Hypobetalipoproteinaemia and truncated forms of human apolipoprotein B

By

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A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Doctor of Philosophy

in

Biochemistry

Lincoln University

1992

Something Pooh said to Rabbit that reminded me of Science:

'We might find something that we weren't looking for, which might be just what we were looking for, really!' 'I don't see much sense in that,' said Rabbit. 'No,' said Pooh humbly, 'there isn't. But there was going to be when I began it.'

Taken from THE HOUSE AT POOH CORNER by A. A. Milne

To Mum and Dad

ABSTRACT

A new variant of human apolipoprotein B has been identified in a subject with decreased low density lipoprotein cholesterol and apolipoprotein B concentrations. Although no clinical signs of fat malabsorption were observed the subject was diagnosed, on the basis of his low cholesterol and apolipoprotein B level, as having hypobetalipoproteinaemia.

The variant of apolipoprotein B was first identified by Western blot analysis. The analysis revealed an abnormal low molecular weight form of apolipoprotein B as well as normal apolipoprotein B-100 indicating that the subject was heterozygous for a truncated form of apolipoprotein B. The new variant (apo B-32) was a result of a C→T transition at nucleotide 4548 in exon 26 of the apolipoprotein B gene. This mutation changes a CAG codon which codes for glutamine into a TAG stop codon resulting in translation of a truncated apolipoprotein B protein approximately 32% the length of normal apolipoprotein B-100.

Although only 32% of the length of apolipoprotein B-100, apo B-32 was still capable of forming lipoprotein particles as indicated by its presence in both the low density and high density lipoprotein fractions. This density distribution is unique since apo B-32 is the shortest known truncated apolipoprotein B to be found in the low density lipoprotein fraction. This finding clearly indicates that the region of apo B-32 is important in the lipid binding characteristics of apolipoprotein B-100. The binding of apo B-32 to heparin confirmed three heparin binding sites previously predicted to be in the amino-terminal 30% of apolipoprotein B-100.

Isolated lipoproteins formed from apo B-32 appeared to be similar to high density lipoproteins in size and composition. However, unlike high density lipoproteins, the apo B-32 lipoproteins in plasma were partially precipitated by polyanion/cation reagents normally used to precipitate very low density and low density lipoproteins. The presence of both apolipoproteins A1 and E on the apo B-32 lipoproteins suggested that apolipoprotein A1 or E may mediate the

metabolism of apo B-32 since apo B-32 does not posses the receptor binding region for the low density lipoprotein receptor.

Four further subjects were identified as having reduced low density lipoprotein and apolipoprotein B concentrations. However a lack of any truncated apolipoprotein B in their plasma made it difficult to link their hypobetalipoproteinaemia with the apolipoprotein B gene. The cause of the hypobetalipoproteinaemia in these subjects remains uncharacterised although future linkage analysis studies in these individuals and family members will at least establish whether their hypobetalipoproteinaemia is related to the apo B gene.

ACKNOWLEDGMENTS

Many people have been of assistance during the work for my thesis so to those not mentioned, thank you.

I sincerely thank my supervisors Dr Peter George and Dr Roy Bickerstaffe, Peter for your many helpful discussions and encouragement, Roy for you help with things administrative and constructive criticism during the writing of this thesis.

I have been fortunate enough to work with Drs Stephen Brennan and Maurice Owen both of whom have given me great instruction on the techniques of protein chemistry

I am especially grateful for the help given me by Andrew Fellowes. Your expert guidance in the field of molecular biology and on the computer! has been invaluable.

I am very grateful to the biochemistry team at Princess Margaret Hospital, Trevor, Janet, Jill etc. Thanks for the many lipoprotein isolations and lipid measurements.

I am sincerely grateful to Drs Stephen Young and Zemin Yao of the Gladstone Foundation Laboratories for supplying me with the monoclonal antibodies and apolipoprotein B expression vector for this work. I especially thank Dr Stephen Young for his interest and expert advice during this work.

The contribution made by Tony Day who performed the electron microscopy analysis is gratefully acknowledged.

I am grateful to the Cytogenetics Research group at Christchurch Hospital for the use of their tissue culture facilities. Also a special thanks to Dr Martin Kennedy for his expert advice on the art of tissue culture.

The subject involved in this study deserves a special mention. This thesis would not have been possible without his interest and willingness to participate.

I am very grateful to Associate Professor Robin Fraser for his constant interest and for giving me the opportunity to give the odd talk to medical students.

Thanks to Liz, Tania and Alison who have been a pleasure to work with, Liz for saving me time on many occasions, Tania for your positive attitude and Alison for sprucing up my notice board

I would like to sincerely thank the Canterbury Medical Research Foundation for their financial support toward the end of this study.

Finally I would like to thank Graeme for giving me the time, space and love that I needed throughout this study and our wee son Todd who has brought so much joy and perspective into my life.

PUBLICATIONS ARISING FROM THIS THESIS

1 McCormick, S. P. A., Fellowes, A. P., Walmsley, T. A., Brennan, S. O., and George, P. M. (1991) A point mutation of apolipoprotein B causes hypolipidaemia. *NZ. Med. J.* 104:320.

2 McCormick, S. P. A. (1991) Cholesterol: a two-faced molecule. Invited article for *Chem NZ*:45:18–19.

3 McCormick, S. P. A., Fellowes, A. P., Walmsley, T. A., and George, P. M. (1992) Apolipoprotein B-32: a new truncated mutant of human apolipoprotein B capable of forming particles in the low density lipoprotein range. *Biochem. Biophys. Acta.* 1138:290–296:

Other:

McCormick, S. P. A., Fellowes, A. P., Brennan, S. O., and George, P. M. (1989) Apolipoprotein E variation in patients with hyperlipidaemia: DNA and protein phenotyping. *NZ. Med. J.* 102:534–536.

ABBREVIATIONS

apo	apolipoprotein
ATP	adenosine 5′-triphosphate
bis-acrylamide	N,N′-methylene-bis-acrylamide
BES	N,N-bis-[2-hydroxyethyl]-2-aminoethanesulfonic acid
bp	base pairs
cDNA	complementary deoxyribose nucleic acid
dATP	2′-deoxy-adenosine-5′-triphosphate
dCTP	2′-deoxy-cytidine-5′-triphosphate
dGTP	2′-deoxy-guanosine-5′-triphosphate
dTTP	2′-deoxy-thymidine-5′-triphosphate
ddATP	2′, 3′-deoxy-adenosine-5′-triphosphate
ddCTP	2′, 3′-deoxy-cytidine-5′-triphosphate
ddGTP	2′, 3′-deoxy-guanosine-5′-triphosphate
ddTTP	2′, 3′-deoxy-thymidine-5′-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribose nucleic acid
dNTP's	dinucleotide triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
HDL	high density lipoproteins
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobases
LDL	low density lipoproteins
LPL	lipoprotein lipase
LRP	low density lipoprotein receptor-related protein

mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
RER	rough endoplasmic reticulum
RFLPs	restriction fragment length polymorphisms
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SER	smooth endoplasmic reticulum
TEMED	N,N,N',N'-tetramethylethlyenediamine
TLCK	tosyl-L-lysine chloromethyl ketone
TPCK	tosyl-L-phenylalanine chloromethyl ketone
Tris	Tris-(hydroxymethyl)-methylamine
USE	unique site elimination
UV	ultraviolet
VLDL	very low density lipoproteins
v/v	volume per volume
w/v	weight per volume
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Other symbols used are those of the International System of Units

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CHAPTER 1 INTRODUCTION

Lipoproteins are complex particles of lipid and protein (known as apolipoprotein) in the bloodstream that deliver cholesterol and triglyceride to tissues for metabolism and storage. Increasing awareness of the role of lipoproteins in controlling lipid levels within a population has prompted a number of structural studies on the major apolipoproteins. The majority of these studies have demonstrated the existence of biochemically defective apolipoproteins which contribute to the variation in lipid levels. In many cases the defective apolipoprotein is associated with the development of disease usually in the form of a specific hyper- or hypolipidaemia. Apolipoprotein (apo) B is a good example of this since molecular defects in this protein have a major effect on triglycerides and cholesterol levels.

Hypobetalipoproteinaemia is a disease in which the plasma concentrations of low density lipoprotein (LDL) cholesterol and apo B are abnormally low. Recently, this has been shown to be due to mutations in the apo B gene that prevent full length translation of the protein. Homozygotes have extremely low levels of apo B and LDL cholesterol and suffer a variety of clinical problems arising from malabsorption of dietary fat and fat soluble vitamins. Heterozygotes, who typically show apo B and LDL cholesterol levels of less than half the levels found in normal subjects, are usually clinically asymptomatic. Heterozygotes for hypobetalipoproteinaemia are actually protected from the development of coronary heart disease by their low LDL cholesterol levels.

Family studies of patients with hypobetalipoproteinaemia have revealed a number of defects in the apo B gene that interfere with the translation of the full length apo B protein (4536 amino acids). The truncated variants range in size, the smallest being apo B-2, a protein 2% the normal length of apo B-100, the largest being apo B-89. Variants less than 30% the full length of apo B are not detected in plasma presumably because they are too short to package lipids into lipoproteins for secretion into plasma. Variants greater than 30% are found in plasma and are big enough to form lipoproteins. The lipoproteins formed from these variants vary in size and density according to the size of the variant, in that the longer the truncated

apo B variant, the bigger and more buoyant the lipoprotein particles formed. Variants near full length appear to package lipoproteins normally. However their metabolism differs from that of the apo B-100 lipoproteins since they react with the LDL receptor with greater affinity and are cleared from plasma at a greater rate. This in part explains the association of the near full length truncated variants with low cholesterol levels. Variants intermediate in length do not react with the LDL receptor since they do not have the LDL receptor binding region. The metabolism of the lipoproteins formed from these variants and their association with low cholesterol levels remains elusive.

The aim of this thesis was to focus on subjects with abnormal lipid profiles, in particular those pointing to a defect in the apo B protein. More specifically those subjects with lipid profiles indicative of hypobetalipoproteinaemia (low LDL cholesterol and low apo B). Individuals having LDL cholesterol levels below the lower limit of the normal range for their sex and age were identified for more detailed investigation.

A 65 year old male was identified as having low LDL cholesterol during a routine lipid level check at a diabetes outpatient clinic at the Christchurch Lipid Clinic in 1989. A repeat blood sample in 1990 showed an LDL cholesterol of 31mg/dL, well below the lower limit of the normal range (105mg/dL) indicating possible hypobetalipoproteinaemia. Further analysis showed a new truncated apo B variant. The finding of this variant which had a unique density distribution, prompted an in depth study of its structure and function.

The main objective then was to elucidate the defect behind, and the altered structure, of the truncated variant. Once this was established, existing protein chemistry and molecular biology techniques were employed to characterise as fully as possible the lipoprotein particles formed from the truncated variant both *in vivo* and in cell culture. This information along with that obtained from other naturally occurring truncated variants has increased existing knowledge on the critical regions, in particular the lipid binding regions, in the structure of apo B. These regions are important in determining the function of the major apolipoprotein involved in cholesterol metabolism. Characterisation of the lipoproteins formed with the

truncated apo B variant has also given insight as to their metabolism.

CHAPTER 2 LITERATURE REVIEW

2.1 The plasma lipoproteins

Lipoproteins are the complexes of lipids and proteins that carry triglycerides and cholesterol in the bloodstream. They are roughly spherical particles with a core of non-polar lipids (triglyceride and cholesterol) surrounded by a mono-layer of polar lipids (mainly phospholipids) and apolipoproteins. The apolipoproteins, with the exception of the large apolipoprotein B, are water soluble and have the ability to exchange between particles (Havel and Kane, 1989a).

Table 2.1 Physical Properties of Human Plasma Lipoproteins[a]

Lipoprotein class	Electrophoretic position	Particle size (nm)	Molecular weight (Da)	Density (g/mL)
Chylomicrons	origin	75–1200	400 000 000	0.93
VLDL	pre-β	30–80	10–80 000 000	0.93–1.006
LDL	β	18–25	2 300 000	1.006–1.063
HDL	α	5–12	175–360 000	1.063–1.21

[a]adapted from Gotto *et al* (1986).

There are four main classes of plasma lipoproteins the physical properties of which are given in table 2.1. Several methods exist for the isolation of the different classes (Mills *et al*, 1984) however the most widely used is that of ultracentrifugation where the particles are separated on the basis of their density. The plasma lipoproteins differ in chemical composition. Table 2.2 shows the chemical composition of the major lipoproteins found in man. The major lipid in chylomicrons and very low density lipoproteins (VLDL) is triglyceride. LDL are rich in cholesterol while the high density lipoproteins (HDL) contain more phospholipid and protein.

Table 2.2 Chemical Composition of Human Plasma Lipoproteins

Lipoprotein Class	Cholesterol	Triglyceride	Phospholipid	Protein
		(% weight)		
Chylomicrons[a]	2.5	84.0	9.0	2.5 (A,B,C)
VLDL[b]	21.6	49.9	18.6	7.7 (B,C,E)
LDL[b]	47.0	11.2	22.1	20.9 (B)
HDL[b]	17.9	8.0	22.7	51.9 (A)

[a]adapted from Skipski (1972)
[b]adapted from Chapman (1986)
The major apolipoproteins involved with each lipoprotein class are placed in brackets

2.2 Lipoprotein assembly and secretion

Evidence suggests there are only two types of cells that assemble and secrete lipoprotein particles, namely the absorptive cells of the intestine and hepatocytes (Gotto *et al,* 1986). Lipoprotein assembly and secretion appears to follow the same route as other secretory proteins. This has been demonstrated by electron microscopy studies using radio- and immuno-labelled precursors (Glaumann *et al,* 1975; Alexander *et al,* 1976) and more recently by pulse-chase and cell fractionation (Bamberger and Lanes, 1988; Borchardt and Davis, 1987). Figure 2.1 shows the general scheme of lipoprotein assembly and secretion using VLDL as an example. The apolipoproteins, with a signal peptide (Protter *et al,* 1986) which is co-translationally cleaved, are synthesised on the ribosomes of the rough endoplasmic reticulum (RER). The apolipoprotein is then associated with lipid from the smooth endoplasmic reticulum (SER) to form a nascent lipoprotein particle. Transport to the Golgi apparatus then occurs and further lipid, mainly phospholipid (Higgins and Fieldsend, 1987), is added and the apolipoproteins undergo glycosylation. Finally the particles are concentrated within the secretory vesicles of the Golgi and transported to the plasma membrane to which they fuse releasing the lipoprotein into the extracellular space. Chylomicrons enter the blood via the intestinal villi and VLDL through the sieve plate fenestrae of the liver. Once in the circulation the lipoprotein particles undergo extensive metabolism and modification.

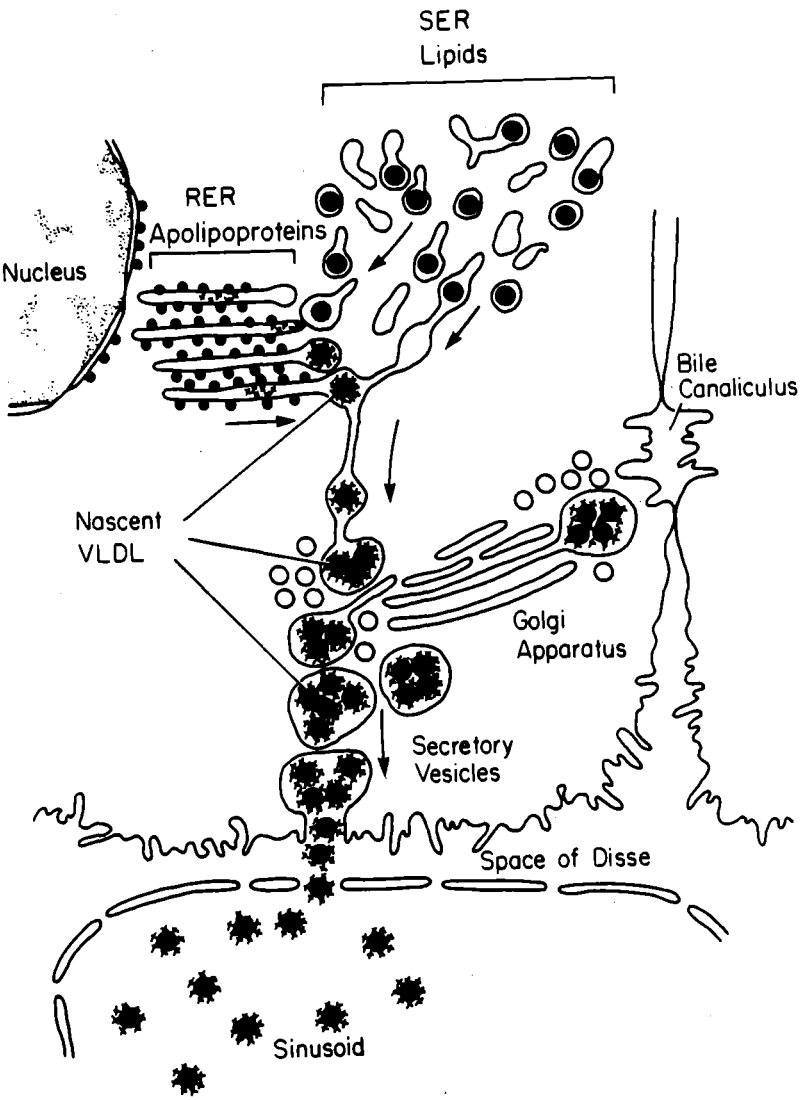


Figure 2.1 General scheme for the assembly and secretion of VLDL from hepatocytes (from Gotto *et al*, 1986).

2.2.1 The crucial role of apo B

The importance of apo B in lipoprotein assembly and secretion is emphasised in the rare disease abetalipoproteinaemia (Havel and Kane, 1989b). Patients with this recessive disorder have no detectable plasma apo B and as a consequence are unable to form the apo B-

containing particles (chylomicrons, VLDL, LDL). Plasma cholesterol levels in these patients are very low (<50mg/dL total cholesterol) as are plasma triglyceride levels (<18mg/dL total triglyceride). Subjects with abetalipoproteinaemia suffer an array of symptoms associated with severe fat malabsorption (Salt *et al*, 1960; Malloy and Kane, 1982). Genetic studies have shown that the primary defect is not in the apo B gene (Talmud *et al*, 1988). Identification of apo B mRNA and protein in the hepatocytes of affected patients (Lackner *et al*, 1986) suggest that the defect is in the assembly and secretion of apo B containing particles. Recent work by Wetterau *et al* (1992) has shown the lack of a microsomal triglyceride transfer protein, thought to be important in lipoprotein assembly, in subjects with abetalipoproteinaemia. This finding suggests that it is a defect in the microsomal transfer protein that is the basis for abetalipoproteinaemia.

2.3 Metabolism of apo B-containing lipoproteins

2.3.1 Chylomicron metabolism

The pathway of chylomicron metabolism is shown in figure 2.2. Chylomicrons are formed in the intestine from absorbed dietary cholesterol and triglycerides. After secretion from enterocytes, the triglyceride-rich chylomicrons containing apo B-48 and apo A are delivered to the bloodstream via the thoracic duct. Chylomicrons are rapidly metabolised in the circulation by lipoprotein lipase (LPL) which hydrolyses 80-90% of the triglyceride in the particle's mass (Young, 1990). LPL resides on the endothelial surface of capillaries and is activated by apo C-II (Gotto *et al*, 1986) which is acquired by chylomicron particles along with apo E from circulating HDL. Triglycerides are utilised by extrahepatic tissues as a source of energy (skeletal muscle) or stored (adipose tissue). The particle at this stage with its shrunken core is termed a chylomicron remnant. Apo E on the surface of the remnant particle binds to receptors on the surface of liver cells that recognise apo E. There is now increasing evidence that the receptor involved in chylomicron remnant metabolism is the LDL-like receptor related protein (LRP) described by Beisiegel *et al* (1989). It has been well

demonstrated that LRP recognises the apo E on chylomicron remnants (Biesiegel *et al,* 1989; Kowal *et al,* 1989) and that this interaction is greatly enhanced by the presence of LPL (Beisigel *et al,* 1991).

