generates apoB26 and apoB74 are marked.

apoB100. In order to determine whether this was due to a mutation in the 5′ flanking region of her paternal apoB allele, the polymerase chain reaction was used to amplify from apoB nucleotides +34 to -635, using four oligonucleotides (see methods) and producing two sequential amplified segments.

Cloning the two segments into M13, followed by sequencing 12 random clones of each segment (see methods) however, revealed no abnormality in this region in any clones sequenced (Figure 15). The consensus sequence of this region is shown in Figure 14.

(a)

-660 GAGGTTGCTC
-650 TTCCCCAGAGGCCTCTCCTCGCTGGGGTTTCTTGAAGACAGATACTTGGA
-600 CTCCTGCTGGGACCAGGCAGGCCACCCATCCTCAGGGGCAGTGACTGGTC
-550 ACTCACCAGACCTCCCTGCATCCCCCTTCTCTCTCCTCCCCCAGCACGGG
-500 CTGAACCCCGCAGCCACAGATTCTGATCAGGATTAGGGTGTGGGTGCAAA
-450 TCCAAGGTCCACCAAAATGGAAAAGAAGTAACCGATGGGAACACGTCTCC
-400 ACCAAGACAGCGCTCAGGACTGGTTCTCCTCGTGGCTCCCAATTCAGTCC
-350 AGGAGAAGCAGAGATTTTGTCCCCATGGTGGGTCATCTGAAGAAGGCACC
-300 CCTGGTCAGGGCAGGCTTCTCAGACCCTGAGGCGCCGGCCATGGCCCCAC
-250 TGAGACACAGGAAGGGCCGCGCCAGAGCACTGAAGACGCTTGGGGAAGGG
-200 AACCCACCTGGGACCCAGCCCCTGGTGGCTGCGGCTGCATCCCAGGTGGG
-150 CCCCCTCCCCGAGGCTCTTCAAGGCTCAAAGAGAAGCCAGTGTAGAAAAG
-100 CAAACAGGTCAGGCCCGGGAGGCGCCCTTTGGACCTTTTG**CAATCCT**GGC
-50 GCTCTTGCAGCCTGGGCTTCC**TATAAAT**GGGGTGCGGGCGCCGGCCGCGC
+1 ATTCCCACCGGGACCTGCGGGGCTGAGTGCCCTTCTCGGTTGCTGCCGCT
+51 GAGGAGCCC

(b)

-660 CTCCAACGAG
-650 AAGGGGTCTCCGGAGAGGAGCGACCCCAAAGAACTTCTGTCTATGAACCT
-600 GAGGACGACCCTGGTCCGTCCGGTGGGTAGGAGTCCCCGTCACTGACCAG
-550 TGAGTGGTCTGGAGGGACGTAGGGGGAAGAGAGAGGAGGGGGTCGTGCCC
-500 GACTTGGGGCGTCGGTGTCTAAGACTAGTCCTAATCCCACACCCAGTTTT
-450 AGGTTCCAGGTGGTTTTACCTTTTCTTCATTGGCTACCCTTGTGCAGAGG
-400 TGGTTCTGTCGCGAGTCCTGACCAAGAGGAGCACCGAGGGTTAAGTCAGG
-350 TCCTCTTCGTCTCTAAAACAGGGGTACCACCCAGTAGACTTCTTCCGTGG
-300 CGACCAGTCCCGTCCGAAGAGTCTGGGACTCCGCGGCCGGTACCGGGGTG
-250 ACTCTGTGTCCTTCCCGGCGCGGTCTCGTGACTTCTGCGAACCCCTTCCC
-200 TTGGGTGGACCCTGGGTCGGGGACCACCGACGCCGACGTAGGGTCCACCC
-150 GGGGGAGGGGCTCCGAGAAGTTCCGAGTTTCTCTTCGGTCACATCTTTTC
-100 GTTTGTCCAGTCCGGGCCCTCCGCGGGAAACCTGGAAAAC**GTTAGGA**CCG
-50 CGAGAACGTCGGACCCGAAGG**ATATTTA**CCCCACGCCCGCGGCCGGCGCG
+1 TAAGGGTGGCCCTGGACGCCCCGACTCACGGGAAGAGCCAACGACGGCGA
+51 CTCCTCGGG

Figure 14. Consensus sequence of the 5' flanking region of the apoB gene sequenced in subject DD (a) sense and (b) antisense strands.

The position of the oligonucleotides used in the amplification are underlined. The CAAT and TATA boxes are shown in bold. No significant deviation from this consensus sequence was found in any of the DD clones sequenced. Part of two sequencing gel autoradiographs of antisense clones from the region are shown in Figure 15.

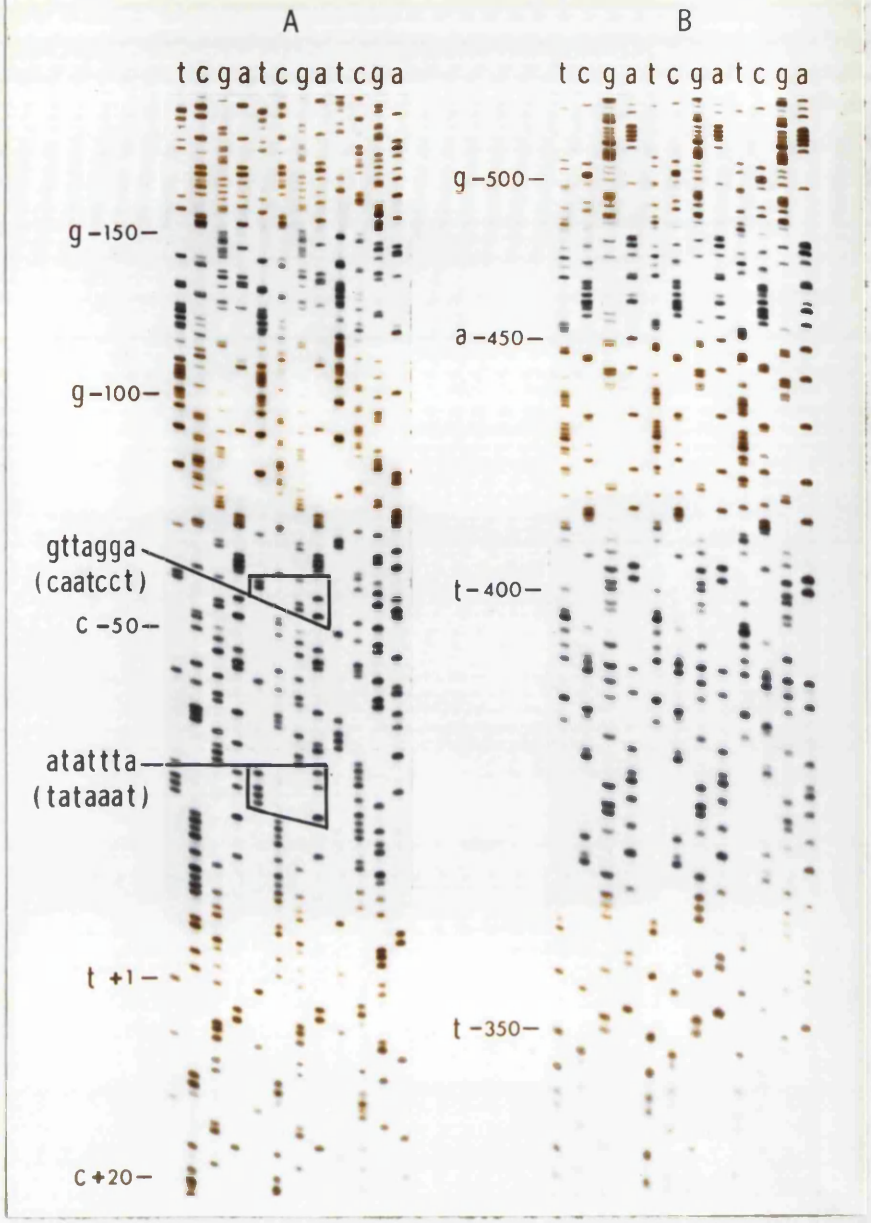


Figure 15.

Sample sequence gel autoradiographs of the 5′ flanking region of the apoB alleles of subject DD. A. Part of the sequence amplified using oligo's DD9 and DD10. B. Part of the more 5′ sequence amplified using oligo's DD7 and DD8. Three antisense clones from each region are shown. The position of the CAAT and TATA boxes are marked. No significant deviation from the consensus sequence (Figure 14) could be found in any of the clones examined.

3.2 Abetalipoproteinaemic individuals.

Unlike the hypobetalipoproteinaemic patients, none of the patients with abetalipoproteinaemia showed any novel polymorphisms with *Taq*1, nor any other restriction enzyme. Also unlike the hypobetalipoproteinaemic patients, since they had a virtual absence of apoB in their plasma, no apoB protein studies could be undertaken. The restriction fragment length polymorphisms (RFLP's), obtained in the J and the M abetalipoproteinaemic families however, did enable a linkage study to be carried out.

The J and M families, each had a pair of siblings with classical, recessively inherited abetalipoproteinaemia. In the J family, there was one unaffected older daughter. The plasma lipid, apoB and vitamin E concentrations in the J and M family members are shown in Table 2.

Southern Blotting

The *Xba*1 and *EcoR*1 Southern blots of the J family members are shown in Figure 16 and the *Msp*1 blots of the M family members, in Figure 18. The pedigrees of the two families together with their apoB RFLP's are shown in Figures 17 and 18. The RFLP's obtained made it possible to clearly distinguish the four parental chromosomes. In the J family, five apoB RFLP's were used to deduce the parental haplotypes. In this family, both affected children have inherited the apoB gene defined as haplotype IV from their father. However, one affected child (MJ), has inherited haplotype II, whilst the other (SJ), has inherited haplotype I from their mother. The unaffected older sister (NJ) has inherited haplotype III from the

Subject	Total Cholesterol (4.0-6.5 mmol/l)*	Triglyceride (0.3-1.8 mmol/l)*	ApoB (60-140 mg/dl)*	Vitamin E (μmol/l)*
Mr.J.	6.6	1.4	112	23.6
Mrs. J.	4.8	0.6	74	24.3
N.J.	3.5	1.1	83	17.4
M.J.	0.9	<0.1	0.24	1.5 ♦
S.J.	0.6	<0.1	0.09	0.6 ♦
Mr. M.	4.0	2.0	84	25.1
Mrs M.	3.7	0.7	61	23.0
C.M.	0.9	<0.1	0.57	4.5 ♦
P.M.	0.9	<0.1	0.31	3.4 ♦

* Normal ranges are 5-95 percentile ♦ Receiving vitamin E supplementation

Table 2:- Plasma lipid, apoB and vitamin E concentrations of abetalipoproteinaemic individuals.

(From Talmud et al 1988)

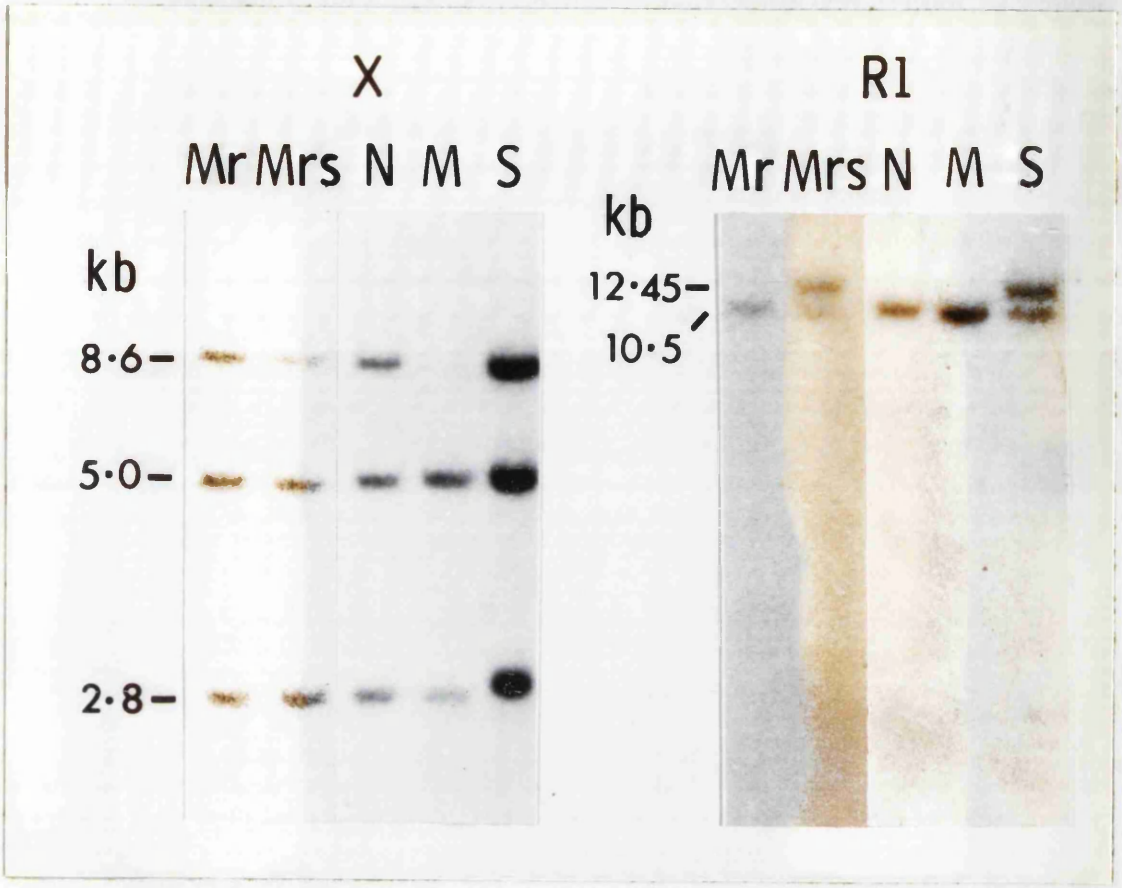


Figure 16.

Southern blots showing *Xba*1(X) and *Eco*R1(R1) RFLP's of the J family members. Genomic DNA was digested with either *Xba*1 or *Eco*R1 restriction enzymes and hybridised with a mixture of two cDNA probes, AB1 and AB7, from the 5' end of the apoB gene (Figure 3).

Both parents and their two daughters S and N, show both X1 (8.6 Kb; restriction site absent) and X2 (5.0 Kb; site present) *Xba*1 fragments, whilst the son M shows only the X2 fragment. The mother and daughter S show both R1 (10.5 Kb; site present) and R2 (12.45 Kb; site absent) *Eco*R1 fragments, whilst the father, daughter N and son M show only the R1 fragment.

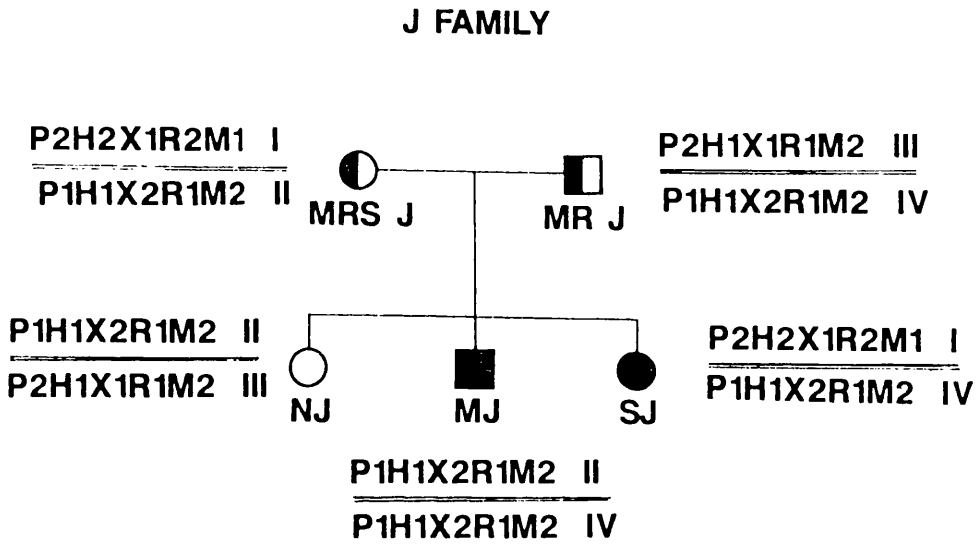


Figure 17.

Pedigree of the J family showing apoB RFLP's. Genomic DNA was digested with the restriction enzymes *Pvu*II (P), *Hinc*II (H), *Xba*1 (X), *Eco*R1 (R1) and *Msp*1 (M) and hybridised with cDNA probes 959, AB1 and AB7 (Figure 3). The Southern blots corresponding to the *Xba*1 and *Eco*R1 RFLP's are shown in Figure 16. The deduced haplotypes are designated I-IV.

□, male; ○, female. (From Talmud *et al.*, 1988).

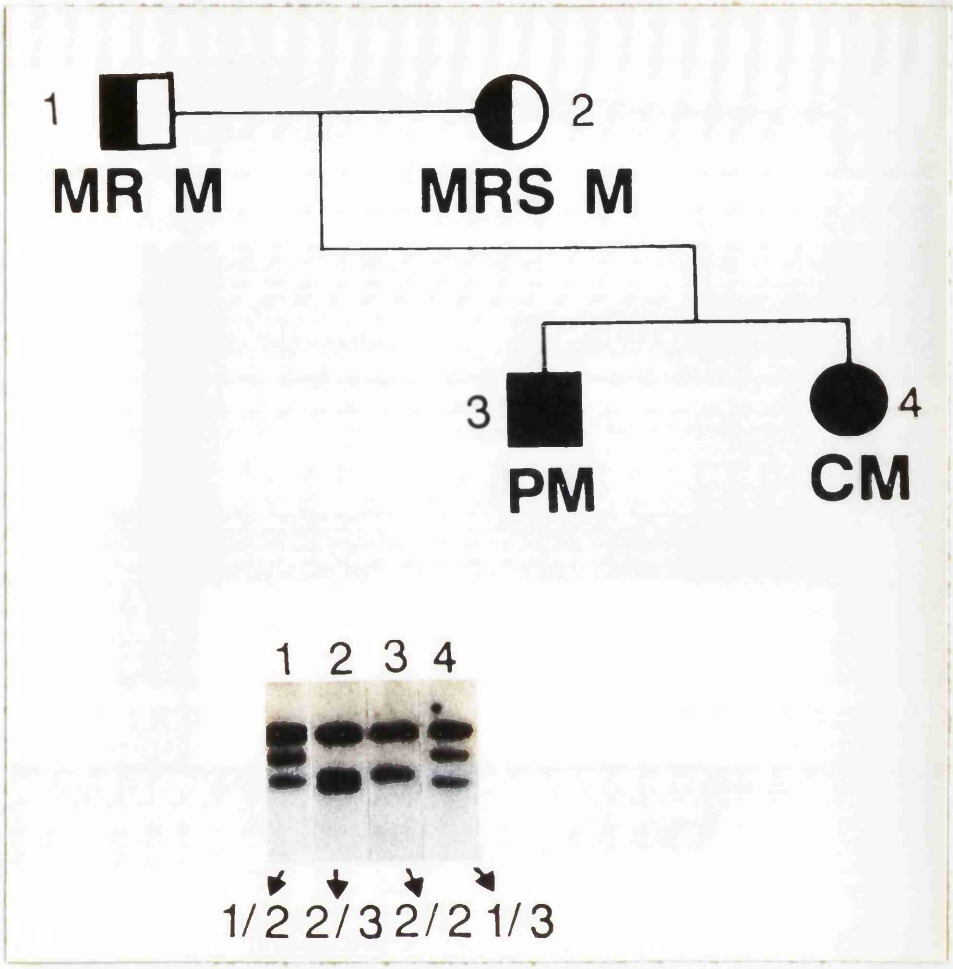


Figure 18.

Pedigree of the M family showing apoB *Msp*1 RFLP's with corresponding Southern blot. Genomic DNA was digested with the restriction enzyme *Msp*1 and hybridised with the genomic probe BH2 (Figure 3). *Msp*1 alleles in this family are numbered 1-3. There is an invariant band of 2.6 Kb. Alleles M2 and M3 have only slightly different sized fragments. Family members are numbered 1-4.

□, male; ○, female. (From Talmud *et al.*, 1988).

father and haplotype II from the mother. The fact that the affected children have inherited different pairs of alleles of the apoB gene from their mother is incompatible with the hypothesis that the disorder in this family is due to a mutation in the apoB gene.

In the M family, the RFLP's obtained with the restriction enzyme *Msp*1 and probe BH2, which lie in the hypervariable region of the 3' untranslated region of the apoB gene, were alone able to distinguish all four parental chromosomes. The RFLP's detected are due to two *Msp*1 sites lying in this region, which vary in position due to a varying number of AT-rich, 30 bp consensus sequence. The mother has alleles M2 and M3 and the father M1 and M2. Examination of the two affected children shows that whilst PM has inherited allele M2 from the father and allele M2 from the mother, CM has inherited allele M1 from the father and M3 from the mother. Thus, in this family the affected children have inherited different apoB alleles from both their mother and their father. As with the J family, the pattern of inheritance of apoB alleles from the normal parents to the affected children, is incompatible with the hypothesis that a mutation in the apoB gene is responsible for the abetalipoproteinaemic phenotype.

Chapter 4: Discussion

The results of the investigations presented in this thesis demonstrate, that whilst familial hypobetalipoproteinaemia can be caused by mutations within the apoB gene, recessive abetalipoproteinaemia, in the families studied, is not caused by a defect in the apoB gene.

Examination of the genomic DNA and lipoproteins in four individuals with familial hypobetalipoproteinaemia has lead to the elucidation of two different apoB mutations. In contrast, no abnormality has been detected in the apoB gene of any patient with abetalipoproteinaemia and linkage analysis based on the inheritance of RFLP's in two families show that the apoB gene is discordant with the disease.

4.1 Hypobetalipoproteinaemia mutation: ApoB(Arg_{1306}-->Term).

In one family, hypobetalipoproteinaemia is caused by a single base change in arginine codon 1306 (CGA:4125-4127). A C to T transition at nucleotide 4125 in the cDNA apoB sequence converts the arginine codon, CGA into a translational termination signal, TGA. This termination signal, represented by UGA in the mRNA, predicts the production of a truncated gene product of only 1305 amino acids in contrast to the full length product of 4536 amino acids (Knott *et al.*, 1986). The truncated protein is only eight amino acids longer than the shortest of the two apoB100 kallikrein cleavage products (Hardman *et al.*, 1986). This product has been found by SDS polyacrylamide

gel electrophoresis, to have a molecular weight 0.26 times that of the full length protein apoB100, and is thus referred to as apoB26 on the centile system introduced by Kane (Kane *et al.*, 1980; Kane., 1983). Because of the close similarity in the amino acid length of the two proteins, the truncated apoB species apoB(Arg_{1306}-->Term), might be predicted to run also as an apoB26, were it actually secreted. For this reason, in the original published report of the mutant (Collins *et al.*, 1988), it is also referred to as apoB26. The protein's predicted number of residues is actually 29% of the number in apoB100 however. Since it is only a theoretical product, not available for sizing in an electrophoretic system, it will in future be referred to as apoB29.

The apoB29 mutation arises as a result of a C to T transition within a CpG dinucleotide. It is now clear that CpG dinucleotides are a common site for mutation within the human genome. Cooper *et al* (1988) have collated reports of single base-pair mutations within the coding regions of human genes that are associated with genetic diseases. Their study showed that 35% of such mutations occurred within CpG dinucleotides. More than 90% of these mutations were C to T or G to A transitions, the latter being due to C to T changes on the non-coding strand.

These findings can be explained by spontaneous deamination of 5-methyl cytosines present in the CpG dinucleotides of coding regions (Figure 19). 5-Methyl cytosines probably represent the most common modification present in eucaryotic genomes. In a study of methylation of DNA in devel-

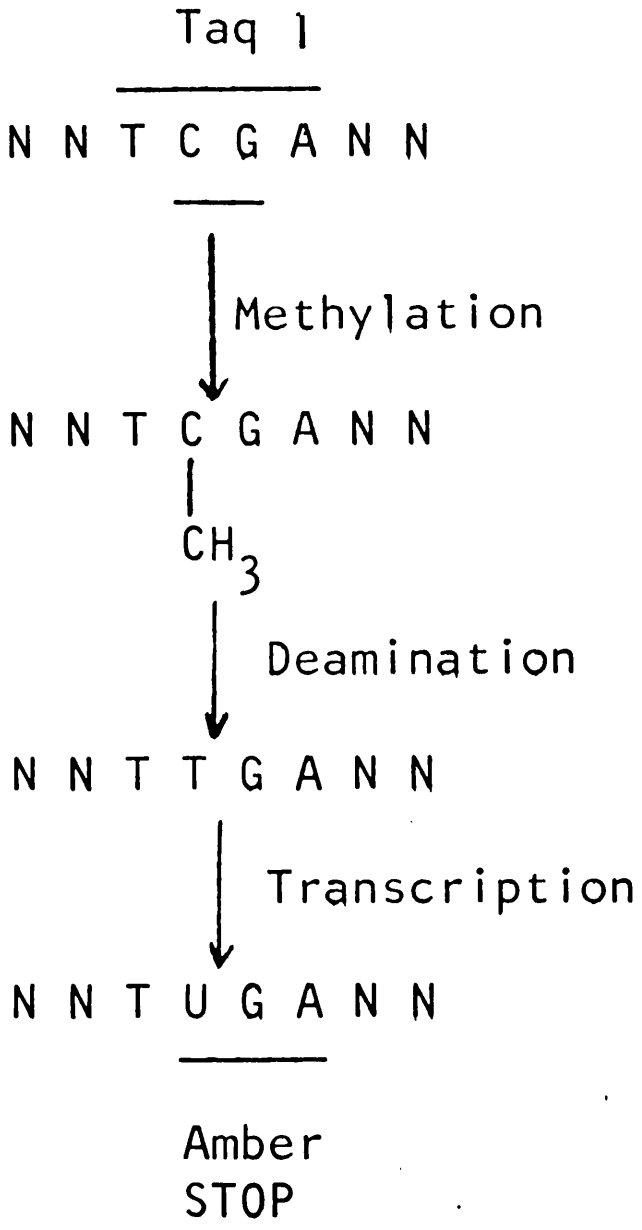


Figure 19.

Production of stop codon by mutation in CG dinucleotide in *Taq*1 site. Spontaneous deamination of methylated cytosine generates thymidine in the DNA, represented by uracil in the mRNA, creating an amber translational stop codon. (Figure, Courtesy of Dr. E. Tuddenham, Director of Haemostasis Research, Northwick Park Hospital).

-oping sea urchins, Grippo *et al* (1968) found that 90% of 5-methyl cytosines were in CpG dinucleotides. It is estimated that about 1% of the genome consists of unmethylated domains and that such regions contain 15% of the total number of CpG dinucleotides (Cooper *et al.*, 1983).

Overall, CpG dinucleotides are greatly under-represented in vertebrate genomes, being present at approximately a quarter of the level predicted from observed mononucleotide frequency (Josse *et al.*, 1961). In man the proportion of CpG in sequenced genes appears to be about 37% of that expected (Nussinov., 1981). It appears that the extent of CpG deficiency correlates well with the level of CpG methylation in different animal genomes. It is also inversely correlated with an excess of the dinucleotides TpG and CpA, that is, those dinucleotides generated by the deamination of 5-methyl cytosines in methylated CpG dinucleotides (Bird., 1980).

The small fraction of DNA which consists of stably non-methylated sequences, contains a relatively high proportion of correspondingly non-methylated CpG dinucleotides. The fraction is detected by its cleavage into tiny fragments with the methyl-sensitive restriction enzyme *Hpa*II (Cooper *et al.*, 1983). Such sequences occur as discrete "islands",1-2 Kb long, with a spacing of approximately one per 100 Kb, but with a very large variation about this mean (Brown and Bird., 1986). In total there are about 30,000 of these islands per haploid vertebrate genome. It is now clear that a large proportion of CpG islands are associated with genes, especially those regions where

transcription begins (Bird., 1987).

Compared to the CpG rich islands, not susceptible to mutation because of their lack of methylation, the inter-island DNA is abnormally CpG poor. Where they do occur however, they are likely to be methylated and hence at risk of deamination, with the possibility of generating genetic disease. Numerous authors have suggested that CpG dinucleotides could be mutation "hotspots" (Coulondre *et al.*, 1978; Bird., 1980) and a particularly large proportion of RFLP's in the human genome are revealed with enzymes such as *Taq*1 and *Msp*1, with CpG dinucleotides in their recognition sequence (Barker *et al.*, 1984).

There is now a steadily increasing catalogue of human genetic diseases which have clearly been generated by mutations within CpG dinucleotides. Youssoufian *et al* (1986) have analysed the genomic DNA of 83 patients with haemophilia A. They screened for mutations within the factor VIII gene by restriction analysis of DNA with *Taq*1, *Sst*I and *Eco*RI and successive hybridisation with two factor VIII cDNA probes spanning exons 1-12 and 14-26. The strategy detected 6 deletions and 10 point mutations. Of the point mutations, 9 were detected using *Taq*1, which has a CpG dinucleotide in it's recognition sequence. For two of the *Taq*1 mutations, present in exon 18 and 22, they used specially constructed oligonucleotide probes to determine the precise nature of the altered *Taq*1 sites. The probes were end-labelled with ^{32}P-ATP and constructed with the normal sequence flanking the respective *Taq*1 sites and with a C to T substitution in the *Taq*1 sites.

Differential hybridisation of the probes to *Hind*III digested genomic DNA from the patients and their relatives showed that the exon 18 and 22 *Taq*1 mutations were indeed a result of C to T transitions, creating in-frame termination codons at positions 1960 and 2135 respectively. They also demonstrated that the same mutations had arisen independently in other unrelated families. Based on their observed recurrence frequency for these mutations, on an estimated 2-4 generation lifespan for haemophilia A mutations (Haldane *et al.*, 1935) and on a disease incidence of 1 in 10,000 males, the authors estimate that over 1,000 individuals are alive with different recurrent origins of each mutation. In one family, RFLP analysis showed that the exon 18 mutation must have arisen in a germ cell of the maternal grandfather, who was 32 years old at the time his carrier daughter was conceived. Similar analysis of one of the families with the exon 22 mutation, showed that the mutation must also have arisen in the maternal grandfather, who was 34 at the time of conception. It is of interest that both the mutations had arisen *de novo* in relatively older fathers. It is presumed that the mutant genes result in the production of truncated inactive factor VIII molecules.