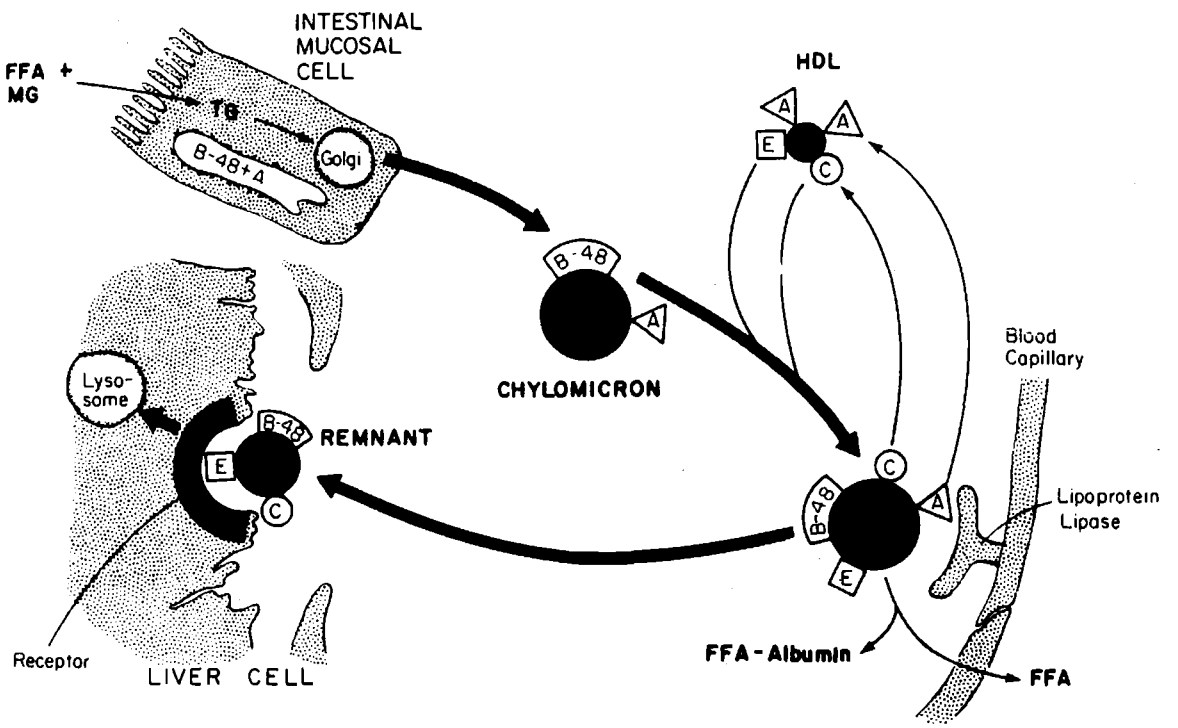


Figure 2.2 Pathway of chylomicron metabolism (from Havel and Kane, 1989a).

After receptor-mediated uptake and subsequent endocytosis the remnant particles are broken down to their constituents in lysosomes. The cholesterol component is generally esterified and either stored or used in the synthesis of bile acids and hepatic lipoproteins (Havel and Kane, 1989a).

2.3.2 VLDL metabolism

VLDL is formed in the liver from endogenously synthesised cholesterol and triglycerides. Figure 2.3 shows the pathway of VLDL (and LDL) metabolism. VLDL particles enter the circulation via the hepatic vein. Initially VLDL is metabolised in a similar way to chylomicrons. The particle's triglyceride core is hydrolysed by LPL to form a VLDL remnant. VLDL is metabolised more slowly than chylomicrons having an average residence time of 15-60 minutes compared to 5-10 minutes for chylomicrons (Havel and Kane, 1989b). A fraction of the VLDL remnants (approximately one half) are removed from the circulation almost entirely by hepatocytes (Havel and Kane, 1989a). It is thought that the LRP involved in chylomicron remnant metabolism is also the receptor responsible for the metabolism of VLDL remnants (Biesiegel *et al*, 1991).

2.3.3 LDL metabolism

Any VLDL remnants remaining in circulation are further hydrolysed by a hepatic lipase (Jones *et al*, 1984) to form the denser cholesterol-rich LDL. Independent secretion by the liver of LDL particles has been implicated (Havel and Kane, 1989b) although most studies indicate that LDL arise solely from VLDL. The majority of LDL (approximately 80%) is removed from the circulation by the interaction of the sole protein component, apo B-100, with the LDL receptor on liver and peripheral cells (Brown and Goldstein, 1986). The remainder is removed by non-receptor pathways (Attie *et al*, 1982; Kesaniemi *et al*, 1983). The residence time of LDL (approximately 3 days) is much longer than that of VLDL. LDL plays an important role in the delivery of cholesterol to tissues. In man approximately 60%-70% of the total cholesterol is delivered to hepatic and extra-hepatic cells in the form of LDL cholesterol (Scott, 1989).

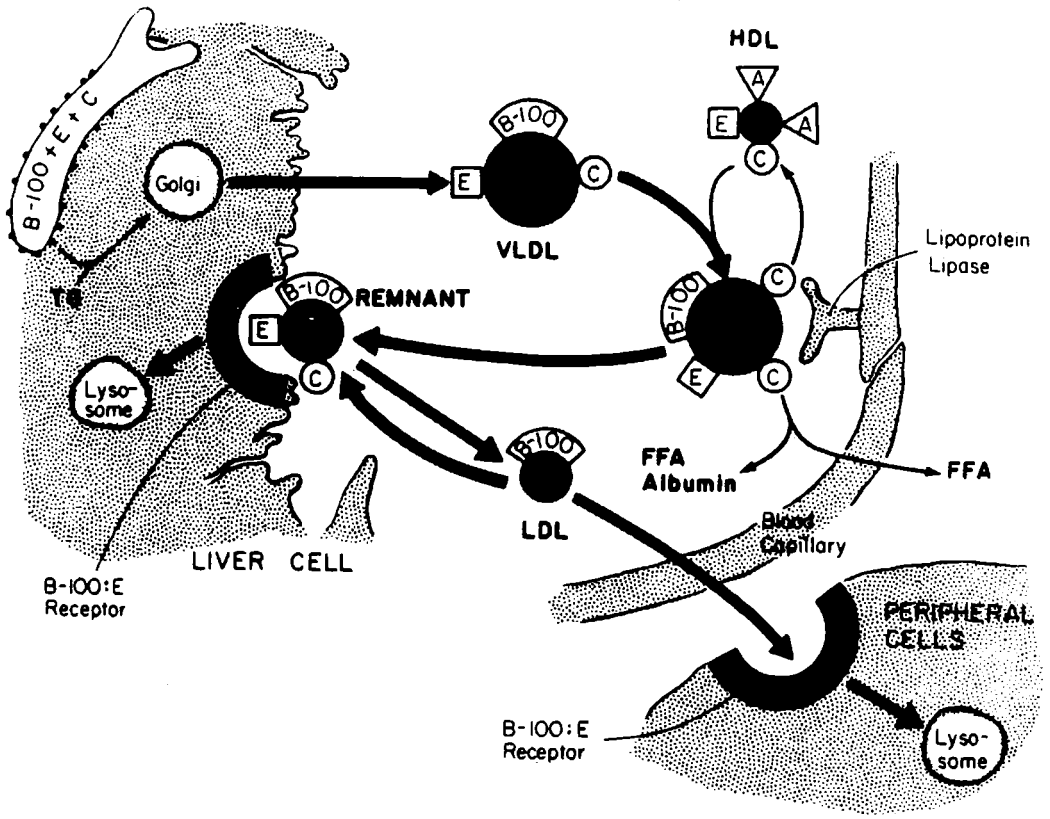


Figure 2.3 Pathway of VLDL and LDL metabolism (from Havel and Kane, 1989a).

A number of proteins are involved in the metabolism of the apo B-containing lipoproteins. Defects in any one of these proteins can result in disease.

2.4 Defects in lipid transport

Genetic defects have been identified in both the exogenous and endogenous pathways of lipid transport. These are summarised in tables 2.3 and 2.4. Defective chylomicron and VLDL secretion are characteristic of the two disorders, abetalipoproteinaemia and homozygous hypobetalipoproteinaemia (Havel and Kane, 1989b). Both disorders are rare and are inherited in an autosomal recessive fashion. In both conditions total cholesterol and triglyceride levels are very low (see section 2.2.1). A virtual absence of apo B and apo B-containing lipoprotein particles is observed together with severe fat malabsorption. Some cases of homozygous

hypobetalipoproteinaemia are an exception to this since some apo B is present and clinical symptoms absent (see section 2.7). In the heterozygous state both abetalipoproteinaemia and hypobetalipoproteinaemia are asymptomatic. However, there is a difference in their effect on LDL cholesterol levels. In abetalipoproteinaemia, heterozygotes have relatively normal LDL cholesterol levels whereas in hypobetalipoproteinaemia, heterozygotes have LDL levels less than half that of normal (Farese *et al,* 1992). Studies have shown that hypobetalipoproteinaemia is due to defects in the apo B gene (see table 2.5). The genetic defect in abetalipoproteinaemia is thought to be in a microsomal triglyceride transfer protein that is required for lipoprotein assembly (Wetterau *et al,* 1992).

Two disorders that interrupt the processing of chylomicrons and VLDL in plasma are LPL and apo C-II deficiency (Brunzell, 1989). Both conditions are characterised by elevated chylomicron and VLDL triglyceride levels, pancreatitis and eruptive xanthomas. The inheritance mode of LPL and apo C-II deficiency is autosomal recessive and due to defects in the genes of both proteins (Fojo and Brewer, 1992). Both disorders are rare with a frequency of less than one in one million (Breslow, 1989).

A more common disease is seen in type III hyperlipidaemia. Type III hyperlipidaemia is associated with genetic variation in apo E, the ligand responsible for the receptor-mediated clearance of chylomicrons and VLDL remnants. In this disorder there is an accumulation of remnant particles in the fasting plasma of affected individuals which greatly increases the risk of premature atherosclerosis (Mahley and Rall, 1989). The most common variant associated with type III hyperlipidaemia is apo E2 (Weisgraber *et al, 1*982) in which a single amino acid substitution yields a protein with only 1% normal receptor binding activity. Other apo E2 variants with reduced receptor binding activity and associated with type III have been reported (Rall *et al,* 1983; Wardell *et al,* 1987; Wardell *et al,* 1990). Homozygosity for E2 seems to be a prerequisite for type III hyperlipidaemia, although it appears a further yet unknown defect is necessary for the development of the disease (Utermann, 1979). The frequency of type III hyperlipidaemia is estimated at one in five thousand (Breslow, 1989).

Table 2.3 Defects in Exogenous Fat Transport[a]

	Defect	Disease
Chylomicron secretion	Apo B	Hypobetalipoproteinaemia Abetalipoproteinaemia
Chylomicron processing	LPL Apo CII	LPL deficiency Apo CII deficiency
Chylomicron remnant clearance	Apo E	Type III hyperlipidaemia

Table 2.4 Defects in Endogenous Fat Transport[a]

	Defect	Disease
VLDL secretion	Apo B	Hypobetalipoproteinaemia Abetalipoproteinaemia
VLDL processing	LPL Apo CII	LPL deficiency Apo CII deficiency
VLDL remnant clearance	Apo E LDL receptor	Type III hyperlipidaemia Familial hypercholesterolaemia
VLDL remnant processing	HTGL	HTGL deficiency
LDL clearance	Apo B LDL receptor	Familial defective Apo B-100 Familial hypercholesterolaemia

[a] both tables were adapted from Breslow (1989)

The processing of VLDL remnants to LDL is defective when there is a deficiency in hepatic lipase (Breckenridge *et al,* 1982; Carlson *et al,* 1986). Symptoms of this disorder include an increase in the triglyceride concentration of VLDL remnants and the presence of triglyceride-enriched LDL particles.

Lastly there are two diseases associated with the defective clearance of LDL. The first of these diseases is familial defective apo B, a disorder characterised by elevated levels of LDL cholesterol (Innerarity *et al,* 1990) and an increased incidence of coronary heart disease. This disease is caused by a single base substitution (3500Arg→Gln) in the apo B gene which produces a protein with only 2–4% the normal activity towards the LDL receptor (Innerarity, 1987). The second disease involves a mirror-image defect where mutations in the LDL receptor (Brown and Goldstein, 1986) cause a similar rise in LDL cholesterol and a predisposition to coronary heart disease. This condition is known as familial hypercholesterolaemia. Both of these disorders are inherited in an autosomal dominant fashion and are reasonably common with a frequency of 1 in 500 (Breslow, 1989).

2.5 Apo B structure

The structure of the apo B-100 protein has been difficult to determine because of its large size and insolubility once delipidated. In 1986 four groups deduced the primary structure of apo B-100 using apo B cDNA (Cladaras *et al,* 1986; Knott *et al,* 1986; Law *et al,* 1986; Yang *et al,* 1986). Human apo B mRNA consists of 14,121 nucleotides which code for a mature protein of 4536 amino acids and a 27 amino acid signal peptide which is co-translationally cleaved (Protter *et al,* 1986). The predicted molecular weight of apo B, excluding glycosylation, is 512 kDA. It seems there is only one molecule of apo B per LDL particle since LDL only contains 500 kDA of protein (Scott, 1989).

Amino acid sequence analysis has revealed that apo B is unique with there being only slight similarities to other apoproteins (De Loof *et al,* 1987). Figure 2.4 shows some of the important structural features of apo B. There are 19 potential N-glycosylation sites in apo B,

16 of which are known to be utilised (Yang *et al*, 1989a). Analysis of secondary structure suggests that the amino-terminus of apo B is highly cross-linked (Yang *et al*, 1989b) corresponding with a region rich in cysteines.

2.5.1 Lipid binding potential

Numerous hydrophobic regions throughout the structure of apo B are thought to be important in lipid binding (Olofsson *et al*, 1987). The amphipathic α helices in apo B (see figure 2.4) which are similar to those found in the putative lipid binding regions of other apolipoproteins (Knott *et al*, 1986; De Loof *et al*, 1987) are also thought to be important. Another structure with high lipid binding potential and which is unique to apo B is the amphipathic β-sheet. It has been suggested that the many proline rich regions throughout apo B contribute to the β-sheet arrangement (Young, 1990). Thus apo B has many potential lipid binding sites throughout its structure. This would help to explain its non-exchangeability between lipoproteins and its absolute requirement for the formation of chylomicron and VLDL particles.

The lipid binding ability of apo B has been demonstrated by many investigators. Chen *et al* (1989) showed that many peptides throughout apo B had the ability to recombine into lipoprotein-like particles when exposed to lipid. Yang *et al* (1989a) demonstrated that the regions between amino acids 1701 and 3070, and 4101 and 4536 were the main regions of apo B that were associated with lipid. This was concluded when digestion of the apo B on LDL left these regions largely undigested or "non-releasable" by trypsin and hence thought to be lipid associated. These regions correspond to sequences in apo B structure which are highly hydrophobic.

The above studies which suggest that apo B binds lipid throughout its entire length are consistent with observations from naturally occurring truncated forms of apo B. Young *et al* (1989) discovered a truncated form of apo B (apo B-46) which was located mainly in the VLDL and LDL density range. Another shorter apo B (apo B-37), although detected in

VLDL and LDL, primarily floated in the HDL density range (Young *et al,* 1987a) and an even shorter variant (apo B-31) was found only in the denser HDL and d>1.21 fractions (Young *et al,* 1990). All these findings suggest that the shorter the apo B, the less lipid is bound and the denser the lipoprotein particle formed.

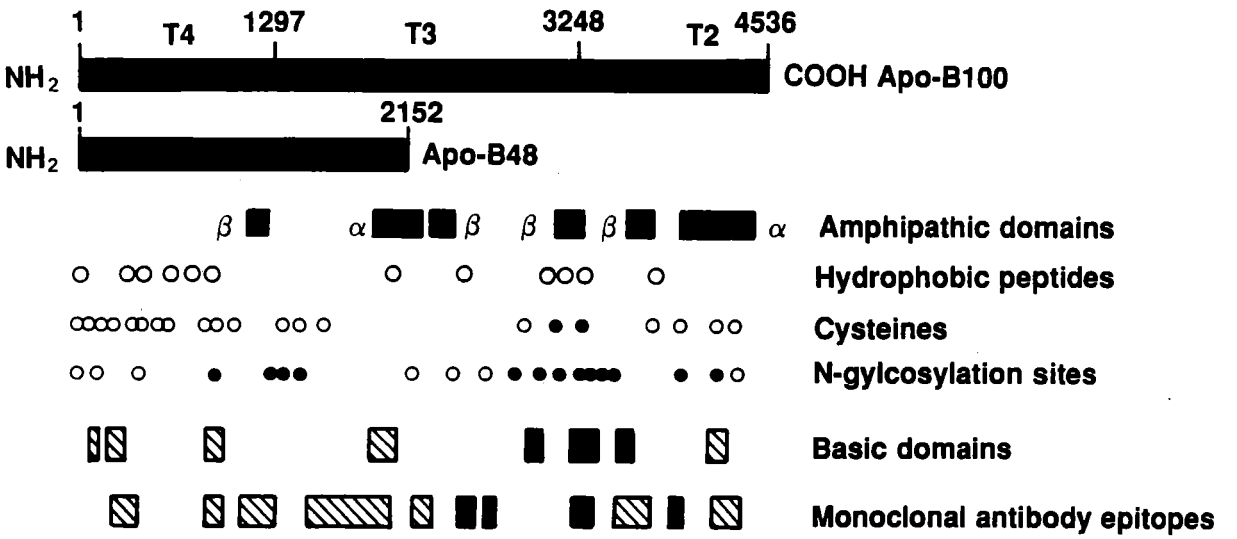


Figure 2.4. Structural features of apo B (from Scott, 1989). Thrombin cleavage fragments T2, T3 and T4 are shown. Amphipathic α-helical domains are designated α and proline-rich β-sheets β. Cysteine residues shown as filled circles are known to be cross-linked. N-glycosylation sites shown as filled circles are known to be utilised. Three basic domains that bind to the LDL receptor are shown as filled boxes. Monoclonal antibody epitopes for antibodies that block binding to the LDL receptor are shown as filled boxes.

2.5.2 Receptor binding

The receptor binding region of apo B has been well defined mainly by using monoclonal antibodies to apo B to block the binding of LDL to the receptor (Milne *et al*, 1989). This technique showed that by blocking the region between amino acids 2980 and 3780 (see figure 2.4) the receptor binding activity of LDL was abolished. Part of this region (amino acids 3357 to 3367) had sequence homology with the region of apo E known to react with the LDL receptor (Knott *et al*, 1986). This area is known to be well conserved through evolution and rich in positive charges. Such positively charged amino acids on apo B are thought to interact with the negatively charged amino acids of the ligand binding domain of the LDL receptor (Brown and Goldstein, 1986). Finally there is a mutation at amino acid 3500 involving a single amino acid substitution (Arg→Gln) which yields a protein defective in binding to the LDL receptor and which is associated with hypercholesterolaemia (Innerarity *et al*, 1990).