Similar recurrence of *Taq*1 mutations has been observed in unrelated patients with ornithine transcarbamylase (OTC) deficiency (Maddalena *et al.*, 1988). *De nova* mutations of the OTC gene in the same Taq1 restriction site, containing an arginine codon, were found in three unrelated individuals. In two unrelated males with neonatal onset of severe

OTC deficiency, there was a C to T transition on the antisense strand, giving a G to A transition on the sense strand, resulting in a glutamine for arginine substitution at amino acid 109 in the mature protein. In the third case, a symptomatic female had a C to T transition on the sense strand at the same site, converting arginine 109 into a premature termination codon. Their results support the view that *Taq*1 restriction sites, with their internal CpG dinucleotides, are particularly susceptible to mutation by deamination of 5-methyl cytosine, on either the sense or antisense strands. In the case of the OTC gene mutations described above, *Taq*1 site loss by deamination on either strand produces disease.

The apoB mRNA contains 12 CGA arginine codons (Table 3) presumably all at risk of deamination of their contained CpG dinucleotides, to produce termination codons and further truncated proteins. Antisense mutations, producing arginine to glutamine substitutions will not of course give rise to truncated apoB proteins and unlike similar mutations occurring in proteins with enzyme or cofactor function, are unlikely to produce disease. They may however have more subtle effects on the function of the apoB molecule.

Of the 12 CGA arginine codons at risk of mutation to termination codons in the apoB mRNA, only that at position 1306, which gives rise to the apoB(Arg_{1306}-->Term) mutant described here and that at position 2058, are located within *Taq*1 restriction sites. The occurrence of the latter mutation has now been described by Young *et al*

Nucleotide	Amino acid	Restriction site change by C-->T
261	18	----
1407	400	----
1443	412	PvuII gain
1800	531	----
4125	1306	Taq1 loss
6162	1985	----
6380	2058	Taq1 loss
7665	2486	----
7686	2493	----
7692	2495	----
7728	2507	----
12657	4150	----

Table 3 - CGA arginine codons in the apoB sequence.

CGA arginine codons in the apoB sequence. To date, two of these codons have been found to have mutated in individuals with familial hypobetalipoproteinaemia. A C to T transition in codon 1306 gives rise to the P family mutation (Figure 11) and in codon 2058, to the apoB46 mutant reported by Young et al (1989).

(1989). The mutation was actually detected by the discovery of a truncated species of apoB, B-46, in members of a family with heterozygous hypobetalipoproteinaemia. The truncated species was present in the VLDL, along with full length apoB100. It was also found in the LDL and HDL density fractions. Antisera to synthetic peptides containing apoB100 residues 1712-1728 and 2008-2024, bound to the apoB46 as well as to apoB48 and apoB100. Antisera to peptides containing residues 2100-2129 and 2140-2151 bound only to apoB48 and apoB100, suggesting that apoB46 was a truncated apoB species with a carboxy terminus between residues 2000 and 2100.

Subsequent PCR amplification of the relevant portion of the apoB gene from genomic DNA of the family members defined the mutation more precisely. Restriction endonuclease studies showed that affected family members were heterozygous for the loss of both a *Taq*1 site at position 6381 and a *Hinf*1 site at position 6378 of the apoB cDNA sequence. The amplified fragments from two affected subjects were cloned into M13 and sequenced. Clones containing both the normal sequence, with CGA Arg_{2058} and the mutant sequence, with TGA $Stop_{2058}$ were identified. The apoB46 variant can be referred to as apoB(Arg_{2058}-->Term).

Thus, as with apoB29, the mutant species apoB46 is also caused by a mutation in a CpG dinucleotide within an arginine codon and likewise results in the loss of a *Taq*1 site.

The only other apoB mRNA CGA arginine codon which, if

mutated to a TGA stop codon, would produce a restriction site change is codon 412. In this case, such a mutation would lead to the gain of a *Pvu*II site. At 411 amino acids long, this protein would be apoB9 on the centile system (Kane *et al.*, 1980; Kane., 1983) and being so short, it is presumably highly unlikely that the product would be secreted in lipoproteins. In recent studies by Graham *et al* (1989), where HepG2 cells were transfected with an apoB9 cDNA construct cloned into a eucaryotic expression vector, no apoB9 secretion was observed.

4.2 Hypobetalipoproteinaemia Mutation;

ApoB(His_{1795}-->Met-Trp-Leu-Val-Thr-Term)

In the second family studied, hypobetalipoproteinaemia is caused by a deletion of a single G nucleotide at position 5591 of the apoB mRNA. The result is a translational frameshift which leads to the substitution of His_{1795} by a short hydrophobic peptide, Met-Trp-Leu-Val-Thr, before termination at a nonsense codon, TAA at position 1800. Patient DD and here mother PD, both possessed a truncated species of apoB with a relative mobility entirely consistent with this mutation. From it's termination position, the protein can be referred to as apoB39 using the centile system of Kane (Kane *et al.*, 1980; Kane., 1980).

The mutant protein apoB39 could be detected in the chylomicron/VLDL/IDL density fraction of both PD and DD and in the LDL density fraction of PD but not DD. There was no detectable apoB39 in the HDL density interval or the infranatant fraction, from either patient.

Although both individuals shared the mutant apoB allele,

B39, there were important differences between them, both clinically and biochemically. DD was an obvious homozygote for hypobetalipoproteinaemia, with malabsorption, 50% acanthocytosis, virtually no LDL cholesterol, a plasma apoB level of only 1.5 mg/dl and with both parents exhibiting features of the disorder. Interestingly however, her triglyceride level was just within the normal range. In contrast, PD was clearly a heterozygote for the disorder, with no symptoms, 10% acanthocytosis, a significant, but reduced amount of LDL cholesterol and plasma apoB level of 17 mg/dl. She also had a normal plasma triglyceride level. Sequencing however, revealed that although DD was a homozygote for the disorder, she was only a heterozygote for the apoB39 allele. The defect in her paternal allele is clearly of a different nature, making her a compound heterozygote for the disorder. Prolonged exposure of the Western blots of the Chylomicron/VLDL/IDL density interval of DD revealed trace amounts of apoB100. This suggests that the paternal allele directs the synthesis of very low levels of full length apoB. The father, AD, was asymptomatic, but had 10% acanthocytosis, a significant, but reduced amount of LDL cholesterol and a plasma apoB level of 28 mg/dl, characteristic of a heterozygote.

The paternal allele could presumably have a mutation that affects gene's transcription, the splicing or stability of the apoB mRNA, or the translation, secretion or degradation processes. If the defect in this allele acts by a reducing the level transcription, an obvious site for a

mutation would be in the 5′ flanking region of the gene, where promoter and enhancer sequences lie. The apoB gene contains a TATA box and a CAAT box, both of which are considered to be essential for the efficient transcription of mammalian genes, within 60 bp upstream of the transcriptional start site (Blackhart *et al.*, 1986). Positive elements that acts to increase liver specific expression are contained within nucleotides -128 to -86 and -86 to -70. A negative regulatory region lies more distally (-261 to -128) (Das *et al.*, 1988). Recently Metzger *et al* (1989) have shown that the hepatic transcription factor AF1 binds to the sequence between -81 and -69 as well as to the promoters of apoAI, apoCIII, and apoCII genes. In the same study, another hepatic transcription factor, C/EBP, was found to bind to a sequence contained within -69 to -52. Mutations generated within the AF1 and C/EBP elements were shown to reduce transcription rates by 50-fold and 2-4-fold, respectively.

Polymerase chain reaction of the 5′ flanking sequences of the apoB alleles of DD, followed by cloning into M13 and sequencing, however, failed to demonstrate any abnormality of these transcriptional regulatory elements.

The compound heterozygote DD, with one apoB allele directing the synthesis of a truncated protein, apoB39 and the other, small amounts only of apoB100, is very similar to an individual described by Young *et al*(1987 (a); 1987 (b); 1987 (c); 1988). The proband, HJB had previously been studied in detail by Steinberg *et al* (1979). At that time he was 67 years old, had mild fat malabsorption, a very

low level of LDL cholesterol and a plasma apoB of only 5.8 mg/dl, suggestive of homozygous hypobetalipoproteinaemia, but a normal triglyceride level. His father was not alive at that time, but his mother, HBB, had LDL cholesterol levels characteristic of heterozygous hypobetalipoproteinaemia, as did his daughters CVB and SGS. One brother, ALB and one sister, JYG had hypobetalipoproteinaemia as profound as the proband.

On reexamining the HJB kindred, Young *et al*(1987 (a)) characterised an abnormal apoB species, apoB37. Unlike apoB39 in the D family, apoB37 was present in all of the lipoprotein fractions of HJB. It was also present in several other members of the kindred. In the affected individuals, apoB37 was present in VLDL along with apoB100 and apoB48. HDL contained most of the apoB37, but no other apoB species. The HDL-apoB37 was actually contained within a unique HDL subfraction referred to as Lp-B37. Unlike other HDL subfractions, this contained little if any apoA1 and had a peak density of 1.12-1.15 g/ml, characteristic of HDL_3. The most abundant lipoprotein in the HDL density interval was a smaller particle, containing apoA1, but no apoB. ApoB37 was subsequently purified from HDL by SDS polyacrylamide gel electrophoresis, visualisation of the protein by potassium chloride precipitation and electroelution. Thirteen residues of it's amino-terminal sequence were then determined and found to be identical with the amino-terminal sequence of apoB100. Use of a panel of 18 different apoB specific monoclonal antibodies and polyclonal antisera specific for apoB37

and apoB100 thrombin cleavage products, confirmed that apoB37 contained only the amino terminal domains of apoB100.

Young *et al* (1987 (b)) subsequently isolated Lp-B37 from HDL using an anti-apoB37-Sepharose 4B affinity column. They went on to show that normal human fibroblasts were able to degrade only one tenth of the amount of ^{125}I-Lp-37 relative to ^{125}I-LDL. Unlabelled LDL reduced ^{125}I-LDL, but not ^{125}I-Lp-37 degradation and unlabelled Lp-37 had no effect on ^{125}-LDL degradation. Their data indicate the amino terminal apoB37 protein, when present on it's naturally occurring lipoprotein particle, does not possess a functional LDL (apo-B,E) receptor binding domain. This is consistent with the position of the domain proposed by other workers, in the carboxy terminal half of the molecule (Knott *et al.*, 1986; Yang *et al.*, 1986). The same presumably therefore applies to apoB39. The inability of the truncated apoB species, B37 and B39 to be taken up by the LDL receptor would suggest that they might accumulate in the plasma of the affected individuals. The fact that this is clearly not the case, indicates that some powerful mechanisms must operate to limit the secretion or accelerate the degradation of these proteins.

Analysis of the transmission of familial hypobetalipoproteinaemia over three generations in the kindred (Young *et al.*, 1987 (c)), showed that the proband, HJB and his siblings AYB and JYG are actually compound heterozygotes for the disorder. One allele produces the truncated variant of apoB, apoB37 and the other, a greatly reduced

amount of apoB100, as well as some apoB48. Use of a monoclonal antibody, MB19, which binds apoB allotypes $MB19_1$ and $MB19_2$ with high and low affinity respectively, established that the apoB37 and apoB100 were definitely the products of different alleles. Whilst the apoB37 had the $MB19_1$ polymorphism, the apoB100 and apoB48 had the $MB19_2$ polymorphism.

Measurement of apoB concentrations in heterozygotes for hypobetalipoproteinaemia with apoB37, using monoclonal antibody MB3, which binds to apoB100, apoB48 and apoB37, gave an average value of 32.5 mg/dl. Use of monoclonal antibody MB47, which binds only to apoB100, gave an average value of 22.8 mg/dl in these subjects, indicating the presence of rather small quantities of apoB37 and apoB48 and consistent with the existence of a normal apoB100 allele. In the compound heterozygotes, the apoB100 concentrations measured using antibody MB47, were all less than 3mg/dl, indicating a poorly functioning apoB100 allele.

The similarity of the HJB kindred compound heterozygotes to that of the D family examined in this study, is thus quite striking. In both families, homozygous hypobetalipoproteinaemia results from the possession of one mutant allele producing low plasma concentrations of a truncated apoB and another producing subnormal levels of full length apoB.

Young *et al* (1988) have since characterised the apoB37 mutant allele at the DNA level. The fact that apoB37 consists of the amino-terminal 37% of apoB100 and the

finding that an antiserum to an synthetic peptide containing apoB100 residues 1712-1728, bound to the protein, suggested that the carboxy terminus of apoB37 lay in the vicinity of apoB100 residues 1725-1750. Following construction of a genomic DNA library from an apoB37 heterozygote, clones encompassing the first third of exon 26, which codes for this region were subcloned into M13 and sequenced. In addition to normal clones, mutant clones with a 4-bp deletion (5391-5394) were obtained. Use of the polymerase chain reaction (Saiki *et al.*, 1985) to amplify the region of the apoB gene flanking this deletion and hybridisation of the PCR products to oligonucleotides representing the normal and mutant sequence, showed the same short deletion in other apoB37 heterozgotes in the family. The deletion causes a frameshift, with an asparagine to valine substitution at residue 1728, followed by a premature termination and can thus be referred to as apoB(Asn_{1728}-->Val-Term).

Thus, both the apoB39 mutant of the D kindred and the apoB37 variant of the HJB kindred arise as a result of deletional events, with a consequent frame shift and premature termination, involving a single base in the former and four bases in the latter.

As with the D family studied in this thesis, the nature of the mutations in the HJB kindred which give rise to the allele producing subnormal amounts of full length apoB has yet to be determined. The under-producing apoB100 alleles of these two families, are not the only examples of this type of this type of defect. Berger *et al* (1983) reported

a hypobetalipoproteinaemic kindred with a proband exhibiting the clinical and biochemical features of the homozygous state. Concentration of the d<1.063 lipoproteins of the proband, followed by gradient SDS polyacrylamide gel electrophoresis, showed that they contained a protein which co-migrated with the apoB100 of controls. Immunoelectrophoresis of the d<1.063 g/ml fraction concentrated 3060 fold confirmed the presence of immunoreactive apoB. From the relative intensity of the apoB100 bands of proband and control samples and a knowledge of the relative volumes of plasma from which they were derived, Berger *et al* estimated that the concentration of apoB100 in the proband's plasma was about 0.025% that of normal. Such a phenotype must presumably be due to the presence of two defective alleles capable of producing only very much reduced levels of full length apoB in the plasma.

Other apoB mutations can apparently have a less severe effect on apoB production, but can, none the less, lead to a much lower level of the product of the affected allele in the plasma. Gavish *et al* (1989) have described the inheritance within a family of allele-specific differences in the amount of apoB and LDL in the plasma. Using the monoclonal antibody MB-19 to distinguish between apoB alleles producing high (MB-19+), or low (MB-19-) affinity apoB-MB-19 binding, they were able to demonstrate an imbalance in the apoB production from alleles, with defective production from the MB-19+ allele. They demonstrated that the unequal expression phenotype was inherited in an autosomal dominant manner and using RFLP analysis, showed

linkage to the apoB gene. The plasma level of apoB produced by the allele defined as MB-19+, was considerably more than that produced by the D, HJB or Berger *et al* kindreds, but still only 33% that of the normal allele. As with these families however, the exact mutation causing this underproduction is not known.