2.5.3 Heparin binding

Apo B also has the property of binding to heparin. This was shown by Weisgraber and Rall (1987) who found seven distinct regions in the apo B molecule that bound heparin strongly. These regions correspond to sequences rich in positively charged amino acids. It is thought that heparin binding sites on apo B may facilitate the binding of the triglyceride-rich lipoproteins to the capillary endothelium while they are metabolised by LPL. The capillary endothelium contains surface proteoglycans which are structurally similar to heparin.

2.5.4 Modified forms of apo B

Apo B is very susceptible to proteolytic cleavage. Immediately after a blood sample is taken apo B is cleaved by the serine protease kallikrein to generate the fragments apo B-26 and apo B-74 (Cardin *et al*, 1984). Further breakdown of apo B occurs from the action of another

serine protease, thrombin (Hirose, 1987). It is therefore very important to routinely add appropriate protease inhibitors when working with apo B-100.

Oxidised LDL has been detected in the human circulation (Scott, 1989). *In vitro* studies have shown that monocytes, macrophages, and endothelial cells, all cells prominent in the arterial wall, can induce oxidation of LDL (Steinberg *et al*, 1989). There is now evidence that oxidation of LDL occurs *in vivo* (Palinski *et al*, 1989). Oxidation causes lipid peroxidation and modification of the amino acids of apo B-100. In particular, lysine and arginine residues are destroyed and the apo B protein becomes fragmented to expose domains which bind to a scavenger receptor on the surface of macrophages (Scott, 1989). Macrophages accumulate the modified LDL to form fat laden cells known as foam cells, a process thought to be important in the formation of atherosclerotic plaques (Steinberg, 1991).

2.6 The apo B gene

The gene for apo B is located at the short arm of chromosome 2 (Law *et al*, 1985; Huang *et al*, 1986). Figure 2.5 is a schematic diagram of the human apo B gene showing restriction sites and intron-exon boundaries. The apo B gene spans approximately 43 kilobases (kb) and contains 29 exons and 28 introns (Blackhart *et al*, 1986). Over half of the apo B mRNA is coded for by the extremely large exon 26 which is 7572 base pairs (bp) long, the longest exon ever reported for any mammalian gene.

The apo B gene is only expressed in large amounts in the liver and intestine (Knott *et al*, 1985). The factors responsible for preventing expression of the apo B gene in other tissues are not fully understood. DNA methylation could be one of the factors since it has been reported that the promoter regions of the apo B gene in liver and intestinal cell lines are undermethylated compared with cells from other tissues (Levy-Wilson and Fortier, 1989). Das and co-workers (Das *et al*, 1988) have reported that DNA sequences governing specific expression in the liver appear to be located within 300 bp of the transcriptional start site.

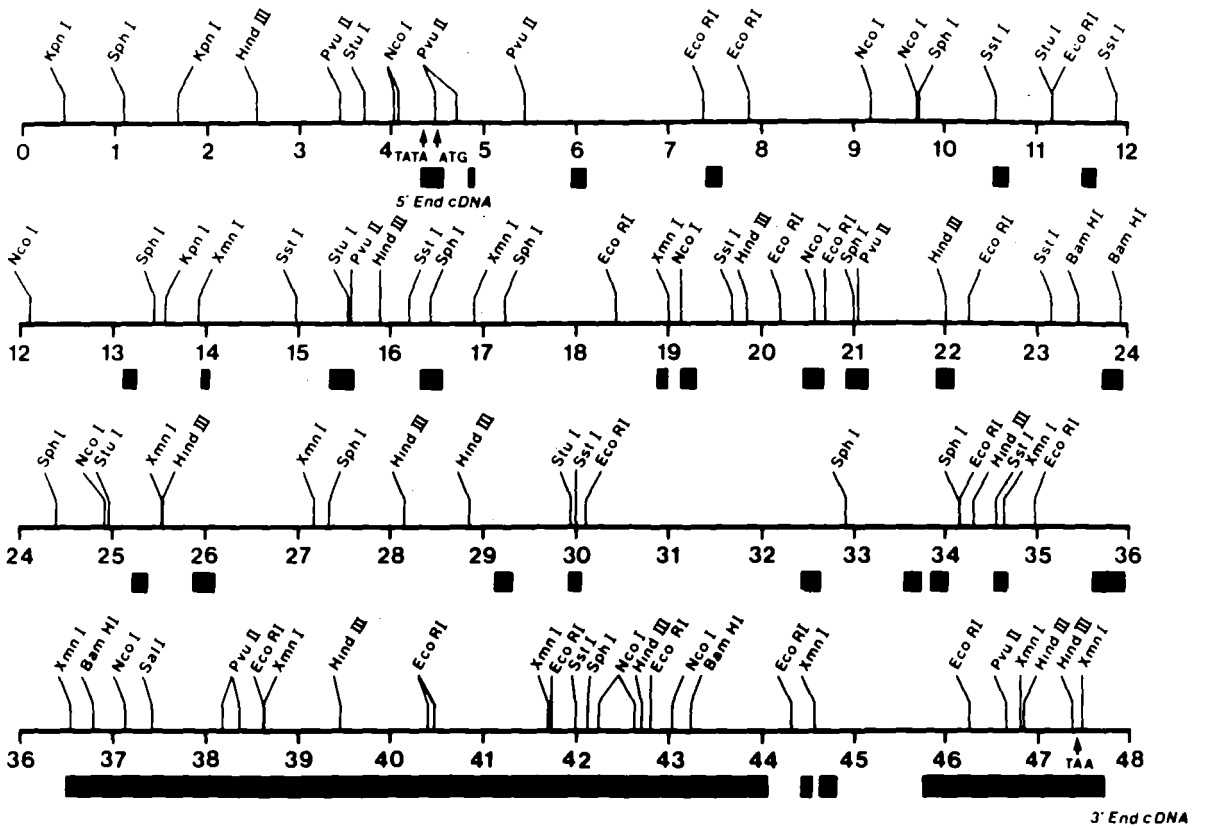


Figure 2.5. Restriction map of the human apo B-100 gene (from Blackhart *et al*, 1986). The scale is in kb, and the filled areas below the restriction map delineate the exon regions.

The promoter region of the apo B gene has been reasonably well defined. Sequences have been located between –128 and –86 that contain a positive element which increases expression (Das *et al*, 1988). Within this region is a 13 bp sequence which is perfectly conserved in the mouse and human gene (Levy-Wilson *et al*, 1988). Metzger *et al* (1989) demonstrated that the binding of two nuclear proteins designated AF1 and CEBP was important in the transcription of the apo B gene. Within 60 bp upstream of the apo B gene transcriptional initiation site is a TATA box and a CAT box, presumably part of the promoter region (Blackhart *et al*, 1986). In the 3' region of the gene, there are two poly-A signals 131 bp apart both of which are utilised in making apo B mRNA (Breslow, 1988).

Current evidence suggests that there is little regulation of hepatic apo B gene expression at the transcriptional stage. Modification of the diet seems to have little influence on the level of apo B mRNA. Sorci-Thomas *et al* (1989) found there was no change in hepatic apo B mRNA in African green monkeys fed a diet high in saturated fats and cholesterol although the diet reduced by 50% the LDL receptor mRNA. Pullinger *et al* (1989) reported that the apo B mRNA levels in human hepatoblastoma cell lines remained unchanged under a variety of metabolic conditions. They did however find that the secretion rate of apo B could be modulated by insulin and fatty acids. Several investigators have reported that some intracellular apo B is degraded and not secreted (Borchardt and Davis, 1987; Bostrom *et al*, 1988). From these results it has been suggested that metabolic conditions, such as lipid availability, determines the amount of intracellular apo B that is secreted. It appears that the secretion of apo B-containing particles is controlled largely by co and/or post-translational factors. Investigation of these factors is currently a major focus of apo B research.

2.6.1 Apo B-48

The intestinal form of apo B is named apo B-48 because it is 48% the size of apo B-100 as determined by Kane *et al* (1980). Analysis of intestinal cDNA clones and protein sequencing have shown that apo B-48 is co-linear with the amino-terminal 2152 amino acids of apo B-100 (Chen *et al*, 1987). Several investigators have shown that apo B-48 is a product of the apo B-100 gene derived by novel editing of mRNA in the intestine (Chen *et al*, 1987; Powell *et al*, 1987; Higuchi *et al*, 1988a). In the intestine nucleotide 6666 of the apo B mRNA is changed from C to U which changes the codon for amino acid 2153 from CAA (which normally codes for glutamine) to UAA which is a stop codon. The exact mechanism responsible for the editing process is unknown. It has been postulated that perhaps a tissue specific enzyme, which recognises a specific sequence within the apo B mRNA, deaminates the cytosine so it is read as a uracil. The fact that the sequence around nucleotide 6666 is well conserved and highly homologous between species (Davis *et al*, 1989) supports this hypothesis.

2.6.2 Apo B gene variation

The apo B gene is a very polymorphic locus. This was first noticed in the 1960's when investigators observed that multiple transfused patients developed antibodies to LDL. These antibodies were used to define the loci of genetic variations in apo B (Berg *et al,* 1986; Breslow, 1988). Additional variations in the apo B gene were revealed by an apo B specific monoclonal antibody (MB19) which detected a two allele polymorphism in apo B (Young *et al,* 1986).

Cloning and sequencing of the apo B gene in 1986 demonstrated that there was considerable variation in the DNA sequence. Yang *et al (*1989a) listed 75 differences in the apo B DNA sequence, 53 of which gave amino acid substitutions. Although some of these substitutions probably resulted from sequencing errors, many have been verified by direct amino acid sequencing and many create restriction fragment length polymorphisms (RFLPs) in the apo B gene. Significant associations between some of the RFLPs and lipoprotein levels have been reported (Young, 1990).

Many repetitive elements occur in the intron sequences of the apo B gene. Introns 4, 14, 15, 20, 21 contain Alu repeats (Scott, 1989). An AT-rich hypervariable region within 200 bp of the poly-A site, which is composed of 11 to 16 bp tandem repeats of varying size, has been reported (Huang and Breslow, 1987; Ludwig *et al,* 1989). Boerwinkle and co-workers through PCR amplification and sequencing, have shown that there are 12 distinguishable alleles in this hypervariable region (Boerwinkle *et al,* 1989). Hypervariable regions in the apo B gene are very useful in epidemiological and family studies.

Further variation in the apo B gene is seen in the form of specific mutations that cause specific hyper- and hypolipidaemias such as the mutation at amino acid 3500 which causes hypercholesterolaemia (see section 2.4) and the truncated forms of apo B which cause hypocholesterolaemia (see section 2.7)

2.7 Familial hypobetalipoproteinaemia

Many investigators have shown that low levels of LDL cholesterol and apo B can be inherited within families in a simple Mendelian fashion. In the past five years, various laboratories have shown that familial hypobetalipoproteinaemia is associated with specific mutations in the apo B gene (Farese *et al*, 1992). All the mutations interfere with the translation of the full length apo B and as a consequence truncated apo B molecules of varying length are produced (see table 2.5). There has also been reports of subjects with familial hypobetalipoproteinaemia that possess an apo B allele which markedly reduces the level of the full length apo B (Berger *et al*, 1983; Collins *et al*, 1988).

An individual rarely inherits two defective alleles and is homozygous or compound heterozygous for familial hypobetalipoproteinaemia. The homozygous state is often clinically severe since the individual is unable to make chylomicrons and cannot absorb fat and fat-soluble vitamins from the intestine. This results in symptoms such as steatorrhea, acanthocytosis, bleeding from vitamin K deficiency and neurological disorder mainly from vitamin E deficiency (Havel and Kane, 1989b). However, homozygotes and compound heterozygotes have been described who have been completely asymptomatic (Hardman *et al*, 1991; Talmud *et al*, 1989). The severity of disease probably depends on the length and amount of apo B formed from the mutant alleles. A recent review on truncated apo B variants (Gabelli, 1992) concludes that homozygotes or compound heterozygotes that have variants greater than apo B-48 will be protected from fat malabsorption since the dietary pathway for the transport of lipids is preserved. Subjects with variants shorter than apo B-48 would therefore be expected to suffer some form of fat malabsorption. This conclusion is supported by the lack of any clinical symptoms in a subject homozygous for apo B-50 (Hardman *et al*, 1991). This is in contrast with a homozygote for apo B-39 in which clinical signs of fat malabsorption were present (Collins *et al*, 1988).

Heterozygotes for hypobetalipoproteinaemia are almost always asymptomatic, because such patients can make some chylomicrons from the normal allele and hence clinical symptoms

from fat malabsorption are absent. Furthermore, because of their low LDL cholesterol levels, it seems that heterozygotes should be protected from developing coronary heart disease. Data from several investigators support this hypothesis (Kahn and Glueck, 1978; Glueck *et al*, 1977). LDL cholesterol levels in heterozygotes are usually less than half that of unaffected family members (Young, 1990). The metabolic basis for the 'less than half' normal levels is not fully understood. Indeed, the majority of the lipoproteins formed from truncated apo B mutants lack the ability to bind to LDL receptors which should produce a higher rather than lower level of LDL. Hypotheses forwarded to explain this disparity include:

1. The LDL receptor is up-regulated which results in an increased clearance of LDL particles formed with the normal apo B allele (Young, 1990).

2. The VLDL formed with the normal allele contains a greater number of apo E molecules which results in an increased clearance of VLDL. This effectively reduces the number of precursors for the formation of LDL (Young, 1990).

Another consistent finding in heterozygotes is the extremely low concentration of the truncated apo B in plasma. Generally the truncated apo B is only 2–10% of the apo B-100 concentration formed with the normal allele (Young, 1990). Impaired synthesis and secretion, and/or increased catabolism of the lipoproteins with the mutant apo B species are possible explanations for this phenomenon.

Studies suggest that familial hypobetalipoproteinaemia may affect 0.1–0.8% of the population (Andersen *et al*, 1979; Laskarzewski *et al*, 1982) as estimated from the frequency of the familial 'low cholesterol' phenotype. It is possible that subjects with this phenotype have defects in a gene other than apo B that causes low cholesterol (Young, 1990). An additional problem with estimating the incidence of hypobetalipoproteinaemia is that some subjects, that have low LDL cholesterol, have total cholesterol levels within the normal range (Young *et al*, 1989).

2.8 Truncated forms of apo B

Studies of patients with hypobetalipoproteinaemia have revealed a number of mutations in the apo B gene that prevent translation of the full length apo B (4536 amino acids). These mutations are summarised in table 2.5. The majority of these studies have included family members to demonstrate the inheritance of the truncated apo B species thus confirming the familial nature of the hypobetalipoproteinaemia. All of these mutations involve the introduction of a premature stop codon through either a single nucleotide substitution or deletion leading to a frameshift. All except two of the single nucleotide substitutions are C→T transitions, a common transition in causing human genetic disorders (Cooper and Youssoufian, 1988). Hence CGA and CAG codons provide potential sites for mutation to stop codons. Collins *et al* (1988) reported 12 CGA codons in apo B mRNA. There are many more CAG codons hence the potential for the production of truncated apo B variants from C→T transitions is large.

The first truncated apo B found in association with hypobetalipoproteinaemia was apo B-37 reported by Young *et al* (1987a). Since then there has been a number of truncated apo B variants reported (see table 2.5). To date each of the reported mutations causing hypobetalipoproteinaemia have occurred only within a single family thus it seems no single mutation predominates. The majority have been found in heterozygotes where there is one normal apo B allele although a few compound heterozygote and homozygote cases have been reported. All of the truncated species confer very low levels of LDL cholesterol and apo B. However the functional properties of the various truncated apo B species differs according to their size.

Variants such as apo B-87 and apo B-89 (Gabelli *et al*, 1989; Krul *et al*, 1989) which terminate near the carboxy-terminus of the protein presumably package lipoprotein particles normally since they are found in VLDL and LDL. Both variants have the LDL receptor binding domain intact. However it was shown that both had increased affinity for the LDL receptor, partially explaining the association with low LDL cholesterol levels (Gabelli *et al*,

Table 2.5 Mutations of the Apo B Gene Associated with Familial Hypobetalipoproteinaemia

Mutation	No. Amino acids	Apo B in plasma	LDL Chol. (mmol/L)	Clinical features	References
Apo B2: G→T at first base of intron 5	—	Absent	0.75—1.21	Compound heterozygote with severe clinical course	Huang *et al* (1991)
Apo B9: C→T at nucleotide 1443	411	Absent	0.41		
Apo B25: 694 bp deletion including all of exon 21	1085	Absent	NA[a]	Homozygote with classical symptoms	Huang *et al* (1989)
Apo B29: C→T at nucleotide 4125	1305	Absent	NA	Heterozygotes	Collins *et al* (1988)
Apo B31: Deletion of nucleotide 4480	1425	HDL, d>1.21g/mL	0.73	Heterozygotes	Young *et al* (1990)
Apo B32: C→T at nucleotide 4557	1449	LDL, HDL, d>1.21 g/mL	0.80	Heterozygote with high HDL	McCormick *et al* (1992)
Apo B37: deletion of nucleotides 5391→ 5394	1728	VLDL, LDL, HDL	1.09	Compound heterozygote	Young *et al* (1987a and b)
Apo B39: deletion of nucleotide 5591	1799	VLDL, LDL	NA	Homozygote with fat malabsorption	Collins *et al* (1988)
Apo B40: deletion of nucleotides 5693→ 5694	1829	VLDL, LDL, HDL	1.27	Compound heterozygotes	Krul *et al* (1989)
Apo B46: C→T at nucleotide 6381	2057	VLDL, LDL, HDL	1.91	Heterozygotes	Young *et al* (1989)
Apo B50: C→T at nucleotide 6963	2251	VLDL, LDL, HDL	NA	Homozygote without symptoms	Hardman *et al* (1991)
Apo B52.8 deletion of nucleotide 7359	2395	VLDL, LDL	NA	NA	Farese *et al* (1992)
Apo B54.8: C→T at nucleotide 7665	2485	VLDL, LDL	1.27	Heterozygotes	Wagner *et al* (1991)
Apo B55: C→T at nucleotide 7692	2492	VLDL, LDL	NA	NA	Farese *et al* (1992)
Apo B61: deletion of nucleotides 8525→ 8561	2784	VLDL, LDL	NA	Compound heterozygote	Pullinger *et al* (1992)

Apo B67: deletion of nucleotide 9327	3040	VLDL, LDL	1.09	Heterozygote with high HDL levels	Welty *et al* (1991)
Apo B75: deletion of nucleotide 10366	3386	VLDL, LDL	1.24	Heterozygotes	Krul *et al* (1992)
Apo B83: C→A at nucleotide 11458	3749	VLDL, LDL	1.34	Heterozygote	Farese *et al* (1991)
Apo B86: deletion of nucleotide 11840	3896	VLDL, LDL	NA	Compound heterozygote	Linton *et al* (1990)
Apo B87: deletion of nucleotide 12032	3978	VLDL, LDL	1.60	Asymptomatic homozygotes	Tennyson *et al* (1990)
Apo B89: deletion of nucleotide 12309	4039	VLDL, LDL	1.91	Compound heterozygote	Krul *et al* (1989)

[a] Not Available

1989; Parhofer *et al*, 1990). Shorter variants which lack the receptor binding domain do not react with the LDL receptor (Young *et al*, 1987b). It is therefore hard to ascertain why these variants are associated with such low levels of LDL cholesterol since there appears to be no clearance pathway. Impaired secretion or alternatively extracellular degradation or rapid clearance of the lipoprotein particles containing the abnormal apo B has been suggested (Collins *et al*, 1988).