As discussed above for the paternal hypobetalipoproteinaemic allele of the D family, a variety of different mutations could conceivably give rise to low levels of full length apoB. They could operate by reducing the level of transcription, or the splicing or stability of the mRNA. Alternatively, they could lead to reduced translational efficiency, impaired assembly or secretion of apoB containing lipoproteins, or an acceleration of either the intracellular or extracellular degradative pathways. In the case of the D family, the possibility of a defect in the 5′ flanking region of the paternal apoB allele, which might reduce the level of transcription, has been explored and no abnormality found.

It is likely however, that at least some of the hypobetalipoproteinaemic apoB alleles producing subnormal levels of full length apoB, have mutations in 5′ flanking sequences which affect transcriptional efficiency.

Certain forms of ß-thalassaemia have been shown to be due to mutations in the 5′ flanking DNA of the beta-globin gene, in the vicinity of promoter sequences involved in the interaction between RNA polymerase and the gene.

One of these is caused by a C to G substitution at position -87 relative to the transcription start site (Treis-

man *et al.*, 1983). The mutation lies 10 nucleotides 5′ to the CAAT box which is thought to be important for the efficient transcription of the globin as well as many other many other mammalian genes. Sequences 5′ to the CCAAT box, particularly the conserved sequence ACAC<u>C</u>C, which includes the nucleotide substituted in this mutation (underlined) have been shown to be required for maximum expression of rabbit and mouse beta-globin genes transferred in tissue culture systems (Grosveld *et al.*, 1982; Dierks *et al.*, 1983). In transient expression systems, this mutant globin gene was initiated and processed normally, but the amount of mRNA produced was only 10% of normal (Treisman *et al.*, 1983).

A second mutation in the same region, a C to T substitution at position -88, has been found in some cases of beta-thalassaemia in blacks (Orkin *et al.*, 1984). Both mutations produce a rather mild thalassaemia phenotype.

Other mutations in beta-globin 5′ flanking sequences, causing variable levels of reduced transcription and severity of phenotype have been found in the vicinity of the TATA or ATA box, which lie about 30 nucleotides upstream of the transcription start site and like the above sequences, also appear to be essential for efficient transcription (Weatherall., 1986).

In their study of two patients with homozygous hypobetalipoproteinaemia, Ross *et al* (1988) documented, by Northern and slot blot analysis of total liver mRNA from the patients, normal sized apoB mRNA, present in greatly reduced quantities relative to controls. Southern blot analysis

of their apoB alleles, using 10 different apoB cDNA probes, failed to reveal any major insertions, deletions or rearrangements. Immunocytochemistry using a pool of anti-apoB mouse monoclonal antibodies, demonstrated diffuse cytoplasmic staining of apoB protein. The intensity of reaction was markedly reduced relative to control hepatocytes however. Whilst control specimens showed dense reaction products along the hepatic sinusoids, consistent with normal apoB levels in the plasma, both hypobetalipoproteinaemic specimens showed no such staining, consistent with the absence of detectable apoB in the plasma.

Given that depressed transcription of apoB would be consistent with these findings, Ross *et al* (1989) went on to examine the transcriptional regulatory sequences in the 5′ flanking region of the apoB gene, in normal subjects and in one of these hypobetalipoproteinaemic probands. They constructed a genomic phage library from an *Mbo*I partial digest of the patient's DNA and screened this, along with a normal human placental library, with apoB cDNA probes. Clones which hybridised with a 5′ cDNA probe were rescreened with a 30 bp oligonucleotide probe from the 5′ untranslated region of the apoB cDNA. Positive clones were digested with *Pvu*II and a 1020 bp *Pvu*II fragment isolated by preparative agarose gel electrophoresis. This was then cloned into M13 and sequenced. Compared to the control sequence, that from the hypobetalipoproteinaemic proband had two base substitutions, one A to G at nucleotide -838 and one T to C at nucleotide -517. Both of these changes are upstream of the known important regula-

tory elements of the apoB gene.

To properly assess the functional significance of these changes, Ross *et al* used the normal and hypobetalipoproteinaemic 5′ flanking regions to construct 5′ apoB-chloramphenicol acetyltransferase (CAT) plasmids. Promoter efficiency was then assessed by transfecting the constructs into the human hepatoblastoma cell line, Hep G2, along with a pRSVßGal construct to normalise for transfection efficiency.

In transient Hep G2 transfections, there was no statistically significant difference between the CAT activities obtained with the CAT constructs containing normal and hypobetalipoproteinaemic 5′ regulatory regions. Thus, the two base substitutions identified in the latter do not appear to have any affect on the function of the apoB regulatory region. The authors point out that in any case, two base substitutions over 1000 nucleotides is within the accepted rate of DNA polymorphisms in the human genome. They conclude that apoB gene transcription is normal in the patient examined and suggest that the low levels of apoB mRNA previously reported may be the result of a mutation within the coding portion of the gene, leading to a structurally abnormal apoB and secondary mRNA instability.

Although theoretically likely, there are, as yet, no proven cases of hypobetalipoproteinaemia where there is abnormal apoB gene transcription due to a 5′ flanking region mutation.

4.3 Other apoB gene mutations causing hypobetalipoproteinaemia.

Of the remaining hypobetalipoproteinaemia mutations characterised to date, all involve coding regions of the apoB gene. The largest mutation described involves the deletion of the whole of exon 21. In the investigation of a proband with homozygous hypobetalipoproteinaemia from a kindred containing seven heterozygous individuals, Huang *et al* (1989), observed a unique *Taq*1 RFLP using a genomic probe from the mid-portion of the apoB gene. In normal individual, the probe identified *Taq*1 fragments of 8.4 and 2.8 kb, but in the proband, only a single fragment of 11 kb. Both parents had all three fragments, indicating heterozygosity for the mutation. The five other heterozygotes in the family all had the same three fragments. Linkage of the allele defined by the novel *Taq*1 RFLP with hypobetalipoproteinaemia was shown to be statistically significant.

Using smaller subclones from their probe, Huang *et al* (1990) localised the *Taq*1 RFLP to within an *Eco*RI fragment running from intron 20 to 23. This fragment was 4 Kb long in normal individuals, but 3.4 Kb in the proband, heterozygotes having both sized fragments. These findings were suggestive of a 0.6 Kb deletion in this *Eco*RI fragment of the mutant allele. The polymorphic *Taq*1 site in the *Eco*R1 fragment was localised precisely, by sequencing the subclones, to a position within intron 21, 351 bp downstream of exon 21.

Subsequent use of the polymerase chain reaction to amplify

this region from the genomic DNA of the proband, followed by cloning and sequencing revealed a deletion, 694 bp long. The deletion started near the end of an Alu sequence in intron 20, included exon 21 and finished near the end of an Alu sequence in intron 21. Alu sequences are present at about 40,000 copies in the human haploid genome (Rinehart *et al.*, 1981) and are moderately repetitive sequences, which, due to homology between members of the Alu family, are prone to homologous recombination. When the two Alu sequences have the same orientation, interstrand recombination can occur and when they have opposite orientations, intrastrand recombination is possible.

Huang *et al* (1990) suggest that the exon 21 deletion arose as a result of unequal crossing over between the Alu sequence in intron 20 from one chromosome to the Alu sequence in intron 21 from the other chromosome. This is the only report to date of an Alu-Alu recombination causing an apoB gene mutation, but the authors point out that there are six Alu sequences that have been reported in the apoB gene (Blackhart *et al.*, 1986) which could be targets for similar mutations.

The deletion of exon 21 results in a frameshift mutation with a premature termination codon (TAG) at codon 1086. Translation of the resultant apoB mRNA would produce a protein of 1085 amino acids, with residues 1014-1085 out of frame, commencing with a valine in place of glycine at position 1014. The mutant protein can thus be referred to as apoB(Gly_{1014}-->Val - - - - $Term_{1085}$). This would be

25% of the length of apoB100 and thus, on the centile system for nomenclature (Kane *et al.*, 1980; Kane., 1983), the mutant apoB could be designated apoB25. As with the apoB29 mutant defined in this study however, no truncated apoB species could be detected in the plasma of the proband.

As with apoB29, the absence of detectable mutant protein in this case could be due, either to non-secretion of the protein because of inadequate length to succeed in the lipoprotein assembly and secretion pathways, or to an extremely rapid metabolism of the product. Huang *et al* (1990) however, note that the exon 21 deletion could lead to a failure of secretion by having other effects on mRNA production such as the splicing of adjoining sequences, or by affecting mRNA stability.

ApoB25 and apoB29 are the smallest apoB variants predicted from hypobetalipoproteinaemia mutations described to date. The next largest variant which has been fully defined, is actually the smallest mutant species of apoB which is detectable in the plasma (Young *et al.*, 1990). The proband with this mutation is the son of the proband with the apoB46 mutation discussed above (Young *et al.*, 1989). Examination of his plasma however, showed that it did not contain any apoB46, but instead contained a different truncated apoB species, with a molecular weight, estimated from SDS gradient polyacrylamide gel electrophoresis, of 165 kDa and designated apoB31 on the centile system. The mutant protein was also found in the plasma of three of the proband's relatives, notably his father,

uncle and cousin. Unlike the larger truncated apoB species described to date, apoB31 was absent from both the VLDL and LDL lipoprotein fractions. This was observed both in fasting plasma and in plasma obtained following a fatty meal. It was present in the HDL ($1.063<d<1.21$ g/ml) fraction with a peak concentration, determined by continuous salt gradient ultracentrifugation, in the d=1.18-1.20 g/ml fraction. Also in contrast to the larger apoB variants, it was present in the lipoprotein deficient ($d>1.21$) density interval.

Use of non-denaturing gradient polyacrylamide gel electrophoresis showed that apoB31-containing HDL particles are smaller than apoB37-HDL particles, which are in turn smaller than apoB46-HDL particles. It also showed that the HDL particles containing the truncated forms of apoB were larger than those containing apoA1. Size fractionation of the plasma lipoproteins of the proband's father, using an agarose column, confirmed the absence of apoB31 from the VLDL and LDL density fractions. The apoB31-HDL peak eluted earlier than the apoA1-HDL peak, as would be predicted from the larger particle size of the former.

In Western blotting experiments, apoB31 was found to react with the monoclonal antibody MB3, which binds to an epitope between apoB100 residues 995 and 1082, but not with antibody 2D8, which binds to an epitope between apoB100 residues 1403 and 1480. From it's electrophoretic size and reactivity with these antibodies, the variant was estimated to contain the amino terminal 1400 amino acids of apoB100. In order to define the precise mutation in

the apoB gene responsible for apoB31, the polymerase chain reaction was used to amplify a 759-bp section of the apoB gene from proband's genomic DNA. The section covered apoB cDNA nucleotides 4369-5127, coding for apoB-100 amino acids 1388-1639. Cloning the product into M13 and sequencing, revealed that the proband was heterozygous for an apoB gene mutation. Whilst about half of the clones obtained had the normal apoB sequence previously reported, the remainder had a deletion of a single cDNA nucleotide, G-4480. The deletion predicts a frameshift, leading to the substitution of the glycine at position 1424 with a valine and tyrosine, before termination at codon 1426. The apoB31 variant can thus be referred to as apoB(Gly_{1424}-->Val-Tyr-Term). Amplification of the same region of the apoB gene from genomic DNA of other family members and probing the products with specially constructed oligonucleotide probes specific for the normal and mutant alleles, identified the other three apoB31 heterozygotes.

Comparison of the phenotypic expression of the apoB31 mutant, with that of the apoB25 mutant discussed above and the apoB29 mutant defined in this study, suggests a minimum length of amino-terminal sequence required for truncated apoB species to appear in the plasma. Whilst apoB29, containing 1305 amino acids and apoB25, containing 1085 amino acids, may fall below a critical molecular length required for the formation and secretion of apoB containing lipoproteins, apoB31, containing 1425 amino acids, may be just long enough for these processes to

occur. As discussed above however, there may be other reasons why apoB25 and apoB29 were not found in the plasma, such as mRNA instability or extremely rapid catabolism.

Comparison of the lipoprotein distribution of apoB31 with that of the larger apoB variants, such as the apo37 and apoB46 variants discussed above and the apoB39 variant of this thesis, gives further insight into lipoprotein assembly and secretion. Unlike the larger forms, apoB31 is not found in the VLDL fraction, suggesting that although the apoB31 molecule is long enough to be secreted successfully, it lacks sufficient length to support the formation of triglyceride rich lipoproteins.

ApoB37 is present in all of the lipoprotein fractions and it may be that the apoB residues running from the amino terminus of apoB31 to the amino terminus of apoB37, that is, residues 1425-1728, may be essential for the assembly and secretion of triglyceride rich lipoproteins. This region includes the terminal 39% of the second apoB100 domain defined by the studies of Yang *et al* (1989). This consists of residues 1001-1700 which form a series of alternating trypsin releasable and non-releasable peptides. An inverse relationship between the abundance of hydrophobic residues and trypsin releasability was observed and it is probable that this domain is the first to enter the lipoprotein core to any significant extent. In addition, apoB37 includes the first 28 residues (2%) of the third apoB100 domain defined by Yang *et al* (1989), which is largely trypsin non-releasable and presumably

mostly lipid associated. It is thus perhaps not surprising that shorter truncated apoB variants, such as apoB31, with only the first 61% of the second domain, are unable to form triglyceride rich lipoproteins.

Although in contrast to apoB31, apoB37 can clearly form triglyceride rich lipoproteins to some extent, it is, nonthe-less, most abundant in the triglyceride poor HDL fraction. The apoB39 variant of this study, on the other hand, was present only in the triglyceride rich VLDL and LDL fractions, and was completely absent from the HDL fraction. ApoB39 consists of the amino terminal 1799 residues of apoB100. It has the whole of the second apoB100 domain defined by Yang *et al* (1989) and the first 93 residues (7%) of the third, hydrophobic domain plus an additional short hydrophobic tail (Met-Trp-Leu-Val-Thr), which, together may ensure that it's exclusive association with the triglyceride rich lipoproteins.

The apoB46 variant discussed above (Young *et al.*, 1989) consists of the amino-terminal 2057 apoB100 residues and thus has the whole of the second apoB100 domain defined by Yang *et al* (1989), plus the first 356 residues (26%) of third, hydrophobic domain. Given that apoB39 was not found in the HDL fraction and following the above reasoning with respect to overall hydrophobicity, it might be expected that apoB46 too, would be absent from HDL. This is not the case however; the protein was found to some extent in the HDL fraction. It may be that the unique hydrophobic tail of apoB39 in some way excludes it from HDL, or there may be factors other than hydrophobicity,

such as an individual's genetic background, which influence the lipoprotein density interval in which an apoB variant appears.

Recently, two further truncated forms of apoB have been demonstrated in a single kindred with hypobetalipoproteinaemia (Krul *et al.*, 1989). The pedigree consists of 11 siblings, five of whom were diagnosed as having hypobetalipoproteinaemia. On examination of their lipoproteins, the three affected females were found, by gradient SDS polyacrylamide gel electrophoresis, to have two truncated apoB species instead of apoB100. One of these had a molecular weight of approximately 90% that of apoB100 and was hence originally termed apoB90. The other truncated apoB was present at a much lower concentration and had a molecular weight of about 40% that of apoB100 and was hence termed apoB40. One affected male was found to have apoB90 only, along with a larger amount of apoB100 and another was found to have apoB40 only, again in the presence of apoB100. Use of RFLP analysis of patient genomic DNA, combined with typing the 3' hypervariable region of the apoB alleles, by the PCR method of Boerwinkle *et al* (1989), enabled the four parental alleles to be haplotyped and their inheritance followed through the sibs. While the affected brothers were single heterozygotes for different abnormal alleles of apoB, the three affected sisters were compound heterozygotes. The apoB90 heterozygote had the least degree of hypobetalipoproteinaemia, with a plasma apoB of 81 mg/dl, compared to 29 mg/dl in the apoB40 heterozygote. The compound heterozy-

gotes had the lowest apoB levels (26, 16 and 12 mg/dl), with apoB90 predominating in the lipoprotein fractions with densities up to 1.124. At densities greater than 1.124, apoB40 was the only detectable species.

Western blots of fresh plasma or LDL preparations using a monoclonal antibody B6C3, which binds to an epitope on apoB100 between residues 4082 and 4348, failed to react with either apoB40 or apoB90. Both antibodies D7.2 and B1B3, with epitopes between apoB100 residues 1878 and 2148 and 3506 and 3635, respectively bound to apoB90, but not to apoB40. Taken together, these findings suggested that apoB40 and apoB90 represent short forms of apoB, with differing amounts of carboxy terminus missing.

In competition experiments using ^{125}I-labelled normal LDL, it was found that apoB40/B90 LDL from compound heterozygotes had a greater than normal affinity for the LDL receptors of human fibroblasts. Use of an anti-apoE antibody and an antibody known to block apoB100-mediated binding of LDL to it's receptor, showed that this increased affinity was not due to apoE contamination, but must have been acting via the apoB receptor binding region of apoB90. ApoB40 was presumed not to be involved in this increased affinity, since apoB48, which is a longer amino terminal molecule, does not react with LDL receptors (Hui *et al.*, 1984).

Krul *et al* (1989) suggest that the hypobetalipoproteinaemic phenotype produced by the apoB90 allele may be due to the increased affinity and more rapid clearance of apoB100 containing LDL.

Talmud *et al* (1989) have since determined the precise mutations responsible for the generation of apoB40 and apoB90. To determine the apoB40 mutation, they used the polymerase chain reaction to amplify, from genomic DNA of one of the compound heterozygotes, a 781 bp section, between cDNA apoB100 nucleotides 5490 and 6271. After cloning the product into M13, ten clones were sequenced. Six of these clones had a deletion of a dinucleotide, TG, at nucleotides 5693 and 5694. The result is a frameshift, where the $Valine_{1829}$ codon, GTT, is replaced by the cysteine codon, TGC, followed by a termination codon, TAA. The apoB40 mutant thus has 1829 amino acids and can be referred to as apoB(Val_{1829}-->Cys-Term). As a percentage of the number of residues in apoB100, 1829 is actually 40.3%, so the original naming of the mutant by Krul *et al* (1989) is maintained by Talmud *et al* (1989).

In order to determine the mutation responsible for the generation of apoB90, a 263 bp section, running between apoB100 cDNA nucleotides 12216 to 12479 was amplified and cloned. On sequencing ten clones, five were found to have a deletion of a single G at nucleotide 12309. This causes a frameshift, where the $Glutamine_{4034}$ codon is replaced by the arginine codon AGG, followed by codons for the sequence, Gln-Leu-Leu-Ala-Cys with a termination codon at position 4040. The apoB90 mutant thus has 4039 amino acids and can be referred to as apoB(Glu_{4034}-->Arg-Gln-Leu-Leu-Ala-Cys-Term). As a percentage of the number of residues in apoB100 however, 4039 residues is actually 89.04%. For this reason Talmud *et al*

rename the protein apoB89.

Polymerase chain reaction amplification of same 181 bp and 263 bp sections of genomic DNA from the other family members followed by probing the products with allele specific oligonucleotides, confirmed the inheritance of the four parental alleles shown by the previous studies. Thus, three of the sibs were indeed compound heterozygotes for the apoB40 and the apoB89 mutant alleles and two were single heterozygotes, one for the apoB40 and one for the apoB89 alleles.

The way in which apoB89 causes the hypobetalipoproteinaemic phenotype is, as discussed above, implied from the human fibroblast LDL receptor binding experiments of Krul *et al*, that is apoB89-containing LDL is cleared from the circulation by the LDL receptor more rapidly than normal, apoB100-LDL. Talmud *et al*(1989) point out that the region deleted from apoB89 includes the fifth apoB100 domain defined by Yang *et al* (1989). This domain was found to be almost exclusively trypsin non-releasable, is significantly more hydrophobic than the other domains and is presumably buried in the lipoprotein core of the LDL particle. Forgez *et al* (1989), found that a monoclonal antibody to a synthetic peptide corresponding to apoB100 residues 4007-4019, binds competitively to the LDL receptor of U937 cells, suggesting that the region may act as a secondary LDL receptor binding domain. It is thus possible that the deletion of the fifth, hydrophobic domain in apoB89, may alter the tertiary structure and availability for binding, of this region in addition to the primary

receptor binding domain. Krul *et al* (1989) however, also point out that they cannot exclude reduced synthesis of apoB89 as a cause of it's decreased level in the plasma.

As with the other apoB mutant apoB species which are definitely secreted, but which lack an LDL receptor binding domain, (apoB31, apoB37, apoB39 and apoB46), the mechanisms whereby apoB40 causes the hypobetalipoproteinaemic phenotype is equally unclear. All of these secreted shorter variants might be expected to accumulate in the plasma by virtue of being unclearable via the LDL receptor pathway. Their universally low plasma levels could be due to accelerated degradation via some other pathway, to a low rate of secretion, or to a combination of both of these mechanisms. A low secretion rate could be secondary to either an instability of the mutant apoB mRNA, or to a reduced ability of the mutant proteins to efficiently enter the lipoprotein assembly pathway.

Although the apoB40 of Krul *et al* (1989) is slightly longer than the apoB39 of this thesis, containing 9% in contrast to 7% of the third, hydrophobic apoB100 domain defined by Yang *et al*, unlike apoB39, it was found in the HDL density interval as well as in the triglyceride rich VLDL and the LDL fractions. Like apoB46 (Young *et al.*, 1989) discussed above, apoB40 may succeed in entering HDL, either because it lacks the unique hydrophobic tail peptide of apoB39, or because of the different genetic background of the kindred involved.

Further truncated variants of apoB continue to be described. Hardman *et al* (1989) have shown that an indi-

vidual previously reported by Malloy *et al* (1981) as having normotriglyceridaemic abetalipoproteinaemia, is actually homozygous for an apoB gene mutation. According to the findings of this thesis and of other workers, the individual should thus strictly, be referred to as having normotriglyceridaemic hypobetalipoproteinaemia. The patient had essentially normal levels of plasma triglyceride and normal intestinal absorption of triglyceride, but the serum was completely devoid of normal LDL.

On examination of the patient's apoB, two components could be resolved, apoB48 and an apoB species corresponding to the amino-terminal 50% of the apoB100 sequence. The variant was estimated to terminate between apoB100 residues 2238 and 2272. Amplification and sequencing of the genomic DNA spanning this interval revealed a C to T substitution at nucleotide 6963. The mutation causes the transformation of glutamine codon 2252 to an in-frame stop codon. The mutant protein can thus be referred to as apoB(Gln_{2252}-->Term). All of the clones sequenced showed the mutation, indicating that the patient is homozygous for the defect. The authors suggest that these findings indicate that some portion of the apoB100 molecule carboxy-terminal to residue 2251, is involved in the normal production of LDL.

Another truncated apoB variant has recently been reported by Gabelli *et al* (1989). The proband is a 41 year old female with a plasma LDL cholesterol level of 4 mg/dl and apoB of 6 mg/dl. SDS polyacrylamide gel electrophoresis and Western blotting revealed an apoB with a molecular

weight of 440 kDa present in the VLDL, IDL and LDL fractions of the proband and her sister. It is referred to as apoB80. Use of four anti-apoB monoclonal antibodies in LDL immunoblots demonstrated that, as with the other short apoB variants described to date, apoB80 lacks a carboxy terminus. In addition, as would be expected, thrombin digestion of the protein yielded normal T1, T3 and T4 fragments, but no carboxy terminal T2 fragment.

In competition experiments using cultured human fibroblasts, apoB80-containing LDL showed a more effective inhibition of uptake and degradation, relative to normal LDL. Thus, as with the apoB90 variant (Krul *et al.*, 1989; Talmud *et al.*, 1989), one way in which apoB80 may cause hypobetalipoproteinaemia is by having an increased rate of metabolism via the LDL receptor pathway. The precise apoB gene mutation responsible for apoB80 however, has yet to be reported.

A final apoB mutant causing hypobetalipoproteinaemia, has recently been identified by Linton *et al* (Unpublished data., 1990). The variant is an apoB86. It is generated by the deletion of apoB cDNA nucleotide 11890, and contains 3896 amino acids. The mutation results in the substitution of valine at position 3894 by a three amino acid peptide Tyr-Ser-Ser, before termination at codon 3897. It can thus be referred to as apoB(Val_{3894}-->Tyr-Ser-Ser-Term). The mutant allele, perhaps not surprisingly, appears to yield normal amounts of apoB48. ApoB86 is present in the VLDL and LDL fractions.

Thus, to date, 11 different apoB mutants have been described in patients with familial hypobetalipoproteinaemia. Following the report of apoB37 by Young *et al* (1987, 1988), the apoB29 and apoB39 variants of this study were reported (Collins *et al.*, 1988). This was rapidly followed by the reports of apoB25 (Huang *et al.*, 1989), apoB46 (Young *et al.*, 1989), apoB40 and apoB89 (Krul *et al.*, 1989; Talmud *et al.*, 1989) and apoB31 (Young *et al.*, 1990). In addition, there are now abstract reports of apoB50 (Hardman *et al.*, 1989), and apoB80 (Gabelli *et al.*,1989) as well as the unpublished report of apoB86. Table 4 lists these truncated apoB species and summarises the causative mutations and the lipoprotein fractions in which they appear.

Comparison of the different apoB gene mutations causing hypobetalipoproteinaemia should enable some general conclusions to be drawn regarding the minimum length of apoB amino-terminus required for the formation of the different classes of lipoprotein and consequently the severity of disease induced by each mutation.

There does appear to be a broad correlation between the length of the truncated apoB predicted from a given apoB gene mutation and the protein's appearance in the plasma and on its distribution between the different lipoprotein fractions and the lipoprotein free fraction. On the evidence available so far, it appears that if the truncated apoB variant predicted from the causative mutation is too short (apoB25, apoB29), the mutant protein is not secreted in a stable form, either in lipoprotein particles

ApoB variant	Residues	Centile size	Mutation	Lipoprotein fraction	Reference
Gly_{1014}-->Val....$Term_{1086}$	1085	B25	Exon 21 del.	Absent	Huang et al., 1989
Arg_{1306}-->Term.	1305	B29	C-->T Nuc. 4125	Absent	Collins et al., 1988
Gly_{1424}-->Val-Tyr-Term.	1425	B31	Nuc. 4480 del.	HDL/Infra.	Young et al., 1990
Asn_{1728}-->Val--Term.	1728	B37	Nuc. 5391-4 del.	V/L/HDL	Young et al.,1988
His_{1795}-->Met-Trp-Leu-Val-Thr-Term.	1799	B39	Nuc. 5591 del.	V/LDL	Collins et al., 1988
Val_{1829}-->Cys-Term.	1829	B40	Nuc. 5693-4 del.	V/L/HDL	Talmud et al., 1989
Arg_{2058}-->Term.	2057	B46	C-->T Nuc. 6381	V/L/HDL	Young et al., 1989
Gln_{2252}-->Term.	2251	B50	C-->T Nuc. 6963	VLDL	Hardman et al., 1989
Not yet defined	----	B80	----	V/LDL	Gabelli et al., 1989
Val_{3894}-->Tyr-Ser-Ser-Term.	3896	B86	Nuc. 11890 del.	VLDL	Linton et al., Unpublished
Glu_{4034}-->Arg-Gln-Leu-Leu-Ala-Cys-Term.	4039	B89	Nuc. 12309 del.	V/LDL	Talmud et al., 1989

Table 4- ApoB variants causing hypobetalipoproteinaemia

or in a lipoprotein-free form. If the variant is a little longer however (apoB31), it can appear in the lipoprotein deficient fraction and triglyceride-poor HDL fraction of the plasma, but is not able to form triglyceride-rich lipoproteins. If longer still (apoB37), it is able to enter all of the lipoprotein fractions, but predominates in the triglyceride-poor HDL. It cannot however be secreted independent of lipid and appear in the lipoprotein deficient fraction. Yet longer (apoB39, apoB40 and apoB46), the variant is clearly able to form triglyceride rich lipoproteins more efficiently, may or may not appear in the HDL, and is not secreted independent of lipid. Longer still (apoB50), the variant can clearly readily form triglyceride-rich lipoproteins, and even if present in double dose, gives rise to normotriglyceridaemic hypobetalipoproteinaemia, but is not, alone, however able to form LDL. The longest variants (apoB80, apoB86 and apoB89) enter the VLDL and LDL fractions and may give rise to LDL particles with a greater than normal affinity for the LDL receptor (apoB80, apoB89).

In order to investigate the regions of the apoB molecule which are required for the assembly and secretion of lipoprotein particles, Graham *et al* (1989) have transfected HepG2 cells with a series of apoB cDNA constructs. The constructs were cloned into to eucaryotic expression vectors and transfected by calcium phosphate co-precipitation.

Six different constructs were transfected, predicted to encode apoB9, B13, B17, B23, B29 and B39. After 24 hours,

the HepG2 cells were pulsed with ^{35}S-methionine and the medium ultracentrifuged into lipoprotein (D<1.25) and lipoprotein-free (d>1.25) fractions. The two fractions were then analysed by immunoprecipitating apoB and apoB containing lipoproteins, running the products on SDS polyacrylamide gels and detecting ^{35}S-labelled proteins by fluorography.

The two largest constructs, apoB29 and apoB39, simulated the two hypobetalipoproteinaemic mutations defined in this study. No patients have been found to date with mutations predicting apoB9, B17 or B23. As discussed above however, an apoB9 mutation might be predicted to occur as a result of a C to T transformation in the CGA arginine codon 412 of the apoB100 cDNA sequence. From what is known about the other hypobetalipoproteinaemic apoB variants however, apoB9 would presumably be far too short to secreted in association with lipoprotein particles and perhaps as with apoB25 and apoB29, if secreted at all, might likewise be prone to rapid degradation.

In the studies of Graham *et al* (1989), no apoB9 secretion was detectable by the transfected HepG2 cells. All of the larger constructs were secreted however, but their distribution between the lipoprotein and lipoprotein-free fractions did vary. The shortest of the secreted constructs, apoB13, was not found at all in the lipoprotein fraction. Since it contains only just over half of the first, trypsin-releasable apoB100 domain defined by Yang *et al* (1989) it may simple lack sufficient lengths of hydrophobic peptide sequences to form any type of lipoprotein parti-

cle, however small.

The three next longest constructs, apoB17, B23 and B29 were found in both the lipoprotein and the lipoprotein-free fractions. ApoB17 contains a little over 3/4 of the first apoB100 domain of Yang *et al* (1989) and apoB23 and B29 contain the whole of the first domain, plus 45% and 65% respectively, of the second trypsin-releasable and non-releasable domain. These results demonstrate that, in this *in vitro* system, apoB molecules consisting of the first 17-29% of apoB100, can enter lipoprotein particles, but that they clearly do not have sufficient length to be exclusive to these particles. On the basis of these findings, it is on first sight surprising that the hypo-betalipoproteinaemic variants apo25 (Huang *et al.*, 1989) and apoB29 (Collins *et al.*, 1988) were not found in the serum. However, as discussed above, their absence in the patients may be the result of very rapid clearance from the circulation. Alternatively, co-existing additional mutations in the apoB25 and apoB29 alleles may prevent secretion.