The class of lipoprotein formed with these shorter variants and hence the density distribution varies according to size. The smallest of these truncated apo B species, apo B-25 (1085 amino acids, Huang *et al*, 1989) was not detected in the lipoprotein fraction of plasma suggesting that this protein is shorter than the critical length required for lipoprotein formation. This was also the case for apo-B 29 (1305 amino acids, Collins *et al*, 1988). Young *et al* (1991) recently described an apo B-31 species which was detectable within the HDL and lipoprotein-deplete fraction (d>1.21g/mL) of plasma. The density distribution of apo B-31 suggested that this molecule, which contains the amino-terminal 1425 amino acids, was adequate to form an abnormal small dense lipoprotein particle but not LDL or the larger, triglyceride-rich lipoproteins. In contrast the apo B-37 mutant (1728 amino acids, Young *et al*, 1987a) was detected in VLDL as well as in LDL and HDL, implying that this molecule was of adequate size to form LDL and the larger triglyceride-rich lipoproteins. Truncated species longer than apo B-37 are all capable of forming VLDL. The length of apo B molecule that is required to form particles in the LDL density class is less clear and appears to lie between the apo B-31 and apo B-37 region.

In conclusion it seems that the longer the apo B species the more lipid is bound and the more buoyant the lipoprotein formed. This is consistent with the finding that apo B-100 has lipid binding regions throughout its length. The exact regions required for the formation of the apo B containing VLDL and LDL particles is becoming clearer but still requires further definition. A number of questions however still remain relating to the synthesis, secretion and catabolism of these truncated apo B species.

2.9 Expression of truncated variants in cell culture

Characterisation of truncated forms of apo B has provided important information on the functional domains of apo B. However the metabolic pathway of the lipoprotein particles formed from the truncated variants has remained elusive. Focus has now turned to the expression of truncated apo B proteins in cell culture in an attempt to further characterise the lipoproteins made and to elucidate the basis of their low concentrations.

Blackhart *et al (*1990) have designed an *in vitro* expression system for apo B. An apo B minigene constructed from cDNA and genomic clones and inserted into a vector carrying the cyclomegalovirus promoter was designed and expressed in a rat hepatoma cell line McA-RH7777. This cell line was chosen over simian and human liver cell lines because of its maximal expression and secretion capabilities. The McA-RH7777 cells act as primary rat hepatocytes and produce apo B-containing VLDL particles (Tanabe *et al,* 1989). This is an advantage over human liver hepatoma cell lines such as HepG2 in which the majority of apo B-containing lipoproteins that are produced are LDL-like (Bostrom *et al,* 1988). Originally both human apo B forms (apo B-100 and apo B-48) were shown to be expressed and secreted as lipoprotein particles using this system.

The same group of investigators (Yao *et al,* 1991) have since expressed the truncated apo B proteins apo B-18, B-23, B-28, B-31, B-37, B-48, and B-53. All of the variants were secreted from the transfected cells. The secretion rates were normal except for the apo B-18 and apo B-23 variants which were secreted at a much slower rate than normal. Both variants were found associated with very little lipid compared to the larger truncated variants in which the longer the apo B the more lipid-rich and more buoyant the particle. Results from this study suggest that secretion rates do not account for the low concentration of truncated apo B seen in human subjects to date. This study did indicate that the ability of apo B to assemble lipids into lipoprotein particles increased as the length of apo B increased. This was reinforced by work by Spring *et al* (1992) in which carboxy-terminally truncated apo B variants of increasing length were associated with increasing lipoprotein core circumference.

Graham *et al* (1991) have expressed the truncated apo B species apo B-9, B-13, B-17, B-23, B-26 and B-39 in HepG2 cells. All were secreted except for apo B-9. Only apo B-39 was found exclusively in the lipoprotein density fraction (d<1.25). Apo B-17, B-23 and B-26 were found in both the lipoprotein and infranatant (d>1.25) fractions whereas apo B-13 was only in the infranatant fraction suggesting no association with lipid. Herscovitz *et al* (1991) have expressed the amino-terminal 17% of apo B in a murine cell line. Characterisation of the expressed apo B-17 showed it was capable of binding some lipid suggesting that the amino-terminus may contribute to the formation of the triglyceride-rich lipoproteins. The secretion of these very short truncated variants is in contrast with the *in vivo* situation where the naturally occurring variants apo B-25 and apo B-29 (Huang *et al*, 1989; Collins *et al*, 1988) were undetectable in patient plasma, suggesting these variants were not being secreted from the liver.

Expression of apo B in cell culture has wide application in the study of truncated apo B variants. As well as studying the naturally occurring variants any region of the apo B gene can be mutated to produce truncated apo B variants of varying length. As well as studying the metabolism of the truncated apo B species, the *in vitro* expression system allows the identification of important functional domains in the large apo B protein.

2.10 Summary

The two forms of apo B play a crucial role in lipid metabolism. Much progress has been made in recent years in understanding this important lipid-carrying protein. Apo B-100 is an extremely large protein synthesised in the liver and is crucial for the assembly of the triglyceride-rich VLDL which carry endogenous triglyceride to peripheral tissues. Apo B-100 is the major protein component of the cholesterol-rich LDL and is responsible for the receptor-mediated uptake of LDL by the liver and extrahepatic tissues. Apo B-48, a product of the apo B-100 gene, is synthesised in the intestine and integral to the assembly of the triglyceride-rich chylomicrons which carry dietary triglyceride to peripheral tissues.

Important regions in the structure of apo B, such as the receptor-binding region and possible lipid binding regions, have been identified. Many different mutations in the apo B gene associated with abnormal cholesterol levels and disease have been identified, some of which have pointed to important functional domains on this large protein.

Familial hypobetalipoproteinaemia is a disease associated with abnormally low levels of LDL cholesterol and apo B. Hypobetalipoproteinaemia is caused by different mutations in the apo B gene which prevent translation of the full length protein (4536 amino acids). Despite the rarity of these truncated forms of apo B, they have been a source of valuable information on the structure-function relationships of apo B and lipid metabolism.

CHAPTER 3 MATERIALS AND METHODS

3.1 Biochemicals

Specialist biochemicals and materials were obtained from the following companies:

American Type Culture Collection, Rockville, MD., USA

Amersham International, Amersham, England.

Bio-Rad Laboratories Pty, Ltd., Richmond, CA., USA

Boehringer Mannheim, Mannheim, Germany.

Calbiochem, La Jolla, CA., USA

Dupont, Boston, MA., USA

Falcon, Lincoln Park, NJ., USA

Fissons, Loughborough, England.

FMC Bioproducts, Rockland, ME., USA

Gibco/BRL, Gaithersberg, MD., USA

Kodak, Rochester, NY., USA

New England Biolabs Inc., Beverly, MA., USA

New England Nuclear, North Ryde, NSW., Australia.

Nunc, Roskilde, Denmark.

Perkin Elmer Cetus, Norwalk, CT., USA

Pharmacia Fine Chemicals, Uppsala, Sweden.

Promega Corporation, Maddison, WI., USA

Schleicher and Schuell, Dassel, Germany.

Sigma Chemical Co., St Louis, MO., USA

United States Biochemicals, Cleveland, OH., USA

Whatman International Ltd., Maidstone, England.

Unless otherwise stated in the text all other biochemicals were of analar grade and obtained from British Drug Houses Chemicals, Poole, England.

Water used in all solutions and reactions was deionised and filtered (0.2μm filter) by the Milli-Q water purification system. Water used for molecular biology techniques was also sterilised by autoclaving.

3.2 Equipment

Major items of equipment used were:

Centrifuges	
Beckman L8-70 ultracentrifuge	Beckman Instruments Inc., Palo Alto, CA., USA
Sorval RC5C	Dupont, Boston, MA., USA
Centra-M microfuge	International Equipment Company, Needham Hts., MA.,USA
Spectrophotometer	
PU 8740 scanning spectrophotometer	Pye Unicam, Cambridge, England
Oligonucleotide synthesiser	
391 DNA synthesiser	Applied Biosystems Pty. Ltd., Victoria, Australia
PCR machine	
DNA thermal cycler	Perkin-Elmer Cetus, Norwalk, CT., USA
Electroluter	
Model UEA	International Biotechnologies Incorporated, CN., USA
Vacuum dryer	
SpeedVac concentrator	Savant Instruments Inc., Farmingdale, NY., USA

Incubators	
G 24 Environmental incubator shaker	New Brunswick Scientific, Co., Inc., Edison, NJ., USA
Sanyo MIR 160	Sanyo Electric Co., Ltd., Japan
Fraction collection system	
Fraction collector: 2210 Super Rac.	
Peristaltic pump: 2232 Microperpex S.	Pharmacia LKB Biotechnology,
U. V. detector: 2238 Uvicord S II.	Uppsala, Sweden
Chart recorder: 2210 2-channel recorder.	
Concentrating cells	
Model 12	Amicon, Beverly, MA., U.S.A.
Water purification system	
MilliQ^{TM}	Millipore Corporation, Bedford, MA., USA

3.3 Human subjects and selection criteria

The individual involved in this study was found to have low LDL cholesterol during a routine check at the diabetes outpatient clinic at Princess Margaret Hospital, Christchurch in 1989. A repeat blood sample in 1990 showed an LDL cholesterol level (31 mg/dL) well below the lower limit of the normal range (105 mg/dL) as determined by a local population study (Janus *et al*, 1981). This individual was selected for further investigation along with four other patients who had LDL levels below 105 mg/dL. Neither the subject or the other four patients showed any clinical signs of fat malabsorption however it was noted that one of the patients was in hospital for liver function tests at the time his hypobetalipoproteinaemia was identified. Investigation of the subject's family was very limited as both of his parents were deceased and he had no siblings or offspring. Blood samples were however, obtained from his first cousins.

Samples were also obtained from normolipidaemic controls at various times during the course of this study.

3.4 Plasma preparation

Blood (20–80 mL) was collected into sterile tubes containing EDTA (1.5 mg/mL). Plasma was separated immediately by centrifugation at 3000 rpm for 10 minutes at room temperature. Isolated plasma was then mixed with the following inhibitors as used by Young *et al* (1987b): 100 kallikrein units/mL aprotinin (Boehringer Mannheim), 1mM TLCK (Boehringer Mannheim), 1 mM TPCK (Boehringer Mannheim), 1 mM PPACK (Calbiochem), 2 mM PMSF (Sigma) and 175 mg/mL sodium azide (Fissons).

Small aliquots of plasma (50μL) were frozen at –80°C for measurement of vitamins A and E (appendix 7.1) and immunoblot analysis. Remaining plasma was kept at 4°C and used for the measurement of plasma apolipoproteins, lipoprotein isolation and the measurement of lipoprotein lipids (appendices 7.2, 7.3 and 7.5). Buffy coat was aspirated from the red cells and stored at –80°C until required for isolation of genomic DNA.

3.5 Lipoprotein isolation from plasma

Analytical lipoprotein isolations were performed at the Lipid Clinic, Princess Margaret Hospital using density gradient ultracentrifugation as described in appendix 7.3.

Initial isolation of the apo B-32 lipoproteins was performed by density gradient ultracentrifugation in a Beckman L8-70 ultracentrifuge using a Beckman Ti 60 rotor according to the method of Havel *et al* (1955). Density solutions were as described in appendix 7.4. Isolation of lipoproteins commenced as soon as the blood was taken. Approximately 25mLs of plasma was adjusted to a density of 1.075 as described in appendix 7.4, added to a polyallomer centrifuge tube, and overlaid with a 1.075 density solution so that the tubes were filled when capped. VLDL and LDL ($d<1.075$) were isolated by

ultracentrifugation at 40 000 rpm at 20°C for 18 hrs. The infranatant was adjusted to a density of 1.291 then overlaid with a 1.291 density solution and ultracentrifugation repeated. After each ultracentrifugation the supernatant lipoproteins were aspirated and stored in the dark under nitrogen at 4°C.

3.6 Isolation of apo B-32 lipoproteins by heparin-Sepharose chromatography

Apo B-32 containing lipoproteins were fractionated from the 1.075–1.291 fraction isolated in section 3.5 by heparin-Sepharose chromatography using a method similar to that of Owen *et al* (1987). Heparin-Sepharose (preswollen and stored in 50mM Tris/HCl (pH 7.4), 1M NaCl, 0.1% sodium azide) was kindly provided by Dr Maurice Owen.

The 1.075-1.291 fraction (2mL) was dialysed for 18 hrs at 4°C in 50mM Tris/HCl (pH 7.4), 10mM sodium citrate before being loaded onto a 6mL heparin-Sepharose column equilibrated with the same buffer. Chromatography with the equilibration buffer was continued until all unbound lipoproteins were eluted. Bound lipoproteins were then eluted with a salt gradient to 0.5M NaCl (in equilibration buffer). Fractions (1.5 mL) were collected with the aid of a fraction collector (flow rate = 20mL/hr). Fractions containing peaks (unbound and bound) were pooled, concentrated to 2mL and screened for their lipoprotein composition by SDS PAGE and immunoblotting with polyclonal anti-apo B, anti-apo A1 and anti-apo E antibodies (see sections 3.10 and 3.11). The unbound and bound lipoprotein peaks along with control lipoprotein fractions were subjected to particle size analysis via electron microscopy as described in appendix 7.7.

3.7 Agarose gel electrophoresis

Agarose gel electrophoresis was performed on plasma and the isolated apo B-32 as described in appendix 7.8. Following electrophoresis, gels were either protein stained or the proteins transferred to nitrocellulose (Schliecher and Schuell) by pressure blotting. This involved the

placement of a strip of nitrocellulose followed by 3 pieces of Whatman 3MM blotting paper (all previously soaked in water) on the gel. On top of this was placed approximately 20 sheets of paper towels and a 500g weight and transfer allowed to proceed for 15 minutes. The nitrocellulose was then subjected to immunoblotting as described in section 3.11.

Alternatively, proteins separated by 1% agarose electrophoresis were electrophoresed in the second dimension by SDS PAGE (section 3.10). For this, lanes were cut from the agarose gel using a scalpel blade and ruler, and placed on the top of the SDS PAGE gel as described in appendix 7.9)

3.8 Composition analysis of lipoproteins

3.8.1 Cholesterol and triglyceride determinations

Total cholesterol and triglyceride determinations on plasma and lipoprotein fractions were performed at the Princess Margaret Lipid Clinic using the Boehringer enzymatic assays (see appendix 7.5). Total cholesterol and triglyceride determinations on the isolated apo B-32 lipoproteins were performed manually using the same assays.

For triglycerides, 20μL of lipoprotein was mixed with 1mL of Test-Combination Triglyceride GPO-PAP reagent and the reaction incubated at room temperature for 15 minutes. Twenty μL of a 2mg/mL triglyceride solution was mixed with 1 mL of reagent and incubated under the same conditions. The absorbance of the sample and standard at 500nm was measured against a reagent blank. The concentration (C) of triglyceride in the sample was then calculated from the following equation:

$$C\ (mg/mL) = 2 \times \frac{A\ sample}{A\ standard}$$

For cholesterol, 20μL of lipoprotein was mixed with 1mL of Monotest Cholesterol CHOD-reagent and the reaction incubated at room temperature for 15 minutes. Twenty μL of a 2 mg/mL cholesterol solution was mixed with 1mL and incubated under the same conditions. The absorbance of the sample and standard at 500nm was measured against a reagent blank. The concentration (C) of cholesterol in the sample was then calculated from the following equation:

$$C\ (mg/mL) = 2 \times \frac{A\ sample}{A standard}$$

3.8.2 Phospholipid determinations

Phospholipid concentrations in lipoprotein fractions were determined using the Test-Combination Phospholipids enzymatic assay (Boehringer Mannheim) based on the method of Takayama *et al* (1977). This method measures the release of choline from phospholipids by the action of the phospholipase D enzyme. Lipoprotein samples were dialysed against 50mM Tris/HCl (pH 7.4), 10mM sodium citrate before analysis. Twenty μL of sample was mixed with 1 mL of phospholipid reagent (1000 units/L phospholipase D, 1400 units/L choline oxidase, 800 units/L peroxidase (all from Sigma) and 8mM 4-aminophenazone in 50mM Tris/HCl (pH 8.0), 20mM phenol) and incubated at 42°C for 30 minutes. The standard was a 0.9mg/mL solution of choline iodide (Sigma) which is equivalent to 3mg/mL phospholipid . Twenty μL of standard was mixed with 1mL of phospholipid reagent and incubated under the same conditions. The absorbance of both the sample and standard were measured against a reagent blank and the concentration of phospholipid calculated from the following equation:

$$C\ (mg/mL) = 3 \times \frac{A\ sample}{A\ standard}$$

3.8.3 Total protein determinations

The total protein in the lipoprotein fractions was determined using the Bio-Rad protein assay based on the method of Bradford (1976). Ten μL of sample was diluted with 10μL of water and mixed with 1 mL of Bio-Rad dye (previously diluted with 4 volumes of water and filtered through Whatman 1MM paper) and the absorbance of the sample at 595nm measured. A standard curve using bovine serum albumin (1mg/mL) as the reference protein was established over a protein concentration range of 50μg/mL to 1mg/mL. The protein concentration of the sample was read from the standard curve and multiplied by the sample dilution factor in order to give a final protein concentration (mg/mL).

3.8.4 Percentage composition calculation

Percentage chemical composition was calculated by dividing each individual chemical component (mg/mL) by the sum of all four chemical components. For example:

$$\% \text{ Cholesterol} = \frac{C \text{ (cholesterol)}}{\Sigma C \text{ (cholesterol, triglyceride, phospholipid, protein)}} \times 100\ \%$$

Where C = concentration (mg/mL)

3.9 Precipitation of apo B-containing lipoproteins

Apo B-containing particles (VLDL and LDL) were precipitated from the plasma of hypobetalipoproteinaemic patients with each of three different reagents commonly used to prepare HDL. Samples from patients with plasma containing apo B-31, apo B-46 apo B-61, apo B-67, and apo B-83 were kindly provided by Dr Stephen Young of the Gladstone Foundation Laboratories, San Francisco, CA., USA. Normal plasma was used as a control throughout the experiment.

After addition of each reagent the mixture was immediately vortexed and then left at room temperature for 15 minutes. The precipitant was sedimented by centrifugation at 2000rpm for 20 minutes. After centrifugation the supernatant was transferred into fresh tubes. The precipitant was then redissolved in 1 mL of 1:1:8 (v/v/v) water:tetramethyl urea:10mM Tris/HCl (pH 8.0), 8M urea. An equivalent amount of plasma, supernatant, and redissolved precipitant was analysed for the presence of the truncated apo B species by western blot analysis using a polyclonal anti-apo B antiserum (section 3.11).

3.9.1 Precipitation with sodium phosphotungstate/magnesium

VLDL and LDL were precipitated from plasma with a sodium phosphotungstic acid/magnesium chloride reagent using a modification of the method of Grove (1979). The precipitation reagent (1mL of 2M $MgCl_2$ and 5mL of 45g/L phosphotungstic acid, 160mM NaOH (pH 7.4)) was freshly prepared and 30μL added to 250μL of plasma at room temperature.

3.9.2 Precipitation with polyethylene glycol

VLDL and LDL were precipitated from plasma with polyethylene glycol (PEG) 6000 following the method of Warnick *et al* (1979). Two hundred μL of an aqueous solution of PEG solution (240g/L PEG 6000) was added to 200μL of plasma.