The longest construct, apoB39, was found only in the lipoprotein fraction. As discussed above, it consists of the whole of the first and second apoB100 domains of Yang *et al* (1989), plus a little of the third, trypsin non-releasable, highly hydrophobic domain. Perhaps it's overall hydrophobicity is too great to allow it to be secreted independently of lipid. This finding is entirely compatible with the studies undertaken here on the apoB39 variant of the D family. The variant was found only in

association with the triglyceride rich chylomicron/VLDL/IDL fraction of patient DD and only in this fraction and the LDL fraction of her mother, PD.

4.4 Truncated apoB length and disease severity.

In heterozygous hypobetalipoproteinaemia, there is no clear relationship between the length of the truncated apoB variant produced by each mutation and the resulting degree of hypobetalipoproteinaemia. This is perhaps not surprising, since the degree to which the normal apoB allele can compensate for the defective allele, is clearly an independent variable.

In homozygous hypobetalipoproteinaemia, there are very few individuals who are homozygous for a single defective allele, so it is hard to assess the clinical impact of individual apoB mutant molecules. For apoB25, where the proband was a homozygote for the mutant allele (Huang *et al.*, 1989), there was no apoB detectable in the serum and the clinical presentation was very early and severe. The patient presented in the first year of life with chronic diarrhoea, failure to thrive and acanthocytosis.

The other variant where a homozygote has been identified is the apoB50 variant of Hardman *et al* (1989). The patient was first reported by Malloy *et al* (1981). She was referred at 8 years of age because she was found to have serum cholesterol and triglyceride levels of 25 mg/dl and 30 mg/dl, respectively. Her serum had alpha and pre-beta mobility lipoproteins, but no beta mobility lipoproteins were present. A chylomicron layer appeared after overnight refrigeration of a serum sample obtained following

an oral fat load and a small intestinal biopsy was normal. The child was obese, with weight above the 95th percentile, mentally retarded and had a wide based ataxic gate. Vitamin E levels were essentially undetectable. As discussed above the patient was originally said to have normotriglyceridaemic abetalipoproteinaemia rather than normotriglyceridaemic hypobetalipoproteinaemia.

The way in which the clinical phenotype of this patient differs from that of the apoB25 homozygote of Huang *et al* (1989) is clearly a direct result of her ability to synthesise apoB48 and to package dietary lipid into chylomicron particles normally. Thus, while the apoB25 homozygote presented early with chronic diarrhoea and failure to thrive, the apoB50 homozygote had normal fat absorption and was obese. As far as gastrointestinal and nutritional effects are concerned, it is clear that those apoB mutants which are large enough to allow chylomicron formation have less severe consequences than the smaller variants. Given that vitamin E transport to the periphery relies on the presence of LDL particles however, unless the variant is large enough to allow LDL formation, then affected patients must still be at risk of neurological and retinal complications.

Of the reported compound heterozygotes, the apoB37 producing individual HJB, of Steinberg *et al* (1977) and Young *et al* (1987, 1988) and patient DD of this study, producing apoB39, are very similar. Not only are apoB37 and apoB39 of very similar size, but the second allele in both individuals contributes to the hypobetalipoproteinaemic pheno-

type by producing only very small plasma levels of full-length apoB100. The clinical severity of the two patients were accordingly very similar, both being normotriglyceridaemic, with only relatively mild malabsorption. In spite of the fact that HJB had only an extremely low plasma LDL cholesterol and DD had no lipoproteins detectable in the LDL density interval, neither had any detectable neurological or retinal degeneration. Unlike the patient of Malloy *et al* (1981) and Hardman *et al* (1989) who was ataxic, both of these individuals are able to produce at least a little apoB100, which may allow sufficient vitamin E delivery to the periphery to stave off such complications.

A similarly mild clinical presentation was seen in the apoB40/B90 compound heterozygote reported by Krul *et al* (1989). Although she presented with chest pain, this did not recur following admission to hospital. Her only other complaint was of occasional episodes of abdominal cramping and flatulence, thought to be due to irritable bowel syndrome. She did not however have any steathorroea. Presumably, apoB48 production proceeds as normal from the apoB90 allele, enabling adequate absorption of fat and the fat-soluble vitamins. She did not have any significant neurological or visual abnormalities. The fact that her apoB90-containing LDL was found to interact with LDL receptors of cultured human fibroblasts with greater than normal affinity (Krul *et al.*, 1989), suggests that her peripheral vitamin E delivery is likely to be adequate to prevent important neurological or retinal changes.

4.5 Abetalipoproteinaemic Individuals.

The apoB gene has been examined in four individuals with abetalipoproteinaemia by Southern blotting experiments, using six different restriction enzymes and probes spanning the coding sequences of the gene. By this technique, no gross alteration in the gene's structure could be detected in any of the individuals, suggesting that their disease might be caused by a defect in another gene.

In the two families examined, with two affected children each, it was possible to identify unambiguously and trace the inheritance of all four parental apoB alleles. Analysis of the apoB RFLP haplotypes in the individuals from the two families, showed that, in both cases, the affected siblings had inherited different pairs of apoB alleles from their normal parents. In a recessive disorder like abetalipoproteinaemia, for a particular gene to be responsible for the condition, affected siblings should inherit identical pairs of alleles from normal parents. The fact that this was not the case, rules out a mutation in or near to the apoB gene as the cause of abetalipoproteinaemia in these kindreds.

These findings are compatible with those of Lackner *et al* (1986). Southern blotting experiments were performed on the genomic DNA of four individuals with abetalipoproteinaemia, using restriction enzymes *Bam*HI, *Eco*RI, *Sst*I and *Xba*I and four cDNA probes from the 5', middle and 3' regions of the apoB gene. The restriction patterns obtained were not significantly different from those obtained with control DNA, indicating no major insertions or

deletions in the apoB gene. The same workers also examined the polyadenylated hepatic mRNA from two of the patients and a normal control by Northern blot analysis. In both patients, hepatocyte contained an apoB100 mRNA which was of similar size to that of the control. In addition, dot blots of total liver RNA from the two patients and from three normal controls were performed using a 5′ cDNA probe. Laser densitometry of autoradiographs of the blots showed that the liver from the abetalipoproteinaemic patients contained approximately six times the quantity of apoB100 mRNA as normal liver. Further, immunohistochemistry was performed on liver tissue from two abetalipoproteinaemic subjects and from control subjects. Using either a polyclonal or a mixture of four monoclonal apoB100 antibodies, apoB100 was readily demonstrable in the cytoplasm of both control and abetalipoproteinaemic hepatocytes, with rather more intensity in the latter. Thus, in these individuals at least, the apoB gene is present without any major insertions or deletions and is transcribed into a normal sized mRNA. The mRNA is present in increased amounts and is translated into apoB or an apoB-like protein, but remains within the cytoplasm of the hepatocytes and is not secreted. The authors suggest that the most likely defect in abetalipoproteinaemia is in the posttranslational processing and secretion of apoB which leads to defective secretion of apoB-containing lipoproteins from the cell. They do not however rule out the possibility that such defects might be secondary to a structural abnormality in the apoB protein,

such as an amino acid substitution which leads to a defect in carbohydrate addition, or even to a single base substitution causing premature termination of apoB100. The results of the studies presented here, indicate that, in the patients studied, any defect in posttranslational modification or secretion must be primary and not secondary to a defect in the apoB gene.

The hypothesis that abetalipoproteinaemia is the result of a defect in the secretion of apoB and apoB-containing lipoproteins is supported by the studies of Dullart *et al* (1986). The presence of apoB in both the liver and intestine of a patient with abetalipoproteinaemia was evaluated by immunohistochemistry with a polyclonal and six monoclonal antibodies to different apoB100 and apoB48 epitopes. Although apoB was not detectable in the patient's plasma, a polyclonal anti-apoB antiserum gave faint but specific staining in her hepatocytes and normal staining in her enterocytes. In addition, the patient's intestinal epithelium labelled with all and the hepatocytes labelled with three of the six monoclonal antibodies. The authors suggest that abetalipoproteinaemia in their patient is due to a defect in the secretion of apoB or in it's association with lipid in both the liver and intestine.

It is possible and perhaps quite likely, that abetalipoproteinaemia might result from a variety of different molecular defects. Although Dullaart *et al* (1986) demonstrated the presence of apoB in abetalipoproteinaemic intestinal epithelium, other workers have found it to be

absent in this tissue. Glickman *et al* (1979) for example, examined peroral biopsy specimens taken from the duodenojejunal junction of five control subjects and two patients with abetalipoproteinaemia, both in the fasting state and after fat feeding. Using an anti-LDL antiserum in an immunofluorescent technique, they demonstrated the presence of apoB in the intestinal epithelium of the controls. The fluorescence appeared in the entire apical region of the cells and in the supranuclear region. Following the fatty meal, there was a marked increase in fluorescence throughout the apical region, along with an increase in intracellular lipid in the same region. More intense fluorescence was seen in the supranuclear area, the known location of the Golgi apparatus in the intestine. In contrast, although fasting samples from the abetalipoproteinaemic individuals were engorged with large lipid droplets, no reaction was seen with the anti-LDL antiserum. Even following a fatty meal in one of the patients, the reaction remained negative.

Although Glickman *et al* (1979) could not rule out the possibility that immunologically altered forms of apoB might be present within the abetalipoproteinaemic intestinal mucosa which were not recognised by the antibody employed, it appears that the disorder in their patients resulted from an inability to synthesis any normal apoB.

In a later study, Green *et al* (1982) used immunofluorescence to localise apoB, apoAI and apoAIV in intestinal biopsy specimens from three patients with abetalipoproteinaemia. They could not detect any staining for apoB or

apoAI, but found staining for apoAIV in two of the subjects, although the staining lined the fat filled spaces and did not resemble that seen in normal biopsy specimens. The authors suggest that the absence in the intestinal cells of apoAI in addition to apoB might be due apoB synthesis serving as a trigger for apoAI synthesis. As they point out however, Schwartz *et al* (1978) had previously found apoAI to be present in the intestine in abetalipoproteinaemia, supporting the view that abetalipoproteinaemia represents a heterogeneous disorder.

Levy *et al* (1987) studied the incorporation of ^{14}C-palmitate into triglyceride, phospholipid and cholesteryl ester and ^{14}C-leucine incorporation into protein in cultured jejunal explants from eight healthy control subjects and two abetalipoproteinaemic girls. They found that the esterification of palmitate into triglyceride, phospholipid and cholesteryl ester was only 50% lower in the patient's explants than in controls. Although substantial amounts of phospholipid and cholesteryl ester were released into the culture media however, only 3-5% of the synthesised triglycerides were recovered from the media. Overall, protein synthesis was reduced by only 40 and 60 % in the two patients, but SDS polyacrylamide gel electrophoresis and immunoblotting of sonicated explants, demonstrated a complete absence of both apoB48 and apoB100. In contrast, a large peak corresponding to apoB48 and a small peak which may have represented some apoB100 was present in control samples.

Thus, although the esterification mechanisms of the three

major classes of exportable lipids were intact in these patients, there was no detectable synthesis of apoB48, nor any significant triglyceride secretion. Like the patients of Glickman *et al* (1979) and Green *et al* (1982), abetalipoproteinaemia in these cases appears to be due to an absence of the synthesis of normal apoB, at least in the intestine. Although these various studies demonstrate heterogeneity in abetalipoproteinaemia with regard to the presence or absence of apoB synthesis, none provide any clear evidence as to whether the apoB gene is involved in the generation of the phenotype. The work described in this study demonstrates for the first time that abetalipoproteinaemia can be caused by a gene or genes other than that coding for apoB.

Very recently, Huang *et al* (1990) in a linkage study involving eight families with abetalipoproteinaemia, provide evidence which strongly supports the work described here. Each family had at least one child with abetalipoproteinaemia. In two of the families, the parents were consanguineous. In order to test the hypothesis that abetalipoproteinaemia is due to rare mutations in the apoB gene, genomic DNA was extracted from family members and two types of apoB RFLPs were used to establish the haplotypes of their apoB alleles. Using Southern blotting, six single base substitution RFLPs were detected with the restriction enzymes *Ava*II, *Hinc*II, *Pvu*II, *Xba*I, *Msp*I and *Eco*RI. A second type of RFLP due to variable numbers of tandem repeats (VNTRs) 3'to the gene were examined with *Xba*I. In cases of apparent homozygosity of

parents using these seven RFLPs, two single base substitution markers at *Alu*I and *Bal*I sites were examined, the former by allele specific oligonucleotide hybridisation because of the small size of restriction fragment involved. If apparent homozygosity persisted, the 3′ VNTRs were cloned and sequenced to look for microheterogeneity.

The results of these studied enabled Huang *et al* (1990) to classify their families into three distinct classes. In one family, referred to as being of class I, affected siblings had different apoB alleles, suggesting that the apoB gene is not linked to the disease in this case. LOD score analysis in fact showed an infinite negative number at $\Theta = 0$. In the two families with consanguineous parents, referred to as class 11, the affected children were heterozygous at the apoB locus. In these cases the disorder should almost certainly be due to homozygosity at the disease causing locus by descent from a common ancestor (Lander and Botstein., 1987). The fact that this is not the case for the apoB gene makes linkage of the disorder to this locus very unlikely; the sum of the LOD scores for these two families being -1.7 at $\Theta = 0$. In a single class III family, one parent appeared to be homozygous at the apoB locus, yet unaffected, again making the apoB gene an unlikely disease causing candidate (LOD score = -0.176 at $\Theta = 0$). In four other families, referred to as class IV, the hypothesis that the apoB gene segregates with the disorder was not violated, but the combined LOD score was not statistically significant (0.977 at $\Theta = 0$).

Although they were unable to conclude that all cases of

abetalipoproteinaemia are not due to defects in the apoB gene, they conclude that in most cases the genetic abnormality is not the apoB gene.

Clearly, mutations in a variety of other genes that are separately involved in the synthetic, assembly and secretory pathways of apoB containing lipoproteins could theoretically be responsible for the disorder. The secretion of these lipoproteins is clearly a multistep process, requiring a substantial amount of time for it's completion. Pulse-Chase studies using HepG2 hepatoma cells, indicate that the apoB molecule is secreted after a lag phase of about 30 minutes, with about 2/3 of this time being taken up by transfer through the Golgi apparatus (Olofsson *et al.*, 1987). During this time, at least eight intramolecular disulphide bonds are formed (Yang *et al.*, 1989). The protein is also modified by N-glycosylation of asparagine residues. All but three of the 19 potential N-glycosylation sites are modified in this way (Yang *et al.*, 1989) to give a carbohydrate content of 4-5% by weight, a high proportion of which has a high mannose structure (Vauhkonen *et al.*, 1985). In addition, there is acylation with palmitate and stearate, by the formation of thiol ester linkages to the molecule, as well as the formation of intramolecular thiol ester linkages between cysteine and acidic amino acid residues (Huang *et al.*, 1988). In the rat liver, phosphorylation of apoB48 has been reported (Davies *et al.*, 1984) and this may represent another important modification involved in the regulation of the apoB secretion. Incorporation of the apoB into

lipoproteins appears to take place at the border between rough and smooth ER (Alexander *et al.*, 1976), but the nascent lipoproteins are apparently modified during their passage through the Golgi, with transfer of phospholipid to the lipoprotein surface (Howell and Palade., 1982).