3.9.3 Precipitation with heparin/manganese

VLDL and LDL were precipitated from plasma with a heparin/manganese reagent according to the method of Bullock *et al* (1980). To 500μL of plasma was added 25μL of heparin (7500 units/L in 150mM NaCl, Fissons) and 25μL of 2.02M manganous chloride.

3.9.4 Precipitation with dextran sulphate

Precipitation of VLDL and LDL with dextran sulphate was according to a modified method of Finley *et al* (1978). To 500μL of sample was added 25μL of a 20g/L solution of dextran sulphate 500 (Pharmacia) and 25μL of 2M $MgCl_2$ solution.

3.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Proteins in plasma and lipoprotein fractions were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) using a modification of the method of Laemmli (1970). Linear gradient gels (3–6% and 3–15%) were made as described in appendix 7.9 and poured into the Mini-Protean II mini-gel system (Bio-Rad). Gel dimensions were 100mm×60mm×0.75mm. Thicker 3–6% gels (1.5mm) were used for immunoblotting for easier handling. Stacking gels (3%) were poured on top of each gradient gel (see appendix 7.9). Samples were incubated for 3 minutes at 100°C in sample buffer (2% (w/v) SDS, 10% (w/v) sucrose, 5% β-mercaptoethanol, 0.001% (w/v) bromophenol blue, in 20mL of tank buffer) before loading.

Electrophoresis was at a constant voltage of 180 V until the dye band had migrated to the end of the gel. The 3-6% gels were run for a further 20-30 minutes before electrophoresis was terminated. Gels were either stained with warmed Coomassie blue protein stain (0.5g Coomassie brilliant blue-R250 dissolved in 300mL of water, 67mL of acetic acid and 300mL of ethanol) and destained in the same solution without Coomassie or subjected to immmunoblotting.

3.11 Immunoblotting

Proteins were transferred to nitrocellulose (Schliecher and Schuell) by electrophoresis using the Mini Trans Blot system (Bio-Rad). Briefly, the gel was placed on top of a gel holder on which was stacked a fibre pad followed by 1 sheet of Whatman 17MM blotting paper

previously soaked in tank buffer (appendix 7.9) without the SDS and containing 5% methanol. On top of the gel was placed the nitrocellulose followed by a further sheet of 17MM Whatman and another fibre pad (also previously soaked). This was then placed in the Mini Trans Blot electrophoresis chamber with the gel side nearest to the cathode plate. The chamber was filled with the above buffer and electrophoresis commenced at 100V for 1 hour.

Immunodetection was as described by Michaelis *et al* (1990). Excess protein-binding sites on the nitrocellulose were blocked with 3% non-fat powdered milk in 50mM Tris/HCl (pH 7.9), 150mM NaCl, 0.05% Tween 20 (solution A) for at least 30 minutes at room temperature. The membrane was incubated with 10μg of a rabbit antiserum to human LDL apo B-100 (supplied by James Yeo, Steroid Unit, Christchurch Hospital) in 30mL of fresh solution A at room temperature for approximately 18 hours. After washing with solution A (3×5 minute washes) the membrane was incubated with a 1:5000 dilution of goat anti-rabbit alkaline phosphatase conjugated antibody (Sigma) for 2 hrs at room temperature. After washing as before, the immunoblots were developed by adding 60μL of Nitro Blue Tetrazolium (Sigma) and 30μL of 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in 25mL of 100mM Tris/HCl (pH 9.5), 100mM NaCl, 5mM magnesium acetate.

At times, blots were incubated with a rabbit anti-human apo A1 antibody (Calbiochem) or a rabbit anti-human apo E antibody kindly provided by Dr Stephen Young of the Gladstone Foundation Laboratories, San Francisco, CA, USA.

A series of immunoblots was performed where the membranes were incubated with three previously characterised monoclonal antibodies to apo B, MB3 which binds between amino acids 994 and 1084, 2D8 which binds between amino acids 1403 and 1480 and MB44 which binds between amino acids 2488 and 2658 (Pease *et al*, 1990). These antibodies were kindly provided by Dr Stephen Young. The second antibody was a 1:5000 dilution of goat-anti mouse alkaline phosphatase conjugated antibody (Sigma).

In some cases, blots were incubated with a 1:5000 dilution of goat anti-rabbit or goat anti-mouse peroxidase conjugated antibody for 1 hr at room temperature and the blots developed with ECL western blot detection reagents (Amersham).

3.12 Molecular weight estimation of the abnormal apo B species

Apolipoproteins in the patient's HDL were separated alongside protein markers (SDS-6H High Molecular Weight Kit, Sigma) on a 3–15% gradient gel by SDS PAGE (described in 3.10) and the proteins stained with Coomassie blue. A graph was established by plotting molecular weight against electrophoretic mobility for each of the protein markers. Electrophoretic mobility was calculated from the distance migrated by the protein band from the top of the gel (cm), divided by the total distance from the top of the gel to the dye front (cm). The molecular weight of the abnormal apo B was read from this graph by plotting the electrophoretic mobility.

3.13 Preparation of genomic DNA

Genomic DNA was prepared using a modification of the method of Kunkel *et al* (1977). Buffy coat (3mL) was thawed at room temperature and transferred to a 50mL Nunc tube to which was added 47mL of Triton Lysis Buffer (0.32M sucrose, 10mM Tris/HCl (pH 7.5), 5mM $MgCl_2$, 1% Triton X-100). The tubes were stored on ice for 30 minutes and centrifuged at room temperature for 10 minutes at 2500 rpm. The supernatant was discarded down to 8mL and Triton Lysis Buffer again added to a volume of 50mL. The tube was centrifuged as before, all of the supernatant was discarded and the pellet resuspended in 2mL of STE (10mM Tris/HCl (pH 7.4), 10mM NaCl, 10mM EDTA). The resuspended pellet was transferred to a 10mL tube with a 1mL wash of STE. SDS (350µL of 10% (w/v)) and proteinase K (150µL of 10mg/mL, Boehringer Mannheim) were added and the tube incubated overnight at 37°C. The next morning 700µL of 6M sodium perchlorate was added and the incubation continued for a further 2 hrs.

Genomic DNA was extracted from the incubation mixture with an equal volume of phenol/chloroform. Two mL of both phenol saturated with 0.1 M Tris/HCl (pH 8.0) and chloroform:isoamyl alcohol 24:1 (v/v) was added and the tube mixed by inversion before being centrifuged at 3000rpm for 2 minutes to separate the phases. The upper aqueous phase containing the DNA was removed to a fresh tube and a repeat phenol/chloroform extraction performed followed by a 4mL chloroform:isoamyl alcohol 24:1 (v/v) extraction. DNA was then precipitated from the upper aqueous phase by addition of one tenth the volume of 3M sodium acetate (pH 4.7) and 2–4 volumes of ice-cold ethanol. The precipitated DNA was removed with a sterile needle to a 1.5mL eppendorf tube where it was washed in 70% ethanol before being air-dried for 30 minutes. The genomic DNA was resuspended at 4°C overnight in TE (10mM Tris/HCl (pH 8.0), 1mM EDTA). The concentration (C) of DNA was determined by measuring the OD_{260} value of a 1 in 200 dilution against the reagent blank and calculation from the following equation:

$C\ (\mu g/mL) = OD_{260}\ \text{sample} \times 50 \times 200$

Where 50 = C (μg/mL) of DNA at an OD_{260} value of 1
and 200 = dilution factor

3.14 Amplification of an apo B gene fragment by the polymerase chain reaction

A 759 bp portion of the apo B gene (encompassing apo B cDNA nucleotides 4369-5127, coding for apo B-100 amino acids 1388-1639) was amplified via the polymerase chain reaction (PCR) from the patient and a control. Genomic DNA (1μg) was added to a 100μL PCR reaction which contained: Ampli-Taq DNA polymerase buffer (50mM KCl, 10mM Tris/HCl (pH 8.3), 1.5mM $MgCl_2$, 0.001% (w/v) gelatin), 0.2mM dNTP's (Perkin Elmer Cetus), 1μM each of oligonucleotide primers B31-1 and B31-2 and 2.5 units Ampli-Taq DNA polymerase (Perkin Elmer Cetus). The primers, B31-1

(5'ACAAGAATACGTTCACACTATCATGTGATG'3) and 31-2 (5'GCACCtGcAGACTACACTTCAAGTTGGTCG3') were those designed by Young *et al* (1990). Two base mismatches (lower case letters) had been included in B31-2 to create a PstI site. Both oligonucleotides were synthesised on an Applied Biosystems 391 DNA Synthesizer. PCR involved amplification for 30 cycles on a DNA thermal cycler (Perkin-Elmer Cetus). Each cycle involved a denaturation step for 1 minute at 96°C, annealing for 1 minute at 60°C and extension for 1 minute at 74°C with a final extension of 3 minutes. The same apo B gene fragment was also amplified from an apo B-31 expression vector (Yao *et al*, 1991) kindly supplied by Dr Stephen Young, using PCR conditions as described above with 0.1 μg of the plasmid DNA as a template.

Products amplified by PCR were analysed by agarose gel electrophoresis alongside DNA molecular weight markers (ϕX174 RF DNA/HaeIII, Gibco/BRL) at 100V for 1 hour in a 3% Nu-sieve (FMC Bioproducts), 1% agarose (Gibco/BRL) gel in 0.5× TBE buffer (45mM Tris/Borate (pH 8.0), 1mM EDTA) containing 0.5μg/mL ethidium bromide. PCR products were visualised over UV light and gels photographed through an orange filter using Polaroid 667 film. The amount of DNA generated by each reaction was estimated by the intensity of the bands over UV light in relation to a known concentration of the molecular weight markers.

3.15 Electroelution of PCR DNA

PCR amplified DNA was purified and prepared for sequencing by electroelution in a commercial electroeluter (International Biotechnologies Incorporated, Conneticut, USA) in accordance with the manufacturers instructions. PCR product (200μL) was electrophoresed on a 3% Nu-sieve, 1% agarose gel as in section 3.14. The 759 bp apo B fragment band was visualised over UV light and quickly exised from the gel using a sterile scalpel blade. The exised band was placed in the electroelution chamber previously filled with 0.5× TBE and DNA electrophoresed from the gel into an adjacent V-shaped channel containing a high salt solution (7.5M ammonium acetate, 0.01% (w/v) bromophenol blue). Electrophoresis

continued at 100V until gel slices were free of DNA, as determined with a hand-held UV light (approximately 30 minutes). The salt solution was then collected from the V-shaped chamber with a 1mL syringe and the DNA precipitated with 2 volumes of ethanol at -20°C for 1 hour. After a wash with 70% ethanol the DNA pellet was left to air-dry before being redissolved in 75μL of TE. Recovery of PCR DNA from electroelution was checked by electrophoresis on a 3% Nu-sieve, 1% agarose gel.

3.16 Sequencing of PCR amplified DNA

PCR amplified DNA was directly sequenced using the method of Higuchi *et al* (1988b) and the Sequenase Version 2.0 sequencing reagents (United states Biochemical Corporation).

3.16.1 Sequencing reactions

For each DNA template there were four sequencing reactions, one for each of the four dideoxy nucleotides (ddGTP, ddATP, ddTTP, ddCTP). All steps were performed on ice and reactions mixed by gentle pipetting so as to avoid air bubbles.

A "premix" of 5× buffer (200mM Tris/HCl (pH 7.5), 100mM $MgCl_2$, 250mM NaCl: 0.37μL per reaction), Sequenase 2.0 enzyme (0.15 μL per reaction) and 0.1M DTT (0.22μL per reaction) was prepared in an 0.5mL eppendorf tube. Dideoxy nucleotide termination mixes (ddGTP, ddATP, ddTTP and ddCTP each containing 80mM of deoxy nucleotide, 8mM of dideoxy nucleotide and 50mM NaCl) were aliquoted into 0.5mL eppendorf tubes to give 2.5μL of dideoxy nucleotide per reaction and premix added (0.75μL per reaction). The dideoxy nucleotide/"premix" solution (3μL per reaction) was then aliquoted to the wells of a Nunclon microtitre plate and the wells labelled G, A, T, C.

The template/primer pair mix was prepared by adding 3μL of a 1μM solution of ^{32}P-end labelled primer (appendix 7.10) to 8μL of electroelution purified PCR DNA (0.15-0.6 pmols). The primer used to sequence PCR DNA was the B31-3 primer (5'TCAAAAGGTTTACTA'3)

which hybridises to the normal apo B-100 allele at the B-31 position. The template /primer mix was denatured by heating to 95°C for 5 minutes and annealing allowed by incubation on ice for 5–10 minutes. This was followed by centrifugation for 10 seconds in a microfuge to spin down any condensate.

To each dideoxy reaction was added 2.5μL of the template/primer mix and the reactions incubated for 15 minutes at 37°C to allow extension. Sequencing reactions were terminated by the addition of 4μL of "stop" solution (95% (v/v) formamide, 20mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (v/v) xylene cyanol). The sequencing reactions were then denatured by heating for 5 minutes at 95°C and kept on ice while 2μL of each reaction was loaded onto the sequencing gel.

3.16.2 Sequencing gel

Sequencing was performed in a 6% polyacrylamide denaturing gel using the Bio-Rad Sequi-Gen nucleic acid apparatus (21x40 cm) with wedge spacers. A sealing gel consisting of 15mL acrylamide (6% (w/v) acrylamide in 1x TBE and 7M urea), 260μL of 10% (w/v) ammonium persulphate and 75μL of TEMED was poured and left to set for 5–10 minutes. The resolving gel consisting of 40mL acrylamide, 400μL of 10% (w/v) ammonium persulphate and 8μL of TEMED was then poured and the straight edge of a 0.25mm sharks tooth comb applied to the top of the gel which was allowed to polymerise for 1 hour.

Gels were pre-run in 1× TBE buffer for 30 minutes at 2000 V before loading. Sequencing reactions were electrophoresed at 50W until the xylene cyanol dye band had migrated to the end of the gel (approximately 2 hours). Sequencing gels were fixed in 10% acetic acid, 10% methanol, backed onto Whatman 3MM paper, vacuum dried and exposed to X-ray film (Kodak X-Omat) for either 24 hours at -20°C or 48 hours at room temperature.

3.17 Cloning of the apo B PCR fragment into the pUC19 plasmid

Electroeluted PCR product as generated in section 3.15 was cloned into the pUC19 plasmid (Yanisch-Perron *et al*, 1985) shown in figure 3.1.

3.17.1 Digestion of PCR and pUC19 DNA

Purified PCR fragment (0.5μg) was subjected to double digestion with 1 unit each of the PstI and XbaI restriction enzymes (Boehringer Mannheim) in buffer (50mM Tris/HCl (pH 7.5), 10mM $MgCl_2$, 100mM NaCl, 1mM dithioerythritol) in a 20μL reaction. The PstI enzyme was allowed to digest for 1 hr at 37°C before the XbaI enzyme was added and the incubation continued for a further two hours. Restriction enzymes were heat denatured after digestion by a 10 minute incubation at 80°C.

Plasmid pUC19 DNA (1μg, Boehringer Mannheim) was double digested with PstI and XbaI in a similar 20 μL reaction. Digestion efficiency was evaluated by agarose electrophoresis in 1% agarose gels (1% agarose in 0.5× TBE buffer containing 0.5μg/mL ethidium bromide) at 100V for 1 hr.

3.17.2 Ligation of digested DNA and transformation into E. coli

Fifty ng of PstI/XbaI digested pUC19 was ligated to 150ng of PstI/XbaI digested apo B PCR fragment in ligase buffer (250mM Tris/HCl (pH 7.6), 50mM $MgCl_2$, 5mM ATP, 5mM dithiothreitol, 25% (w/v) PEG 8000) with 1 unit of T4 DNA ligase (Gibco/BRL) for 16 hours at 16°C. The ligation reaction (2μL) was transformed into E. coli DH5α cells (Gibco BRL) according to the manufacturer's transformation procedure (appendix 7.12). The cells were then pelleted by spinning in a microcentrifuge for 1 minute, resuspended in 200μL of LB media (appendix 7.13) and plated on LB agar containing 125μg/mL ampicillin (appendix 7.13) previously spread with 50μL of 20mg/mL Xgal and 100μL of 100μM IPTG. Plates were placed in a 37°C incubator and cells allowed to grow for 18–24 hours.

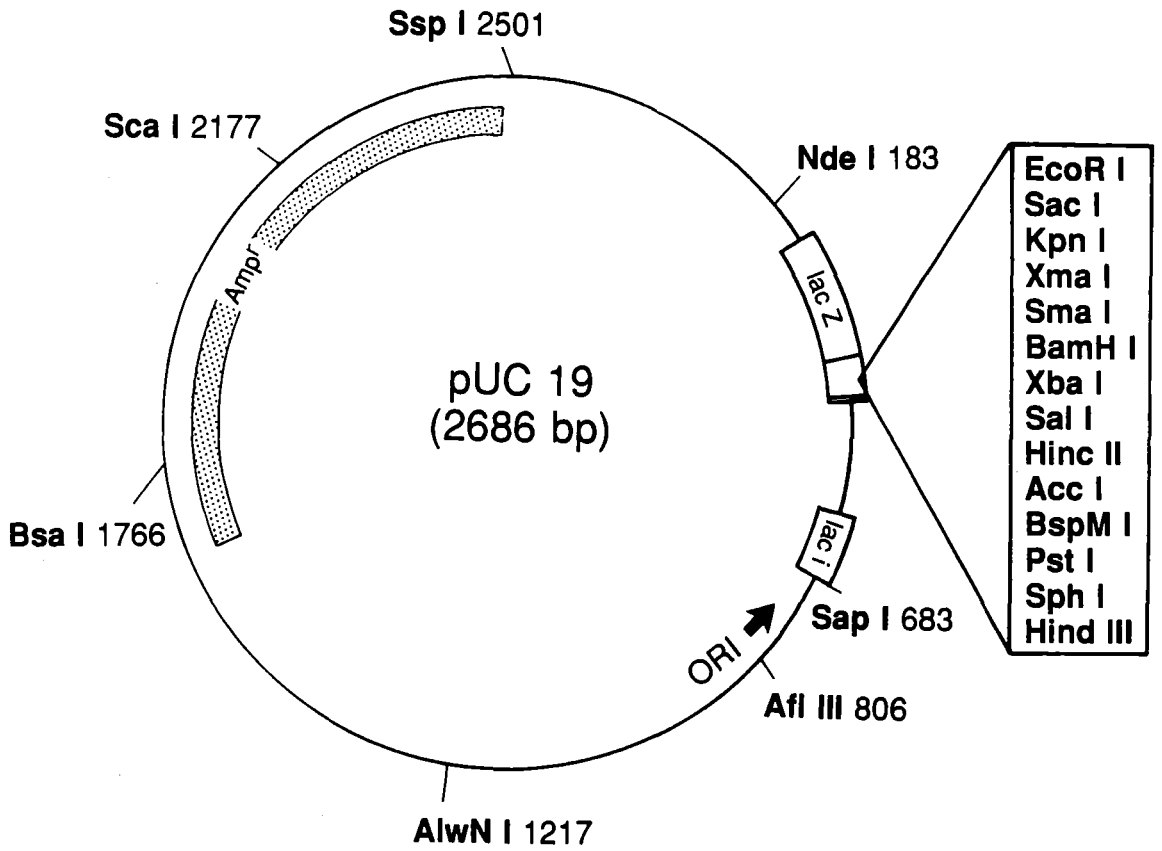


Figure 3.1 pUC19 plasmid circle map.