Clearly any one of these processes, or other, as yet uncharacterised stages of the lipoprotein secretory pathway, could be affected by a non-apoB gene mutation which could interrupt the secretory process and as is the case with abetalipoproteinaemia, might be predicted to be transmitted recessively.

4.6 Future Studies.

More apoB variants will no doubt continue to be identified in patients with hypobetalipoproteinaemia and further artificial apoB constructs synthesised and expressed in *in vitro* liver cell lines. Both of these sources will continue to provide new information about the different functional domains in the apoB molecule. In addition, it is likely that mutations in the 5′ flanking and perhaps other regulatory regions of the apoB gene will be found in some instances of hypobetalipoproteinaemia, thereby giving information about the mechanisms of regulation of apoB synthesis and secretion.

With regard to abetalipoproteinaemia, if the gene or genes responsible for the disorder are identified and the causative mutations defined, a much greater understanding of the lipoprotein secretory pathways and the factors controlling apoB production will inevitably result.

One approach which could prove successful in locating the

gene(s) responsible for abetalipoproteinaemia is that of homozygosity mapping (Lander and Botstein., 1987). This approach is in theory a highly efficient strategy for mapping any human genes that cause rare recessive traits. The method involves the study of DNA from affected children from consanguineous marriages (sibling, first cousin or second cousin). It depends on the detection of the disease locus by virtue of the fact that the adjacent regions will be preferentially homozygous by descent in such inbred children. In practice it requires a complete linkage map of the human genome, containing RFLP's evenly spaced every 10 centimorgans and about a dozen unrelated affected inbred children. With more highly polymorphic markers and a denser linkage map, rather less individuals would be required to achieve an adequate odds ratio in favour of linkage ($\log_{10}$ of the odds ratio; LOD score >3).

4.7 Summary

A study of the apoB gene and protein has been carried out in a series of individuals with familial hypocholesterolaemia. In the case of those individuals with familial hypobetalipoproteinaemia, the disease phenotype has been shown to be the result of mutations within coding regions (exons) of the apoB gene. The mutations predict the production of truncated variants of apoB and in one family such a variant has been identified in the serum of two of the affected individuals. Since the work was performed, these findings have been supported by the work of others. Additional apoB gene coding region mutations have been defined, some of which likewise result in the appearance of truncated forms of apoB in the serum. Each new mutation identified in subjects with hypobetalipoproteinaemia has been different, indicating that the disorder is highly heterogeneous. Although theoretically likely, no mutations in non-coding regions of the apoB gene have to date been found to cause hypobetalipoproteinaemia as a result of either this work or that of others.

In those individuals with abetalipoproteinaemia, it has been shown by a linkage study in two families that the apoB gene is discordant with the disorder, a finding now supported by other workers studying different families. This suggests that in these cases, abetalipoproteinaemia must be due to a mutation in another gene or genes responsible for the synthesis or secretion of apoB containing lipoproteins from both the liver and the intestine. It is hoped that with the development of a complete linkage map

of the human genome it will be possible to identify the gene(s) and the mutations responsible for abetalipoproteinaemia by the study of a relatively small number of inbred affected individuals.

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Statement of work done by the author

Work performed exclusively by myself includes all of the Southern blotting experiments, the preparation of lipoproteins, SDS polyacrylamide gel electrophoresis, the Western blotting and the cloning and sequencing of the apoB 5' flanking region of patients with familial hypobetalipoproteinaemia, plus definitive Southern blots on the patients with abetalipoproteinaemia.

The cloning and sequencing of the two hypobetalipoproteinaemia mutants was performed by Dr.Timothy Knott and many of the Southern blots on the abetalipoproteinaemic patients, were performed by Dr. Philipa Talmud.

Claims to originality

The two apoB mutants, apoB29 and apoB39, were the second two apoB mutations to be fully characterised in patients with hypobetalipoproteinaemia (Collins *et al.*, 1988), appearing in press just one month after the complete description of apoB37 (Young *et al.*, 1988). The mutations fully explain the pathogenesis of the hypobetalipoproteinaemic phenotype in three heterozygous subjects and partly account for the phenotype of a homozygous individual. This is the only description to date of these particular apoB mutants.

The linkage work on the two abetalipoproteinaemic families is the first to demonstrate conclusively that abetalipoproteinaemia is not caused by a defect in the apoB gene. This is important since it points to the specific involvement of a gene or genes other than that for apoB in the apoB secretory process and implies new modalities of regulation of this major apolipoprotein.

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Genetic Evidence from Two Families that the Apolipoprotein B Gene Is Not Involved in Abetalipoproteinemia

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Abstract

Abetalipoproteinemia (ABL) is a recessive disorder in which affected individuals have extremely low or undetectable levels of serum apo B-containing lipoproteins. Using restriction fragment length polymorphisms, we have studied two families, each with two children with classical ABL born of normal parents. In each of these families, the two affected children have inherited different apo B alleles from at least one parent, whereas the siblings would be anticipated to share common alleles if this disorder were due to an apo B gene mutation. This linkage study shows that in these families, the apo B gene is discordant with ABL and therefore the disorder is caused by a defect in another gene, which is important for the normal synthesis or secretion of apo B-containing lipoproteins from both the liver and intestine.

Introduction

Inherited inability to secrete apo B-containing lipoproteins is exemplified by abetalipoproteinemia (ABL)[1] and homozygous hypobetalipoproteinemia (HBL). Patients with classical ABL are characterized by extremely low or undetectable plasma concentrations of apo B and of the apo B-containing lipoproteins (1, 2). The clinical features are fat malabsorption and acanthocytosis, both of which are present from birth, and a progressive spinocerebellar degeneration and retinopathy, which typically develop in the second decade of life. The latter features can be prevented by the administration of large oral doses of vitamin E (100 mg/kg per d) (3). Obligatory heterozygotes are phenotypically normal and have normal concentrations of the serum lipoproteins and apo B. Family studies and a high degree of consanguinity indicate the autosomal recessive inheritance of a rare gene (2). The molecular defect causing ABL remains unknown.

Conversely, HBL is an autosomal codominant disorder; obligate heterozygotes have half the normal plasma apo B and apo B-containing lipoproteins and may be asymptomatic or show some of the features of ABL. Homozygous HBL is, however, indistinguishable both biochemically and clinically from ABL. The difference in the mode of inheritance in ABL and HBL has aided in the differential diagnosis of these two disorders (4). There is good evidence from molecular genetic studies that HBL is caused by a defect in the apo B gene itself. Low levels of apo B mRNA in liver biopsies from patients with HBL have been reported, suggesting a defect in production in these individuals (5). Young et al. have described an individual from a large kindred with compound heterozygous HBL. In this case one apo B allele produced a truncated apo B, apo B-37, and the other allele caused low levels of apo B-100 in the plasma. In this kindred, both alleles cosegregate with HBL (6, 7). We have also studied two patients with HBL and documented the presence of premature termination codons in the coding sequence of the apo B gene, resulting in the production of short apo B proteins (7a).

As raised apo B levels are associated with hyperlipidemia and risk of myocardial infarction (8), factors controlling apo B synthesis in the liver and intestine are important to our understanding of the development of dyslipoproteinemia. It may therefore be possible to improve our understanding of the control of apo B synthesis by studying patients with rare inborn errors in the synthesis and secretion of apo B-containing lipoproteins. In this study, we have used restriction fragment length polymorphisms (RFLP) to perform linkage analysis on two families, each with two children with ABL. Our results provide the first clear genetic evidence that in these two families, ABL is not caused by a defect in the gene for apo B.

Methods

Patients. Two families, each with two affected children, were studied. These patients had the classical recessive form of ABL. The J family has been previously reported (case 1 and 2 of references 3, 9, and 10). Family M has not been previously reported. C.M. presented in infancy with fat malabsorption and acanthocytosis. Her brother was diagnosed at birth by examination of cord blood.

These results were presented in preliminary form at the North Atlantic Treaty Organization Advanced Research Workshop, Limone, Italy, March 1988, and the International Symposium on Intestinal Lipid Metabolism, Hamburg, Federal Republic of Germany, May 1988.

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1. *Abbreviations used in this paper:* ABL, abetalipoproteinemia; HBL, hypobetalipoproteinemia; RFLP, restriction fragment length polymorphism.

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Lipid determinations. Whole blood was collected into EDTA and the plasma was stored at −20°C before estimating the lipid, lipoprotein, and apo B concentrations. For samples within the normal range, apo B was assayed immunochemically using a polyclonal antiserum (Orion Diagnostics, Espoo, Finland) that correlates well with the results obtained with radioimmunodiffusion. For samples with apo B levels < 50 mg/dl, an ELISA assay was used (11). Samples of standards (LDL-apoB) ranged from 0 to 0.5 mg/dl; this accounts for the small amounts of apo B detected in the patients using this method. Total cholesterol and total triglyceride concentrations were estimated by routine enzymatic methods using commercial kits supplied by Boehringer-Mannheim Corp. (London, UK) and Metachem Diagnostics (Rugby, UK), respectively.

DNA extraction and digestion. DNA was extracted by the Triton X-100 method (12) and digested with Hinc II, Pvu II, Xba I, Eco RI and Msp I (2–10 U/μg DNA) under conditions recommended by the manufacturers (Anglian Biotech. Ltd., Colchester, UK). The DNA fragments were separated by size on a 1% (Pvu II, Hinc II, Xba I, and Msp I) or 0.7% (Eco RI) agarose gel and transferred to Hybond-N (Amersham International, Amersham, UK) by Southern blotting as previously described (13).

DNA probes. The probes used were a 3.5-kb Eco RI unique fragment (pAB3.5C) of the apo B gene to detect the Xba I RFLP (14), a 2-kb Hind III unique fragment (BH2) to detect the Msp I and Eco RI RFLP, and a 959-bp cDNA probe to detect the Pvu II and Hinc II RFLP (15). The labeling of probes, hybridization, and washings were carried out as previously described (16). A map of the apo B gene showing the position of the variable sites and fragment sizes of the Pvu II, Hinc II, Xba I, and Eco RI RFLP appears in Fig. 1. The hypervariable region identified at the 3′ untranslated end of the gene was detected using the enzyme Msp I.

Results

Two pairs of siblings with the classical, recessively inherited form of ABL and their parents (in both cases unrelated) were studied. In Family J, there is an unaffected older daughter. The relevant biochemical findings are given in Table I. The diagnosis of the classical form of ABL was made on the basis of the plasma lipid and apo B concentrations of the parents, which were within the normal range and the absence of clinical and hematological abnormalities in the parents. The relatively low cholesterol and apo B concentrations observed in the parents in the M family are at the lower end of the normal range and may result from the low-fat diet adopted by this family.

Figs. 2 and 3 show the pedigrees of the two families studied. In both cases, it is possible to distinguish unambiguously all four parental chromosomes. In the J family (Fig. 2) we have used five RFLP of the apo B gene to deduce the parental haplotypes. The affected son, M.J., is homozygous for all five polymorphisms. This allows us to deduce the four parental haplotypes and hence determine the phase of the RFLP variable sites of the parental chromosomes. Both affected children have inherited the apo B gene defined by haplotype IV from their father, whereas one child (M.J.) has inherited haplotype II and his sibling (S.J.) haplotype I from their mother. The unaffected sister N.J. has inherited haplotypes II and III.

In the M family (Fig. 3) we have made use of the length polymorphism identified by Msp I to distinguish all four parental chromosomes. The mother has alleles *M2* and *M3* and the father has alleles *M1* and *M2*. The two Msp I sites span a hypervariable region in the 3′ untranslated region of the gene, which consists of a varying number of repeats of a AT-rich, 30-bp consensus sequence (17). P.M. has inherited allele *M2* from the father and allele *M2* from the mother; C.M. has inherited allele *M1* from the father and allele *M3* from the mother.

Thus, in both families, the children have inherited different alleles of the apo B gene from one or both parents. These observations are incompatible with the hypothesis that a mutation in, or close to, the gene for apo B causes classical recessive ABL in these families.

Discussion

Previous studies in patients with ABL provide no clear evidence as to whether this condition is caused by a mutation of the apo B gene itself or whether it results from an abnormality in a gene or genes necessary for the biosynthesis and secretion of apo B-containing lipoproteins. Two groups have failed to detect apo B epitopes in intestine (18, 19), which suggests a defect in apo B synthesis, whereas others, using similar techniques, have reported the presence of apo B protein using antibodies in both intestine (20) and liver (20, 21). Lackner et al. have also detected increased amounts of normal-sized apo B mRNA in liver biopsies of ABL patients in addition to the absence of gross deletions or insertions in the apo B gene of these patients (21). We have also examined the gross structure of the apo B gene in the four patients reported here and a further five unrelated ABL patients. We have hybridized Southern blots of DNA from these patients, digested with a number of restriction enzymes, with DNA probes that span the apo B gene. No gross alterations of gene structure could be detected in any of the samples (not shown). Together, these data suggest that ABL may not be caused by a defect in the apo B gene and that it may be a heterogeneous disorder caused by defects of genes that are separately involved in regulating the synthesis, assembly, or secretion of apo B-containing lipoproteins.

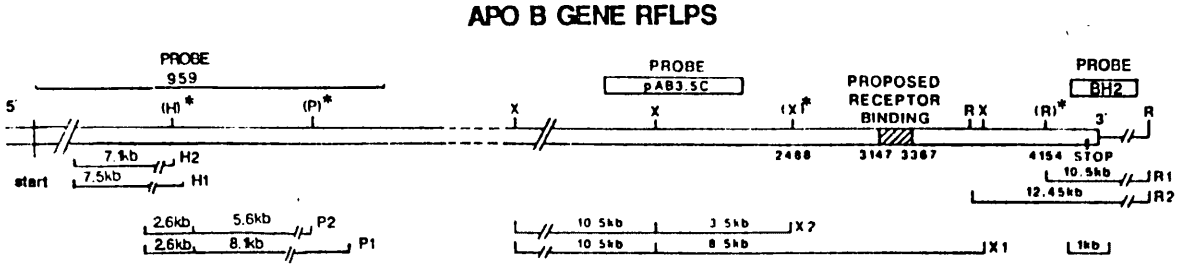

Figure 1. A map of the apo B gene illustrating the DNA probes used in the identification of the RFLP and the variable restriction enzyme sites (•). P, Pvu II; H, Hinc II; X, Xba I, and R, Eco RI. The fragment sizes of the alleles for each RFLP are shown.

Table I. Serum Lipid, Apo, and Vitamin E Concentrations in Patients with ABL and their First-Degree Relatives

	Cholesterol	Triglyceride	Apo B	Vitamin E
	mmol/liter		*mg/dl*	*μmol liter*
Mr. J.	6.6	1.4	112	23.6
Mrs. J.	4.8	0.6	74	24.3
N.J.	3.5	1.1	83	17.4
M.J.	0.9	<0.1	0.24	1.5*
S.J.	0.6	<0.1	0.09	0.6*
Mr. M	4.0	2.0	84	25.1
Mrs. M.	3.7	0.7	61	23.0
C.M.	0.9	<0.1	0.57	4.5*
P.M.	0.9	<0.1	0.31	3.4*
Normal range	3.8–6.8	0.4–1.8	60–140	11.5–35.0

* Patients received vitamin E therapy in addition to other fat-soluble vitamins and a low-fat diet.

This study demonstrates for the first time that ABL can be caused by defects of a gene or genes that do not code for apo B and are not closely linked to it. In a recessive disorder like ABL, affected siblings should inherit the same defective gene from both the mother and father. Analysis of RFLP haplotypes in the affected siblings from both of the families studied here rules out a mutation in or near the apo B gene as the cause of ABL in these kindreds.

Many of the steps in the biosynthesis of apo B-containing lipoproteins in the liver and intestine have yet to be explained. Pulse-chase experiments in a hepatoma cell line (HepG2) show that the time taken from the start of apo B mRNA translation on the ribosome to the secretion of apo B from the cell in a lipoprotein particle is ~ 30 min (22). During this time, protein folding, disulfide bond formation, and lipoprotein assembly must take place. Apo B is O- and N-linked glycosylated and other posttranslational events including acylation and phosphorylation take place (23–25). There are therefore several potential points in this process at which defects in proteins that carry out these steps may block or alter the rate of production of apo B-containing lipoproteins. Such defects would result in the features of recessively inherited ABL found in our patients. It remains to be seen whether in different patients with ABL the defect is in different genes, resulting in the same phenotype. The possibility remains that defects in the coding region of apo B or upstream promoter regions of apo B may account for this clinical syndrome.

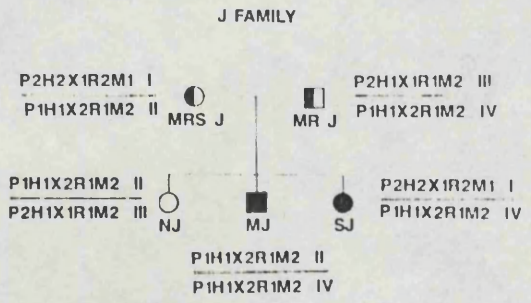

Figure 2. Pedigree of the J family. DNA was digested with the enzymes Pvu II (P), Xba I (X), Hinc II (H), Eco RI (R), and Msp I (M). The unambiguously deduced haplotypes are designated I–IV. □, male; ○, female.

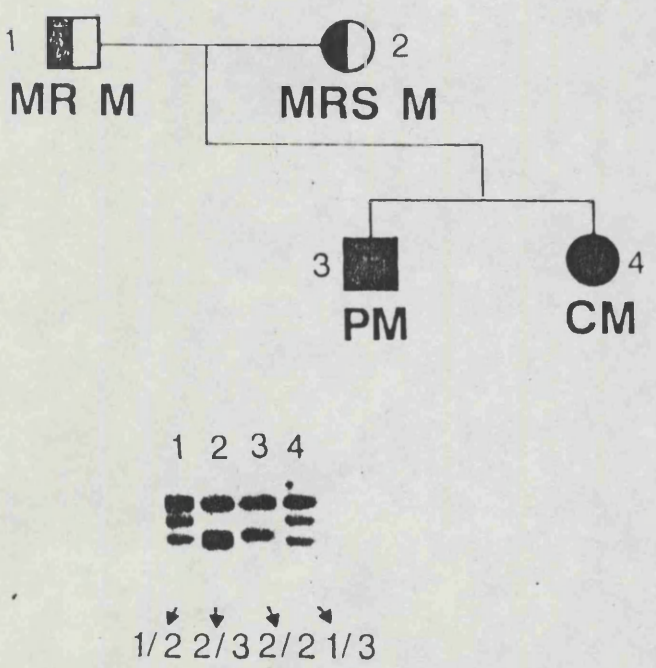

Figure 3. Pedigree of the M family. DNA was digested with the enzyme Msp I and hybridized with the genomic probe BH2. Msp I alleles in this family are numbered 1–3. There is an invariant band of 2.6 kb. Alleles *M2* and *M3* are not easy to distinguish. The broad doublet in the second track, from Mrs. M., indicates that she is *M2M3*. P.M. is homozygous for the *M2* allele, Mr. M. is *M1M2*, and C.M. is *M1M3*. Family members are numbered 1–4. □, male; ○, female.

An efficient strategy has recently been proposed to map defects causing recessive disorders, by studying the affected offspring of consanguinous marriages (26). This approach may be applicable to determine the gene defect in ABL. It is possible that common variations in the gene or genes whose functions appear to be vital for the normal secretion of apo B containing lipoproteins, may also be involved in the abnormal secretion of apo B-containing lipoproteins in patients with some common forms of dyslipoproteinemia.

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Truncated variants of apolipoprotein B cause hypobetalipoproteinaemia

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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

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 ($1.063 < p <$

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

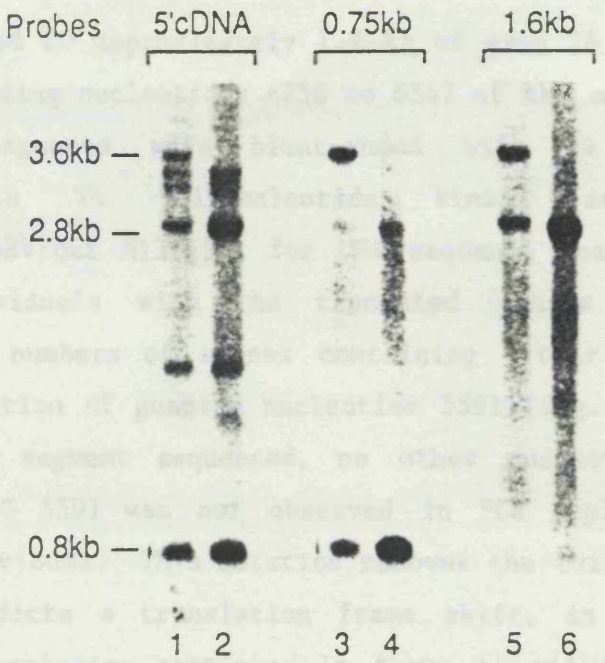

Figure 1: Genomic Southern blots of TaqI digested DNA. Lanes 1, 3 and 5 DNA from patient CP. Lanes 2, 4 and 6 control DNA. Lanes 1 and 2 were hybridised with pABF (4), a 6.5 kb cDNA from the 5' end of the apo-B message. Lanes 3 and 4 were probed with a 0.75 kb genomic XbaI fragment (fig. 2). Lanes 5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

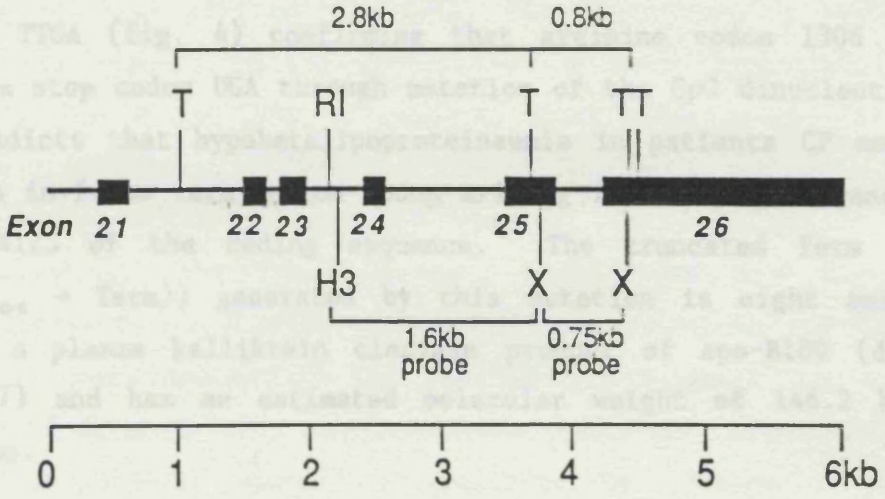

Figure 2: Restriction map of the apo-B gene around the TaqI mutation in patient CP. The positions of two genomic probes and the TaqI fragments which they detect are shown. Loss of the TaqI site in exon 25 joins the two TaqI fragments to give a 3.6 kb band (fig. 1). Abbreviations: T = TaqI, RI = EcoRI,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

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,

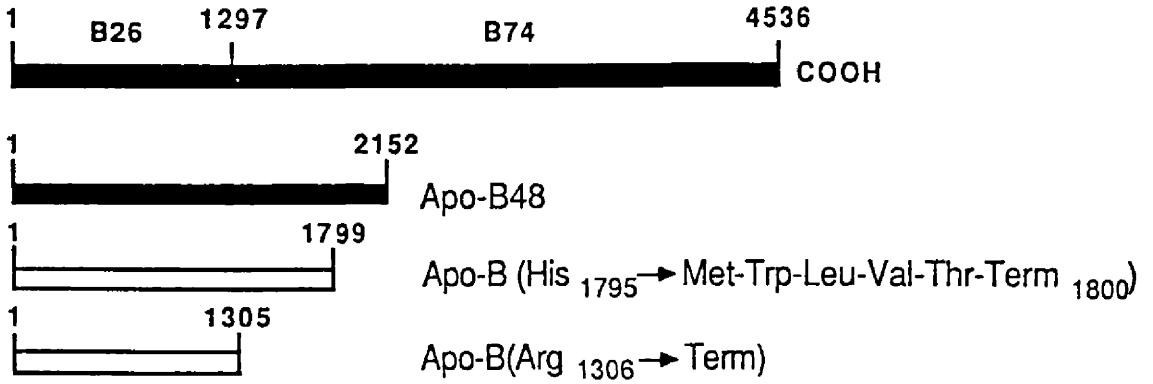

Figure 5: Truncated variants of apo-B which cause hypobetalipoproteinaemia. The normal plasma forms apo-B100 and apo-B48 are also shown. The kallikrein cleavage site (27) at residue 1297 in apo-B which generates apo-B26 and apo-B74 is marked.

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

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-339 are much lower than apo-B100 and apoB(Arg_{3306} → 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.

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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-Al. 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.

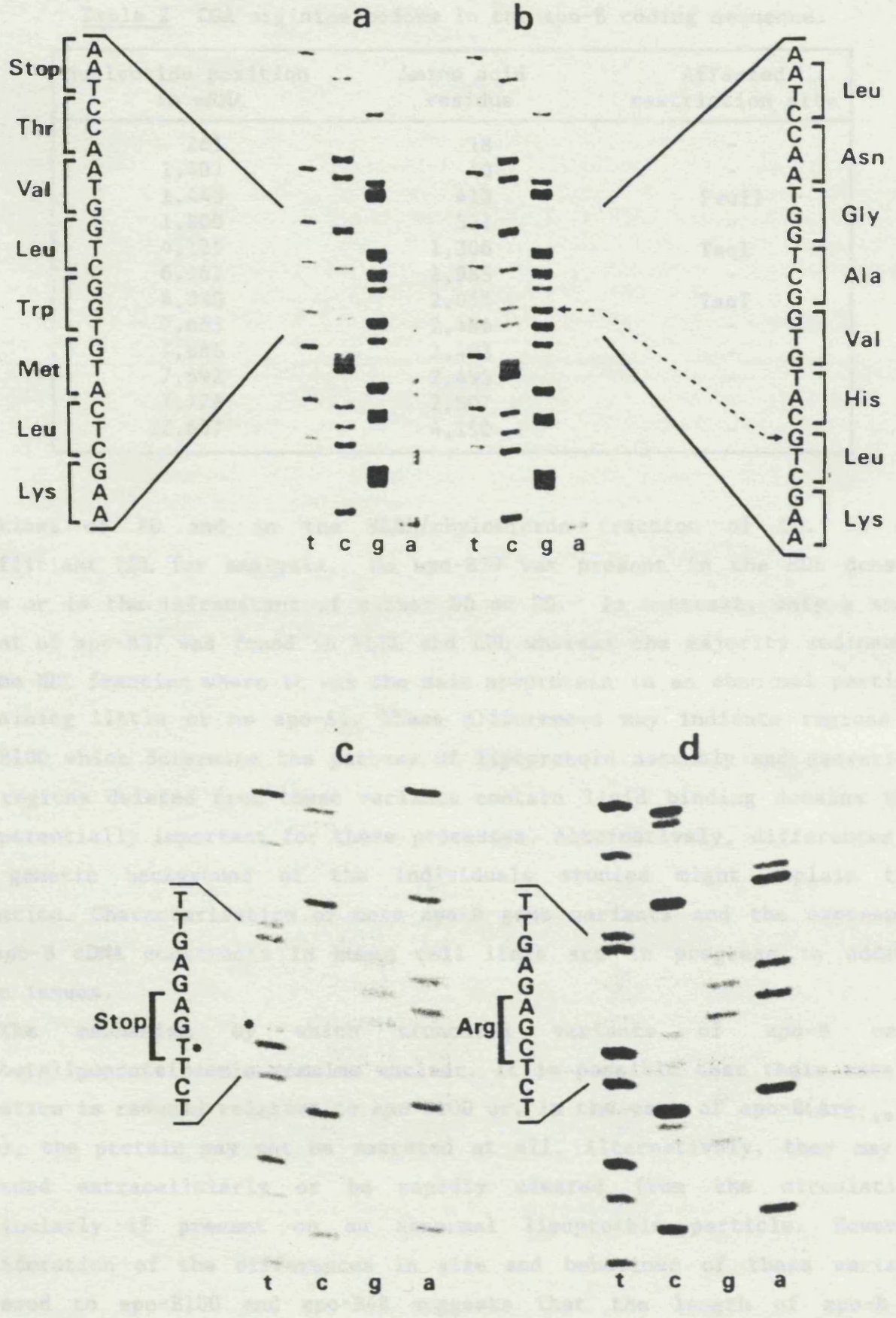

Figure 4: DNA sequence ladders of apo-B gene mutations. (a) mutant and (b) normal sequences from patient DD. The G nucleotide which is deleted from leucine codon 1794 in the defective allele is arrowed. The frame-shifted and normal translations are displayed alongside the ladders. (c) mutant and (d)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

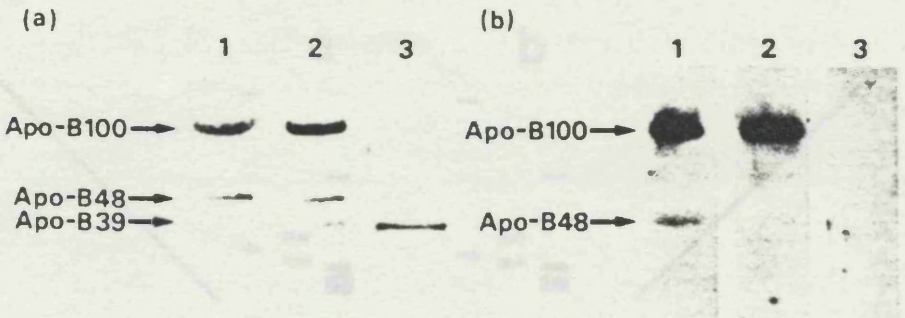

Figure 3: Western blots of lipoprotein sub-fractions from individuals with hypobetalipoproteinaemia. (a) 50 µg of proteins from the VLDL/chylomicron fractions of 1) normolipidaemic control individual, 2) patient PD, 3) patient DD. (b) 50 µg of proteins from 1) VLDL, 2) LDL, 3) HDL of patient CP. Blots 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

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▲	83

+ Normal ranges are 5 - 95 percentile.

* Receiving vitamin E supplement.

▲ 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)

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

(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.