3.18. Identification of cloned DNA and Southern blot analysis

The polycloning site of pUC19 resides within the E. coli *lac* Z gene which codes for a fragment of β-galactosidase (Yanisch-Perron *et al,* 1985) In the presence of IPTG and X-gal this enzyme makes colonies blue. Insertion of DNA into the polycloning site disrupts the *lac* Z gene therefore clones transformed with plasmids containing DNA inserts remain white giving an effective selection for recombinant clones.

Nine white and 1 blue colony were chosen for plasmid DNA preparation. One half of each colony was restreaked onto a LB/Amp master plate and the other half inoculated into 1.5mL of LB/Amp media and incubated for 18 hours at 37°C in a shaking incubator. "Mini-preps" (appendix 7.14) of plasmid DNA were prepared from each cultured colony. To determine the

presence of the 759 bp apo B insert, each mini-prep DNA (0.5μg) was digested with the PstI and XbaI restriction enzymes using the same conditions as described in 3.17.1. The digest (10μL) was electrophoresed on a 1% agarose gel and visualised over UV light.

The DNA on the above gel was transferred to two filters using a combination of the original techniques of Southern (1975) and the sequential blotting technique of Grosveld *et al* (1981). The gel was treated with 200mL volumes of the following solutions: 0.25N HCl for 25 minutes with one change of solution followed by 0.5N NaOH, 1.5M NaCl for 40 minutes with one change of solution and finally, 0.5 M Tris/HCl (pH 7.0), 3M NaCl for 60 minutes with one change of solution. The gel was placed on top of four pieces of Whatman 3MM which was placed on a sponge in a reservoir of stock 20× SSC (0.3M sodium citrate/NaOH (pH 7.0), 3M NaCl). A Nylon filter (Hybond-N, Amersham) presoaked in 2× SSC was placed on top of the gel followed by four pieces of Whatman 3MM presoaked in 2× SSC, approximately 20 sheets of Hygenex Handitowels and a 500g weight. DNA was allowed to transfer for 15 minutes when the filter was replaced and transfer continued for a further 15 minutes. The blotted filters were rinsed in 2× SSC and allowed to air-dry at room temperature.

The membranes were prehybridised for 4 hours in 6× SSC, 1% SDS and 100μg/mL herring salmon sperm DNA (preboiled for 10 minutes, Boehringer Mannheim) at 30°C. The membranes were then probed overnight with two ^{32}P-end labelled oligonucleotide probes labelled as described in appendix 7.10. One membrane was probed with an allele-specific probe for the normal apo B-100 allele, B32-1 (5'AAGAAACAGCATTTG'3) the other with an allele-specific probe for the mutant apo B-32 allele, B32-2 (5'CAAATGCTATTTCTT'3). The labelled probe was added directly to the prehybridising solution and left to hybridise overnight at 30°C. Hybridisation and subsequent wash conditions for this and other hybridisations during this study were based on the calculation of melting temperatures of each oligonucleotide probe. Melting temperatures (Tm) were derived from the following equation formulated by Itakura *et al* (1984):

Tm = No. A + T residues × 2°C + No. G + C residues × 4°C

The following morning the hybridising solution was disposed of and the membranes washed with 2× SSC and 0.1% SDS for 3 washes at 30°C, 34°C and 36°C respectively. The membrane probed with the B 32-1 probe was given an extra wash at 38°C. The two membranes were then sealed in a plastic bag and autoradiographed at −80°C for 1 hour using Kodak X-Omat XAR5 film. The film was developed in the automatic developer at the Christchurch Hospital X-ray Department.

3.19 Sequencing of cloned DNA

The apo B PCR fragment cloned into pUC19 was sequenced according to the protocol described in 3.16. The primer used was a universal sequencing primer Messing *et al* (1983) supplied in the Sequenase kit which anneals to pUC19 40 bp before the polylinker (see figure 3.1). A mutant and a non-mutant clone as identified in 3.18 were sequenced with the plasmid DNA being linearised before sequencing by restriction digestion with the PstI enzyme.

3.20 Dot blot analysis

3.20.1 Preparation of DNA

PCR amplified DNA (100μL, see section 3.14) from the subject, a control and an expression vector carrying the apo B-31 allele was phenol/chloroform extracted and ethanol precipitated as described in section 3.13. The DNA was resuspended in 100μL of water and the DNA then denatured by incubation for 10 minutes in 0.25M NaOH at room temperature. DNA was neutralised by the addition of SSC to 0.25×.

3.20.2 Transfer of DNA to a Nylon filter

One quarter of each of the three denatured samples (approximately 1μg) was applied to 4 wells of a Bio dot apparatus (Bio-Rad Laboratories) containing a Nylon membrane (Hybond-

N, Amersham) assembled according to the manufacturer's instructions. The samples were left for 30 minutes at room temperature before a vacuum was applied for 20 seconds. After dismantling the apparatus the membrane was left to air dry.

3.20.3 Hybridisation of oligonucleotide probes

The membrane was prehybridised as in 3.18 then cut into horizontal strips and each set of the three DNA samples probed with the following probes:

B31-3 (5'TCAAAAGGTTTACTA'3) which hybridises to the normal apo B-100 allele at the B-31 position

B31-4 (5'CTCAAAAGTTTACTA'3) which hybridises to the mutant apo B-31 allele

B32-1 (5'AAGAAACAGCATTTG'3) which hybridises to the normal apo B-100 allele at the B-32 position

B32-2 (5'CAAATGCTATTTCTT'3) which hybridises to the mutant apo B-32 allele

The probes were end labelled with γ-^{32}P as in appendix 7.10 then added directly into the prehybridising solution and left to hybridise overnight at 30°C. The next morning the hybridising solution was disposed of and the membranes washed as in 3.18 at the same temperatures with the membrane probed with B32-1 given an extra wash at 38°C. The four membranes were then sealed in a plastic bag and autoradiographed at -80°C for 1 hour using Kodak X-Omat film.

3.21 Production of an apo B-32 expression vector

A vector (pB32) designed to express the apo B-32 protein was constructed from the apo B-42 expression vector (pB42) described by Blackhart *et al* (1990). The pB42 vector was kindly provided by Dr Zemin Yao of the Gladstone Foundation Laboratories, San Francisco, CA, USA. The pB42 vector (see figure 3.2) was originally constructed from pCMV4 (Andersson *et al*, 1989) an expression vector which utilises the cytomegalovirus promoter-enhancer sequences and the human growth hormone transcription termination and

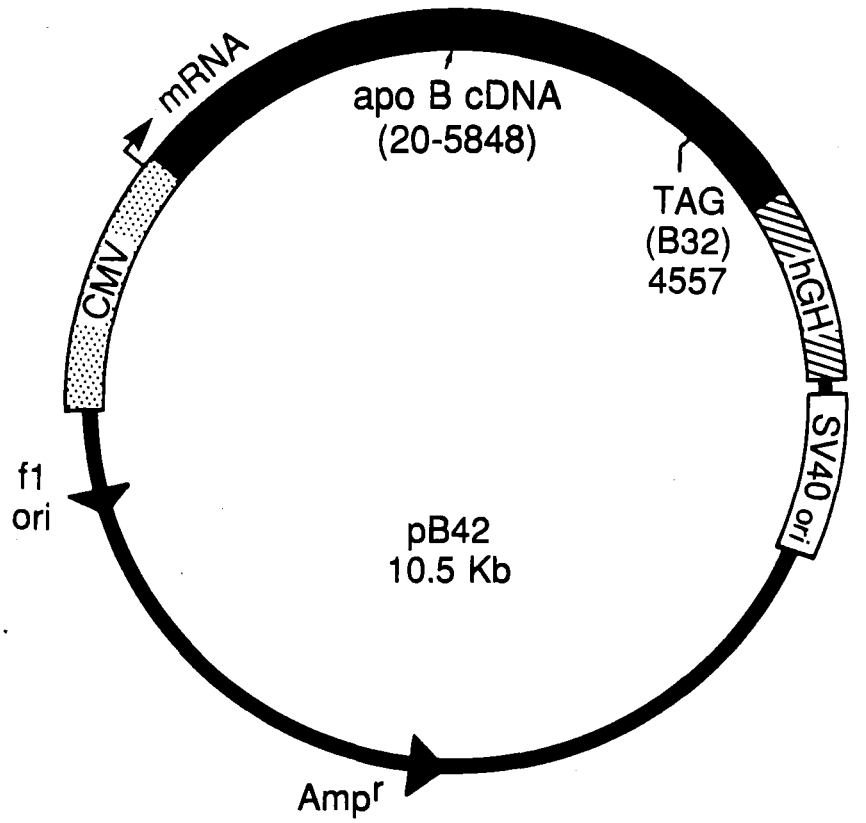


Figure 3.2 pB42 plasmid circle map.

polyadenylation signals. The pB32 expression vector was constructed by introducing the C→T mutation at nucleotide 4557. The nucleotide numbering used in this plasmid was different to that originally used to report the apo B-32 mutation (McCormick *et al*, 1992) where the mutation was reported at nucleotide 4548.

3.21.1 Mutagenesis method

Mutagenesis was performed on pB42 using the unique site elimination (USE) method of Deng and Nickoloff (1992) with slight modification. This method eliminates the need for cloning as mutagenesis is performed directly in the expression vector. USE mutagenesis employs two primers, one carrying the desired mutation, another containing a mutation which eliminates a unique non-essential restriction site. Both primers are annealed to the circular single stranded plasmid (produced by heat denaturation) and a new strand containing both primers is

synthesised. The resultant DNA is then transformed into a negative mismatch repair E. coli strain. This increases the chance of the mutant strand cosegregating from the wild type during the first round of DNA replication. Transformants are selected by growth in an appropriate antibiotic and plasmid DNA prepared and treated with the enzyme which recognises the unique restriction site. The mix of digested and undigested DNA is then retransformed into an appropriate E. coli host Plasmids with a mutation in this site are resistant to digestion and remain circular. Circular DNA is selected for since linearized DNA transform bacteria very inefficiently. The frequency at which the undigested circular DNA also contains the other desired mutation is reported to be about 80%.

3.21.2. The mutagenic primers

Two 21-mer oligonucleotides (Hpa I and B 32, figure 3.3) were synthesised on a Applied Biosystems 391 DNA synthesiser both of which had a single base mismatch (underlined) at the central position.

Hpa I 5' TGGAGCAGTTGACTCCAGAAC '3
B 32 5' CAAAAAGAAATAGCATTTGTT '3

Figure 3.3 Primers for USE mutagenesis

The Hpa I primer was designed to eliminate the unique HpaI restriction site which occurs at nucleotide position 1616 in the apo B cDNA. Because this was in a coding region care was taken so that the mismatch did not change the amino acid being coded for. The B 32 primer was designed to introduce the desired mutation as described above.

Before mutagenesis, both oligonucleotides were phosphorylated by treatment with T4 Polynucleotide kinase (Gibco/BRL). Primers (100pmol) were incubated with 5 units of T4 polynucleotide kinase in 50mM Tris/HCl (pH 7.5), 10mM $MgCl_2$, 5mM dithiothreitol in a

20μL reaction at 37°C for 30 minutes. The kinase was then inactivated by heating to 70°C for 10 minutes.

3.21.3. The mutagenesis reaction

Mutagenesis of pB42 was performed as follows:
A 20μL reaction containing the B 32 and Hpa I primers (25pmol each) and pB42 (0.1μg) in 20mM Tris/HCl (pH 7.5), 10mM MgCl, 50mM NaCl was heated to 100°C for 3 minutes. Primers were annealed by immediately placing tubes in an ice bath. DNA synthesis was performed by the addition of 3μL of 10mM Tris/HCl (pH 7.5), 5mM each dNTP's, 10mM ATP and 20mM DTT; 5μL of H_20; 1μL of T4 DNA polymerase (13 units, Promega) and 1μL of T4 DNA ligase (10 units, Promega). The reaction was incubated at 37°C for 90 minutes and then stopped by heating to 80°C for 10 minutes. Some of the mutagenesis reaction (10μL) was transformed into the E. coli host strain BMH 71-18 *mut* S as in appendix 7.12. After recovery a further 5mL of LB containing ampicillin (125μg/mL) was added and the cells incubated for 18 hours at 37°C. Plasmid DNA was prepared from the 5mL culture as in appendix 7.14 and 0.5μg digested with the HpaI enzyme (3.5 units, Boehringer, Mannheim) in 33mM Tris acetate (pH 7.9), 10mM magnesium acetate, 66mM potassium acetate, 0.5mM DTT for 16 hours at 37°C. Digests were heat denatured for 10 minutes at 80°C then electrophoresed at 75V for 90 minutes on a 0.7% agarose gel. The gel was stained in 0.5μg/mL ethidium bromide and visualised over UV to determine the amount of uncut and cut plasmid DNA. The HpaI digest was retransformed into the E. coli strain DH5α and the transformed cells plated onto LB plates containing 125μg/mL ampicillin and incubated at 37° C overnight. Plasmid DNA was prepared from 10 colonies and the DNA again digested with HpaI. Plasmid DNA resistant to HpaI digestion was sequenced along with the wild type pB42 vector to verify the presence of the B-32 mutation.

3.21.4. Sequencing of the apo B-32 expression vector

Both the pB32 and original pB42 expression vectors were sequenced with the Sequenase version 2.0 kit (United States Biochemical Corporation) using the protocol provided with the kit. Mini-prep DNA (5μg from each of 5 pB32 mini-preps) were alkali denatured by incubation in 0.2M NaOH and 0.2mM EDTA at 37°C for 30 minutes. Denatured DNA was then precipitated with 0.1 volumes of 3M sodium acetate (pH 4.7) and 3 volumes of ice-cold ethanol at -80°C for 20 minutes. Denatured DNA was redissolved in 7μL of water and annealing reactions set up by the addition of 2μL of annealing buffer (200mM Tris/HCl (pH 7.5), 100mM $MgCl_2$, 250mM NaCl) and 1μL of a 2mM solution of the sequencing primer B31-3 described in 3.16.1. The annealing reaction was heated to 65°C for 2 minutes and the reaction allowed to cool at room temperature for 30 minutes. At this stage 2.5μL of each of the four dideoxynucleotides termination mixes (ddGTP, ddATP, ddTTP, ddCTP, see section 3.16.1) were aliquoted into eppendorf tubes and incubated at 37°C.

The annealing reaction was then placed on ice and the following added: 1μL of Mn buffer (United States Biochemicals), 1μL 0.1M DTT, 2μL 1 in 15 dilution of dGTP labelling mix (7.5mM each of dGTP, dCTP, dTTP), 0.5μL [α-^{35}S]-dATP (5μCi, Amersham), 2μL Sequenase Version 2.0 enzyme diluted 1 in 8 with dilution buffer (10mM Tris/HCl (pH 7.5), 5mM DTT, 0.5 mg/mL BSA).

The above labelling reaction was incubated for 3–5 minutes at room temperature and 3.5μL then placed into each of the dideoxynucleotide solutions. The reactions were then placed at 37°C for 5 minutes to allow termination and 4μL of stop solution (95% v/v formamide, 20mM EDTA, 0.05% w/v bromophenol blue and 0.05% (v/v) xylene cyanol) added. Tubes were kept on ice until loading when they were heated to 90°C for 5 minutes. Each reaction (2 μL) was loaded on a 6% denaturing gel and electrophoresed as described in section 3.16.2. Gels were run until the bromophenol blue dye band was near the bottom of the gel (approximately 1.5 hours). Sequencing gels were backed onto Whatman 3MM paper, vacuum dried and exposed to X-ray film (Kodak X-Omat) for 24 hours at room temperature.

Only the C and T dinucleotide reactions were loaded for the sequencing of the miniprep DNA described above. After identification of clones with the B-32 mutation, miniprep DNA from these clones was transformed into E. coli DH5α and plasmid DNA prepared as described in appendix 7.15. This DNA, along with similarly prepared DNA from the pB42 vector, was sequenced as described above with all four dinucleotide reactions being loaded.

3.22. Expression of apo B-32 and apo B-42 in cell culture

The pB32 expression vector along with the original pB42 vector were expressed in a rat hepatoma cell line McA-RH7777 using the protocol of Yao *et al* (1991). All cell culture work was performed with sterile solutions (either autoclaved or filter sterilised through a 0.2μ filter) under sterile conditions.

3.22.1 Maintenance of cell line used in expression

The rat hepatoma cell line McA-RH7777 was obtained from American Type Culture Collection. Cells were grown in Dulbeccco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated fetal bovine serum, 25mM glucose, 100 units/mL Penicillin and 100 μg /mL of streptomycin (all obtained from Gibco). Medium was filter sterilised through a 0.22μ filter and stored at 4°C. Cells were grown in 15mL of medium in Falcon 50mL flasks in a 37°C incubator with 5% CO_2. Cells were maintained by feeding twice a week which involved the aspiration of old medium and addition of fresh medium to the flasks.

Cells were split 1:24 weekly. Splitting was performed when the cells were 60-70% confluent as determined by microscopy. Cells were loosened from the flasks for splitting by the addition of 3mL of 0.05% Trypsin, 0.53mM EDTA (Gibco BRL) and the rolling of flask for 30 seconds. Trypsin was then aspirated before cells were placed in new medium.

Stocks of McA-RH7777 cells were frozen at -80°C in 1mL aliquots in freezing medium (20% fetal bovine serum, 10% DMSO, in DMEM).

3.22.2 Co-transfection of McA-RH7777 cells with the apo B expression and pCMVneo vectors.

Stable cell lines expressing apo B-32 and apo B-42 were generated by cotransfection of the pB32 and pB42 vectors with the pCMVneo vector (kindly provided by Dr Martin Kennedy of the Cytogenetics Laboratory, Christchurch Hospital). pCMVneo contains a gene coding for a phosphotransferase enzyme (Jimenez and Davies, 1980) which, when expressed in eukaryotic cells, confers resistance to the antibiotic Geneticin. Cells transfected with and expressing this gene continue to grow in the presence of Geneticin while untransfected cells die.

Transfection was performed using the calcium phosphate coprecipitation and glycerol shock method previously described by Tanabe *et al* (1989). Cells trypsinised as above at 60–70% confluency were split 1:6 into 50mL flasks with 15mL of medium and allowed to reattach overnight at 37°C, 5%CO_2. Expression vector DNA (5μg of pB32 or pB42) along with 0.3μg pCMVneo in 100μL of H_2O was placed in 14mL Falcon 2057 tubes. To each DNA sample was added 500μL of 2× BES buffered saline (50mM BES (pH 6.95), 280mM NaCl, 1.5mM Na_2HPO_4, Sigma). A 0.32M $CaCl_2$ solution was then made by diluting 63μL of a 2M $CaCl_2$ solution with 337μL of H_2O and this was added dropwise to the samples while vortexing. The tubes were then capped tightly and left to stand at room temperature for 20–30 minutes. The $CaPO_4$-DNA solution was then vortexed vigorously for 30 seconds and immediately added dropwise to the culture medium of each flask and gently mixed. After 4 hours at 37°C, 5%CO_2 a glycerol shock was performed on the cells. Medium was aspirated and 3mL of 15% glycerol in 150mM PBS (pH 7.4) was added to one end of the flask and gently rolled over the layer of cells 3–4 times. After 2 minutes the glycerol was aspirated and the cells quickly washed twice with 10mLs of PBS. Fresh medium (15mL) was added and the cells incubated for 48 hours before being split into two 50mL flasks and exposed to 400μg/mL Geneticin (G418) containing media. Cells were incubated at 37°C, 5% CO_2 with the G418 containing medium changed 2–3 times a week. After 14–21 days, colonies which formed were picked when the colony diameter was approximately 1mm. Medium was

aspirated and 5μL of trypsin added to the top of a colony and the colony transferred to a six well Nunclon multidish. The cells in the colony were loosened by gentle pipetting and 2mL of G418-containing medium added. Cells were incubated and the medium changed as before. Medium was analysed for the expression of apo B when the cells became confluent.

3.22.3 Screening for apo B expression

Media (2mL) was aspirated from the cells and lipoproteins precipitated from the media by the addition of 60μL of a 20mg/mL slurry of fumed silica (Sigma) in PBS. After one wash with PBS the lipoproteins were eluted from the silica by adding 60μL of sample buffer (section 3.10) and heating to 100°C for 5 minutes. Thirty μL of this was then subjected to SDS PAGE electrophoresis and immunoblotting as described in sections 3.10 and 3.11. Immunoblotting was performed with both the polyclonal anti apo B antibody described in section 3.11 and a monoclonal anti-apo B antibody, 1D1 (Pease *et al*, 1990), kindly provided by Dr Stephen Young of the Gladstone Foundation Laboratories (San Francisco, CA, USA). Cells identified from resistant colonies as expressing apo B-32 and apo B-42 were trypsinised as in 3.22.1 and transferred to Falcon 250mL flasks in 30mL of media. Cells were grown as before and on reaching confluency medium from pB32 and pB42 expressing cells were subjected to density ultracentrifugation as described in section 3.5 so as to obtain four fractions: VLDL ($d<1.006$ g/mL), LDL ($d=1.006–1.063$), HDL ($d=1.063–1.21$) and $d>1.21$. The lipoproteins in each fraction were precipitated and subjected to electrophoresis and immunoblotting as described above to determine the density distribution of the expressed B-32 and B-42 apoproteins.

3.22.4 Supplementation of media with oleic acid

The media of cells expressing apo B-32 and apo B-42 was supplemented with 1mM oleic acid for 48 hours before being subjected to density ultracentrifugation. Oleic acid dissolved in 0.1M KOH was added to media which was then stirred for 30 minutes to allow adequate mixing before being filtered.

CHAPTER 4 RESULTS

4.1 Lipoprotein and apolipoprotein concentrations in the proband and relatives

Lipoprotein and apolipoprotein concentrations in non-fasting plasma samples from the subject and three genetic relatives are given in Table 4.1. The proband's LDL cholesterol and apo B concentrations were well below the 5th percentile limits of 105 and 45 mg/dL respectively. One of the proband's first cousins (subject 3) also had a low LDL cholesterol concentration (below 105 mg/dL) and a low apo B concentration while the other two subjects (2 and 4) had relatively normal lipoprotein and apolipoprotein concentrations. The proband, a diabetic, had no clinical symptoms or signs of fat malabsorption. The proband was also characterised as having high HDL and apo A1 levels.

Table 4.1 Lipoprotein and apolipoprotein concentrations in the apo B-32 subject and relatives

Subject	Apo B Phenotype	Age/Sex	Triglyceride	Cholesterol			Apolipoproteins	
				Total	LDL	HDL	B	A1
			(mg/dL)					
1	100/32	64/M	23	143	31	90	20	191
2[a]	100/100	65/M	42	248	155	51	94	139
3[a]	100/100	68/F	77	197	56	69	63	139
4[a]	100/100	67/F	54	283	151	78	95	165
Normal[b]				155-317	105-215		45-125	

[a]Subjects 2, 3 and 4 are first cousins to subject 1, and are free of significant medical problems.
[b]The normal range for LDL cholesterol and apo B concentrations are the 5th to 95th percentile values as determined in two local studies (Janus *et al,* 1981; Walmsley *et al,* 1991).

4.2 Lipoprotein and apolipoprotein concentrations in other unrelated subjects

Table 4.2 shows non-fasting lipoprotein and apolipoprotein concentrations from four other subjects identified at the Christchurch lipid disorder clinic as having low total cholesterol and LDL levels below 105 mg/dL. Plasma apo B concentrations were well below the normal range in all subjects. Subject 6 had lower apo B and LDL cholesterol levels than the proband described in 4.1. LDL cholesterol levels in the other three subjects (5, 7 and 8) were well below the 5th percentile but not as low as those measured in subjects 1 and 6. Like the proband, the subjects in table 4.2 had no symptoms of fat malabsorption. Based on their low LDL and apo B levels, subject 1 in table 1 and all subjects in table 2 were classified as having hypobetalipoproteinaemia.

Table 4.2 Lipoprotein and apolipoprotein concentrations in four unrelated subjects

Subject	Age/Sex	Triglycerides	Cholesterol			Apolipoproteins	
			Total	LDL[a]	HDL	B	A1
		(mg/dL)					
5	33/F	40	151	80	63	36	167
6	40/M	108	94	(25)	45	15	123
7	80/M	214	114	33	38	23	117
8	39/M	41	97	39	50	23	42
Normal				105-215		45-125	

[a] The LDL cholesterol levels given here were calculated using the Friedewald equation (see appendix 7.6). The LDL cholesterol value in brackets is a measured LDL cholesterol as are the values in table 4.1.

4.3 Vitamin A and E concentrations

Plasma vitamin A and E concentrations in the five subjects with hypobetalipoproteinaemia are shown in Table 4.3. Vitamin A levels were low but within the normal range except for subject 5 whose vitamin A level was well below the normal range. Vitamin E levels were normal in subjects 1 and 7. Subjects 5 and 8 had vitamin E levels below the normal range.

Table 4.3 Vitamin A and E concentrations in five hypobetalipoproteinaemic subjects

Subject	Vitamin A (μg/mL)	Vitamin E (μmol/L)
1	470	24.2
5	241	6.3
6	702	—
7	728	14.3
8	561	10.0
Normal[a]	350—800	12—46

[a]The normal adult ranges for both vitamins are taken from Kaplan and Pesce (1989).

4.4 Immunoblot analysis of an abnormal apo B

Immunoblotting of plasma from subject 1 (figure 4.1, lane 1) showed an abnormal apo B band along with the normal apo B-100 band indicating subject 1 was heterozygous for a truncated species of apo B. The truncated protein was found largely in the HDL fraction (lane 4) although a small amount was detected in the LDL and d>1.21 fractions (lanes 3 and 5). Immunoblotting of plasma from relatives (not shown) revealed only normal apo B-100 bands. Further study of these individuals was therefore abandoned.

Immunoblot analysis of plasma from the four individuals in table 4.2 is shown in figure 4.2. Subjects 6 and 7 (lanes 2 and 4) initially showed apo B bands at approximately apo B-74 and apo B-48 positions respectively. Repeat analysis of fasting samples (lanes 3 and 5) however showed only apo B-100 bands in the subjects. Similar results were obtained with subjects 5 and 8 (lanes 1 and 6). Thus there was no evidence of any truncated apo B variants in subjects 5, 6, 7 and 8. Further analysis of these individuals was abandoned because of the lack of substantial evidence linking their hypobetalipoproteinaemia with the apo B gene. A detailed linkage analysis study involving family members would be required to assess the association between apo B and the hypobetalipoproteinaemia in these subjects. Such a study is beyond the scope of this thesis.

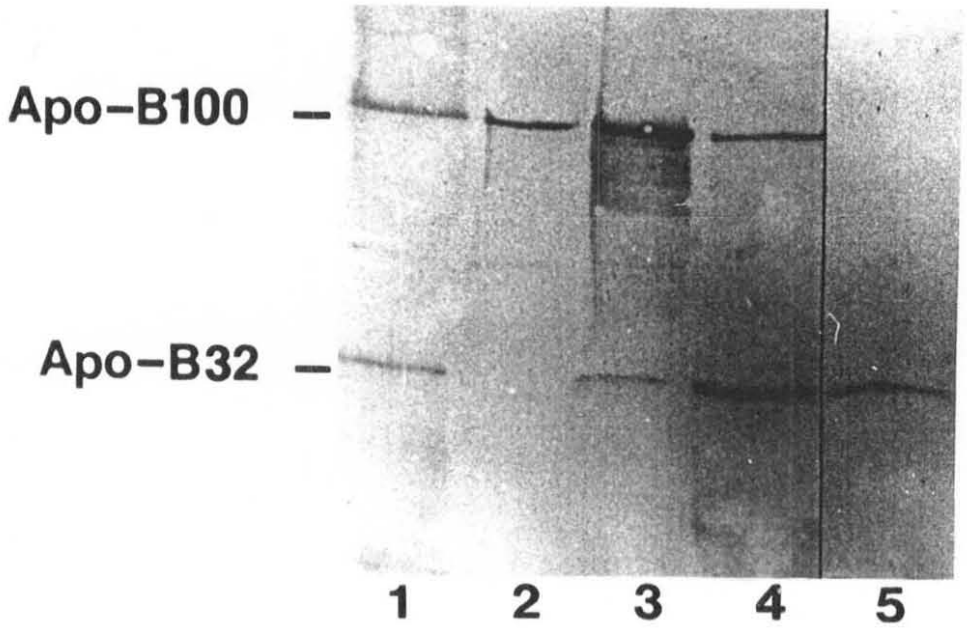


Figure 4.1 Immunoblot detection of a truncated apo B in the subject's plasma and lipoprotein fractions. Lane 1, whole plasma; lane 2, isolated VLDL; lane 3, isolated LDL; lane 4, isolated HDL; lane 5, lipoprotein-deplete plasma (d>1.21 g/mL). The loadings in plasma equivalents are 5, 30, 10, 30, and 30μL. Proteins were electrophoresed on a 3–6% SDS PAGE minigel and immunoblotting performed as previously described by Michaelis *et al* (1991) using a polyclonal anti–apo B antibody.

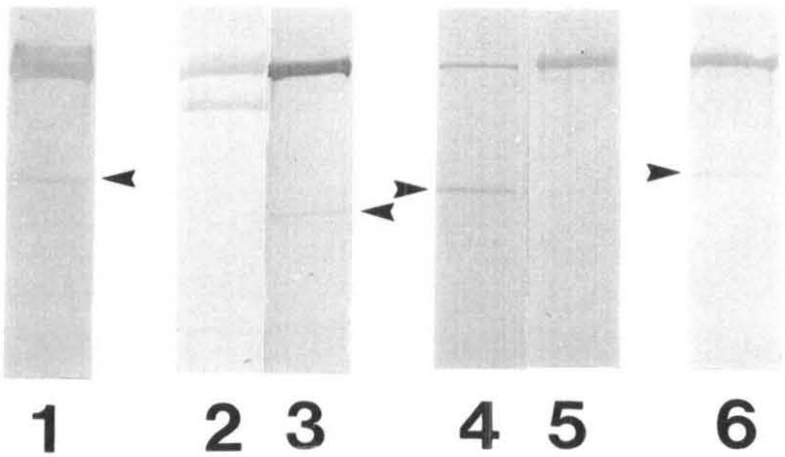


Figure 4.2 Western blot analysis of plasma from four hypobetalipoproteinaemic subjects. Plasma (5μL) was electrophoresed on a 3–6% SDS PAGE minigel and immunoblotting described as in figure 4.1. Lane 1, subject 5; lane 2, subject 6; lane 3, subject 5 (repeat sample); lane 4, subject 7; lane 5, subject 7 (repeat sample), lane 6, subject 8. The top band is apo B-100 while the arrows indicate the position of apo B-48.

Immunoblotting plasma of the proband with apo B-31 and apo B-37 (figure 4.3a) as standards showed that the truncated apo B was similar in length to apo B-31. The latter contains the 1425 amino-terminal amino acids of apo B-100 (Young *et al,* 1990). The reaction of the truncated apo B with three monoclonal antibodies was also similar to apo B-31. Antibody MB3 (figure 4.3b), which binds to an epitope between apo B-100 amino acids 994 and 1084 (Pease *et al,* 1990), reacted with both apo B-31 and the new variant. Antibody 2D8 (figure 4.3c), which binds to an epitope between apo B-100 amino acids 1403 and 1480 (Pease *et al,* 1990), did not react with either apo B-31 or the new variant. Antibody MB44 (figure 4.3d), which binds to an epitope between apo B-100 amino acids 2488 and 2658 (Pease *et al,* 1990), reacted only with apo B-100.

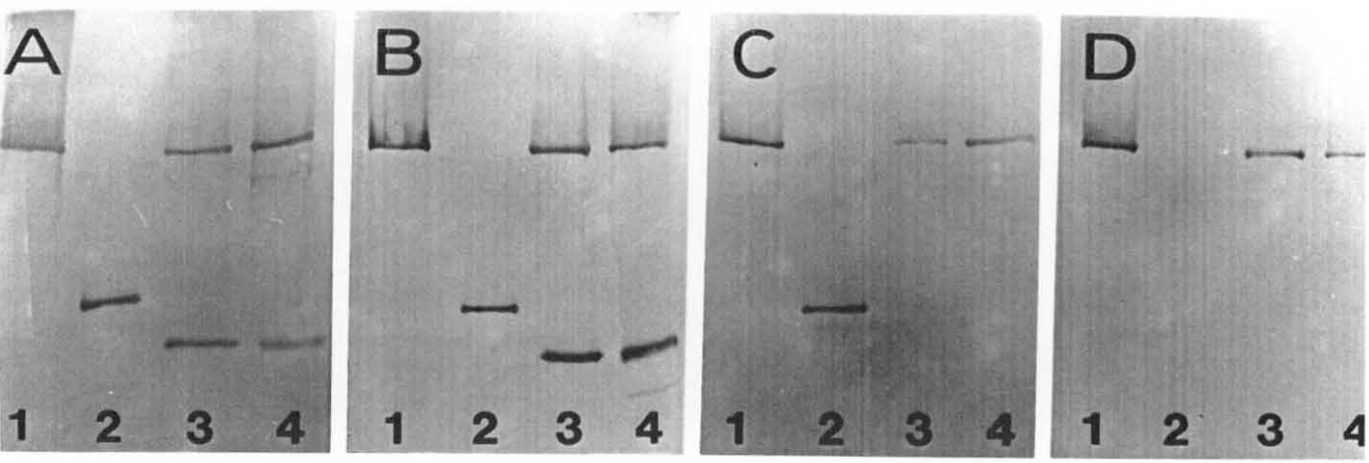


Figure 4.3 Immunoblots demonstrating binding of three apo B specific monoclonal antibodies to apo B-100, apo B-37, apo B-32 and apo B-31. A, B, C and D are immunoblots using a polyclonal anti-B antiserum and monoclonal antibodies MB3, 2D8 and MB44 respectively. Lanes 1–4 are the same in each immunoblot. Lane 1, 1 μL plasma from a normal control; lane 2, 0.3 μL HDL from a patient with apo B-37; lane 3, 3 μL plasma from the apo B-32 subject; lane 4, 2 μL plasma from a patient with apo B-31. The apo B-31 and apo B-37 samples along with the three monoclonal antibodies were generously provided by Dr Stephen Young of the Gladstone Foundation Laboratories CA, USA. Immunoblots were performed as in figure 4.1.

4.5 Determination of molecular weight

The molecular weight of the truncated apo-B was estimated from SDS-PAGE of HDL alongside protein markers (figure 4.4). On this basis, a molecular weight of 165 000 was estimated and the abnormal apo B designated apo B-32 using the nomenclature suggested by Kane *et al* (1980).

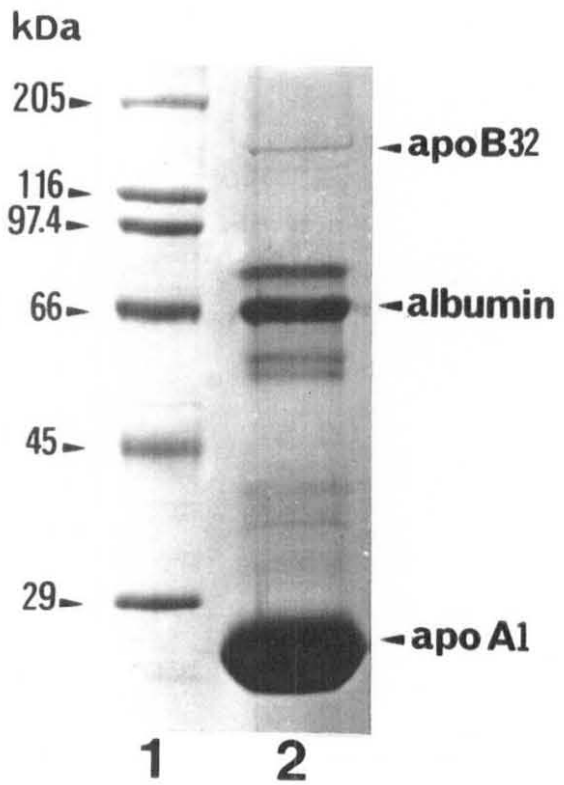


Figure 4.4 Size estimation of the truncated apo B species.
HDL from the subject was electrophoresed on a 3-15% SDS-PAGE mini-gel alongside protein markers. Lane 1, 3 μL protein markers (SDS-6H High Molecular Weight Kit, Sigma, USA); lane 2, 20 μL patient HDL (equivalent to 40 μL plasma). Protein bands were detected by staining with Coomassie blue R-250.

4.6 Two-dimensional analysis of apo B-32 lipoproteins from plasma

Analysis of the apo B-32 particles by electrophoresis on 1% agarose (figure 4.5b) showed no abnormal band with protein staining (1) or Western blot analysis (2). The low LDL (or β lipoprotein fraction) in the plasma from the patient was apparent from the reduced intensity of staining in both the lipid stain (not shown) and Western blot compared to control plasma (figures 4.5a and b, 2). However, further analysis of the electrophoresed proteins in the second dimension followed by immunoblotting revealed the apo B-32 protein (indicated by the arrow) in patient plasma (figure 4.5b, 3). Only apo B-100 (indicated by the arrow) was present in control plasma (figure 4.5a, 3). The B-32 band appeared slightly anodal to the apo B-100 band in the β lipoprotein position indicating the apo B-32 lipoproteins were of pre-β electrophoretic mobility similar to that of the VLDL lipoproteins.

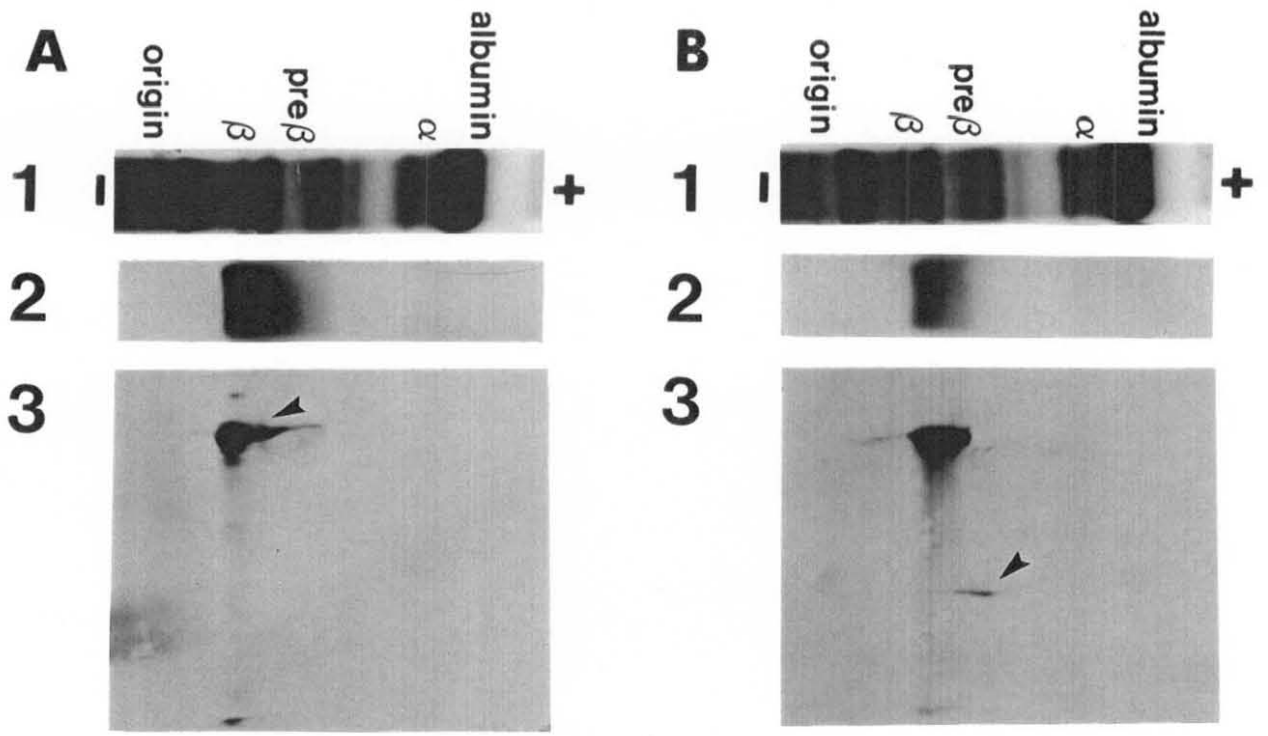


Figure 4.5 Two-dimensional analysis of apo B-32 lipoproteins from plasma. Plasma (3 μL) from a normal control (A) and the apo B-32 subject (B) were electrophoresed in 1% agarose to separate the various lipoprotein classes and the resultant gel either stained with Coomassie blue (1) or immunoblotted with a polyclonal anti apo B antibody (2). The protein component of the separated lipoproteins was then electrophoresed in the second dimension (3) by SDS PAGE in a 3-10% gel and western blotted as in figure 4.1. The arrows indicate the position of the apo B-100 (figure 4.5a) and apo B-32 proteins (figure 4.5b).

4.7 Isolation of apo B-32 lipoproteins

Affinity chromatography of isolated HDL on heparin-Sepharose (figure 4.6) separated the apo B-32 containing lipoproteins from HDL lipoproteins. Analysis of the breakthrough peak (A) by SDS PAGE showed that the HDL was not retarded by the heparin column. Furthermore the apo B-32 protein was not present in this peak indicating that the apo B-32 lipoproteins were bound to the column. A small broad peak (B), which was absent in a control HDL sample, was eluted off the heparin column at approximately 0.1M NaCl. SDS PAGE analysis of the 0.1M NaCl eluted peak showed the presence of apo B-32. This peak also contained some apo E and A1 (confirmed by Western blot analysis).

The possibility of contamination of the apo B-32 lipoproteins with HDL containing apo E was investigated by 1% agarose gel electrophoresis of the isolated apo B-32 followed by immunoblotting with anti apo B, anti-apo A1 and anti-apo E antibodies. Two bands of pre-β mobility which reacted with the anti apo B and A1 antibodies were evident (Figure 4.7a, lanes 2 and 3). The lack of bands in the β and α positions on these blots indicated that the isolated apo B-32 was free of any significant contamination from LDL or HDL lipoproteins. The more cathodal of the two apo B-32 lipoprotein bands reacted with the apo E antibody (lane 4). Immunoblotting with the 2D8 monoclonal antibody whose epitope lies beyond the 1449 amino acids of apo B-32 showed a signal indicating some contamination from LDL apo B degradation. Further two-dimensional analysis of the isolated apo B-32 (figure 4.7b) showed only very slight contamination from LDL apo B breakdown. The isolated apo B-32 fraction shown above was subjected to chemical composition and particle size analysis.

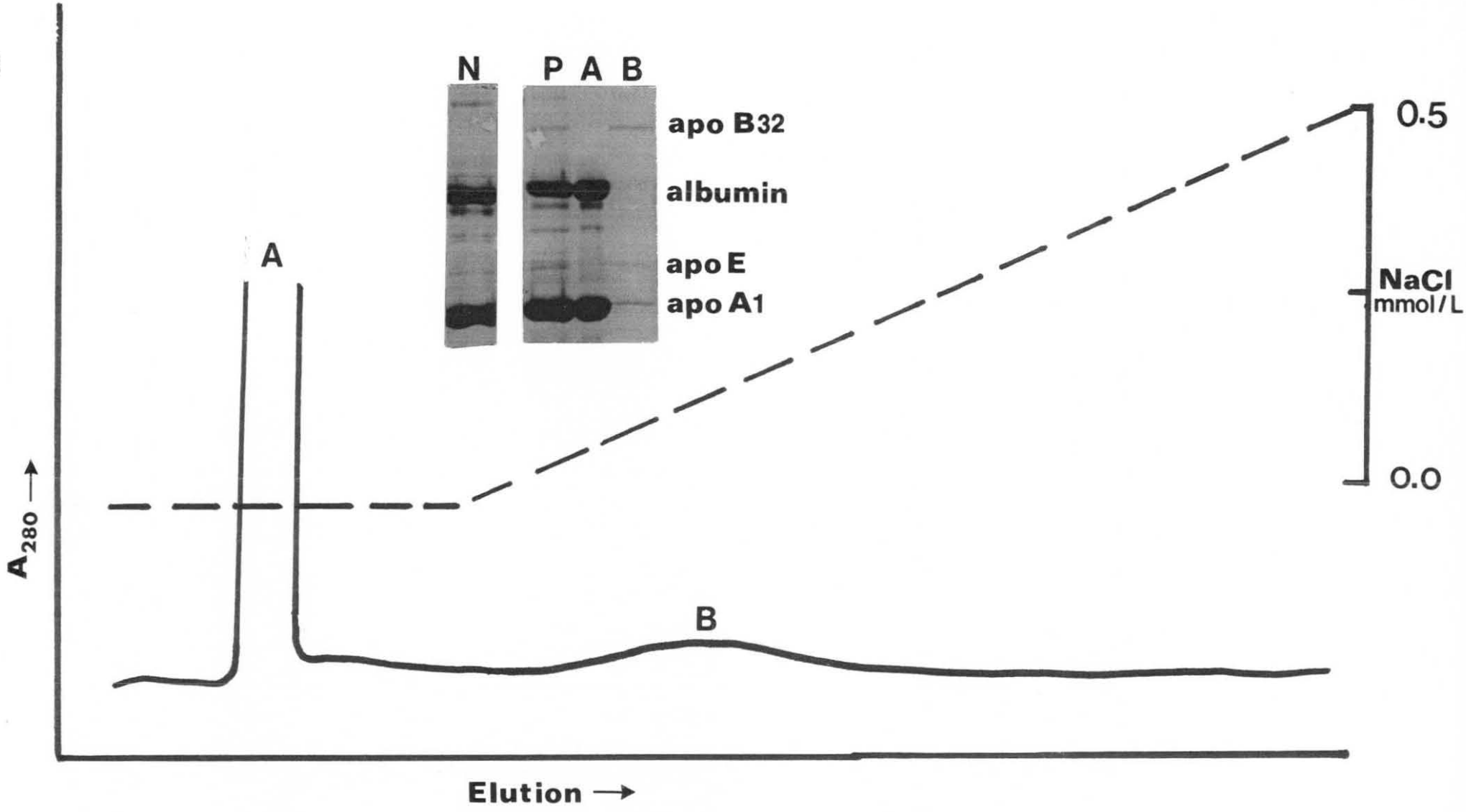


Figure 4.6 Heparin-Sepharose elution profile of patient HDL (d=1.075—1.291 g/mL) and electrophoresis of eluted fractions. HDL (2mL) was applied to the column and the unbound fraction (A) eluted in 50mM Tris/HCl (pH 7.4), 10mm sodium citrate. The bound fraction (B) was eluted with a 0.5M NaCl gradient. The apoprotein composition of the eluted peaks was determined by SDS PAGE electrophoresis on a 3–15% gel (shown in inset). Lane N, HDL from normal subject; lane P, HDL from apo B-32 patient; lane A, peak A (unbound fraction); lane B, peak B (bound fraction).

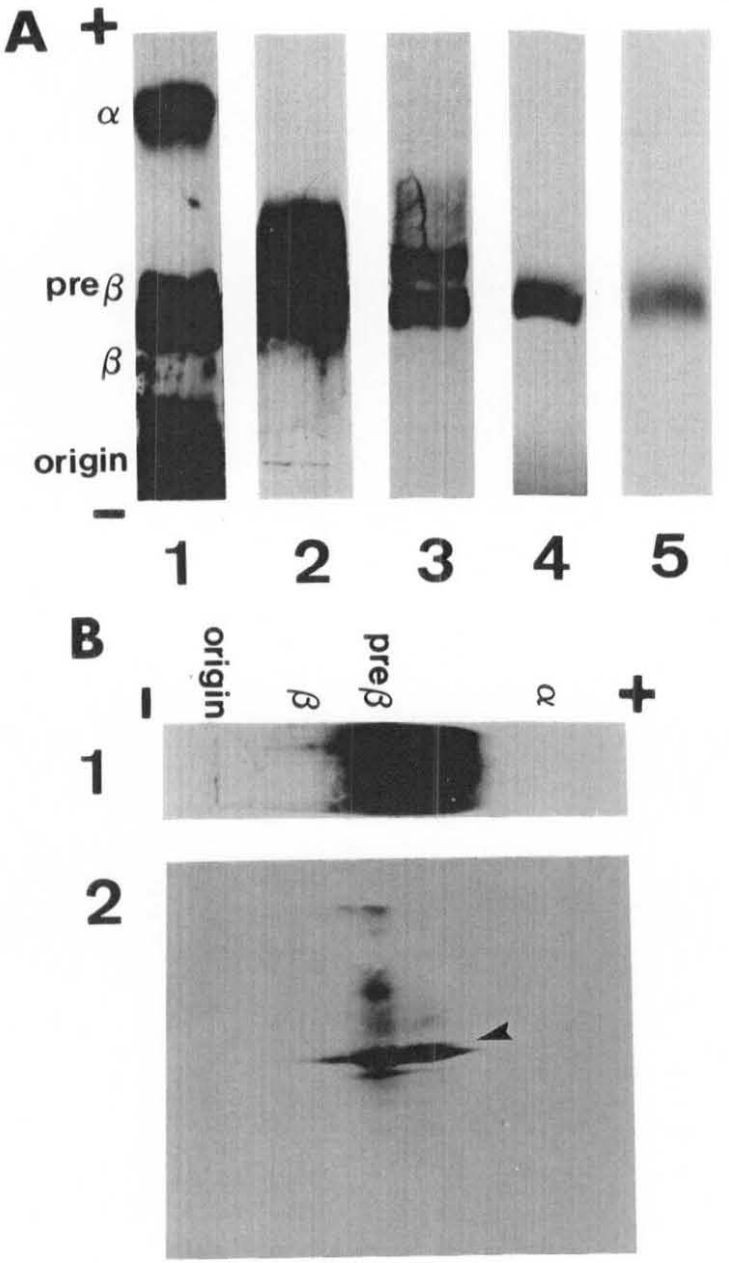


Figure 4.7a Agarose gel electrophoresis and Western blotting of isolated apo B-32 lipoproteins. Lane 1 is a 3μL plasma control incubated with both a polyclonal anti-apo B antibody and an anti-apo A1 antibody to identify the position of the various lipoprotein classes. Lanes 2–5 are loaded with approximately 3μg of isolated apo B-32. Lane 2 was incubated with a polyclonal anti-apo B antibody; lane 3, an anti-apo A1 antibody; lane 4, an anti-apo E antibody; lane 5, a monoclonal anti-apo B antibody which binds to an epitope beyond apo B-32.

b Two-dimensional analysis of isolated apo B-32 lipoproteins. Isolated apo B-32 lipoproteins (approximately 3μg) was electrophoresed in 1% agarose (1) then taken into the second dimension by SDS PAGE in a 3–10% mini-gel (2). Both gels were western blotted and probed with an anti-apo B antibody. The arrow indicates the apo B-32 protein.

4.8 Chemical composition of apo B-32 lipoproteins

The chemical composition of isolated apo B-32 particles is compared with normal lipoprotein classes in table 4.4. Cholesterol, triglyceride, phospholipid and protein analysis of normal VLDL, LDL and HDL showed their composition to be comparable to literature values. The chemical composition of the isolated apo B-32 lipoproteins was most similar to HDL lipoproteins since protein was high (40%) and triglycerides low (7.0%).

Table 4.4 Percentage chemical composition of apo B-32 lipoproteins

Lipoprotein Type	Cholesterol	Triglyceride	Phospholipid	Protein
VLDL	19.4 (21.6)	50.8 (49.9)	16.9 (18.6)	12.9 (7.7)
LDL	50.5 (47.0)	10.5 (11.2)	22.6 (22.1)	16.4 (20.9)
HDL	24.0 (17.9)	4.8 (8.0)	26.0 (22.7)	42.0 (51.9)
B-32	19.0	7.0	24.0	40.0

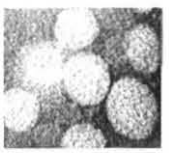
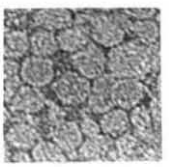
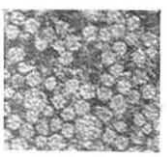
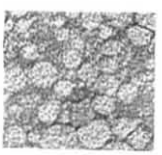
Numbers in brackets indicate reported values (Chapman, 1986)

4.9 Particle size analysis of apo B-32 lipoproteins

Electron microscopy of normal control lipoproteins gave particle sizes within reported normal ranges. Electron microscopy of isolated apo B-32 lipoproteins showed an average particle size intermediate between HDL and LDL with a tendency to HDL.

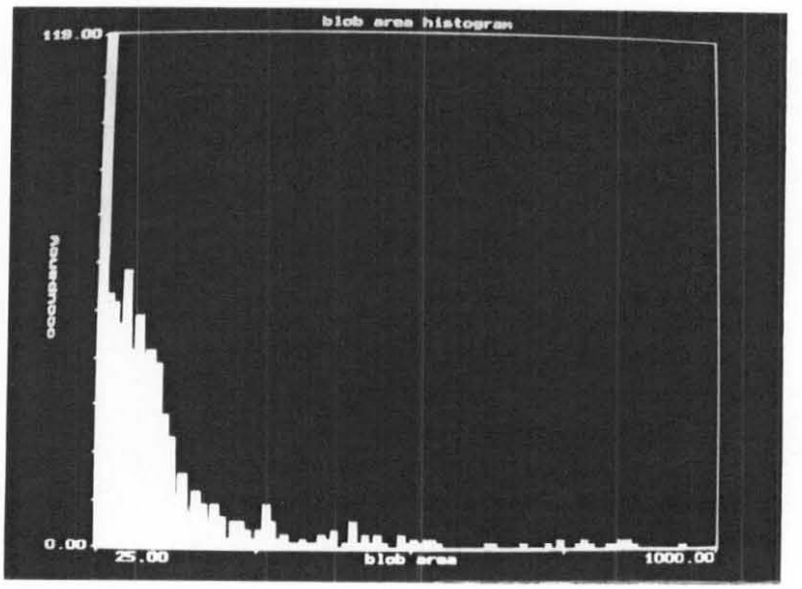
Analysis of particle size distribution by measuring particle area (Figures 4.8a and b) showed a much wider distribution of particle sizes in the apo B-32 patients HDL than normal HDL. The range of particle sizes in the normal HDL (figure 4.8a) was narrower than the range for patient HDL (figure 4.8b), where greater variation was observed.

Table 4.5 Average particle size of apo B-32 lipoproteins

Lipoprotein Type	Average Particle Size[a] (nm)	
VLDL	45.3 (30-80)	
LDL	21.0 (18-25)	
HDL	10.7 (5-12)	
B 32	13.3	

[a] The diameter of 50 free standing particles were measured manually or by the MIA image analysis program. Numbers in brackets indicate reported ranges amongst normal lipoprotein types (Gotto *et al,* 1986). Electron micrographs at 170 000 X magnification are shown for each lipoprotein fraction.

A



B

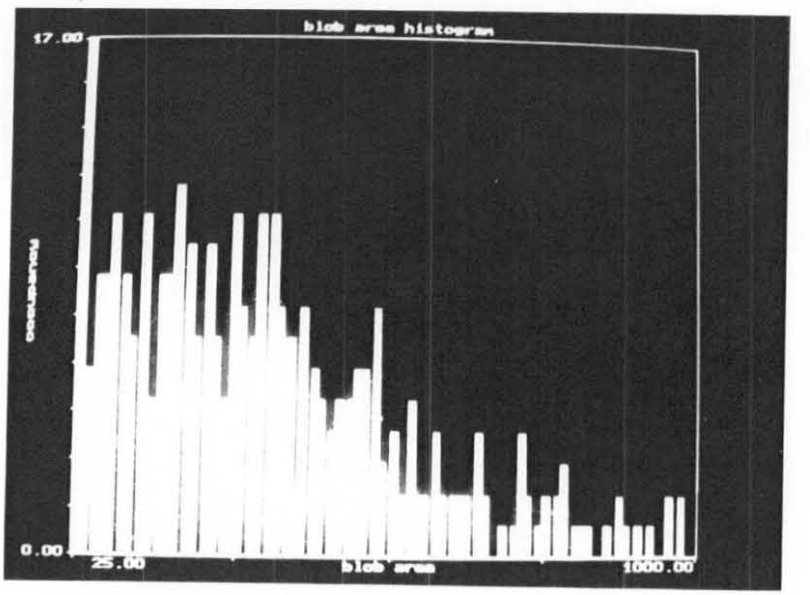


Figure 4.8a Lipoprotein particle size distribution in normal HDL. Areas of lipoprotein particles were measured by electron microscopy of negatively stained HDL using the MIA image analysis program. These areas were plotted on a histogram to show the number of particles in the area range 25–1000 nm (particle diameter 6–36nm).

b Lipoprotein particle size distribution in patient HDL. Areas of lipoprotein particles were measured by electron microscopy of negatively stained HDL using the MIA image analysis program. These areas were plotted on a histogram to show the number of particles in the area range 25–1000 nm (particle diameter 6–36nm).

4.10 Precipitation of apo B-containing lipoproteins

Treatment with four different precipitation reagents gave complete precipitation of apo B-100 but only partial precipitation of apo B-32 as indicated by the presence of apo B-32 in all four supernatants after precipitation treatment (figure 4.9a). Both apo B-100 and some apo B-32 were recovered in redissolved pellets (figure 4.9b) except in the pellet from heparin /manganese treatment which showed poor recovery of apo B-100. This was largely due to the insolubility of this pellet even after repeated heating.

Other truncated apo B variants treated with heparin manganese or dextran sulphate showed differing propensities to precipitate depending on the size of the particular variant (figures 4.10 a and b). The shortest variant (apo B-31) was largely unprecipitated while apo B-46 and apo B-61 were mainly precipitated by both reagents. Apo B-67 and apo B-83 appeared to be completely precipitated as was apo B-100 by both reagents. All the precipitated apoproteins were recovered in the redissolved pellets.

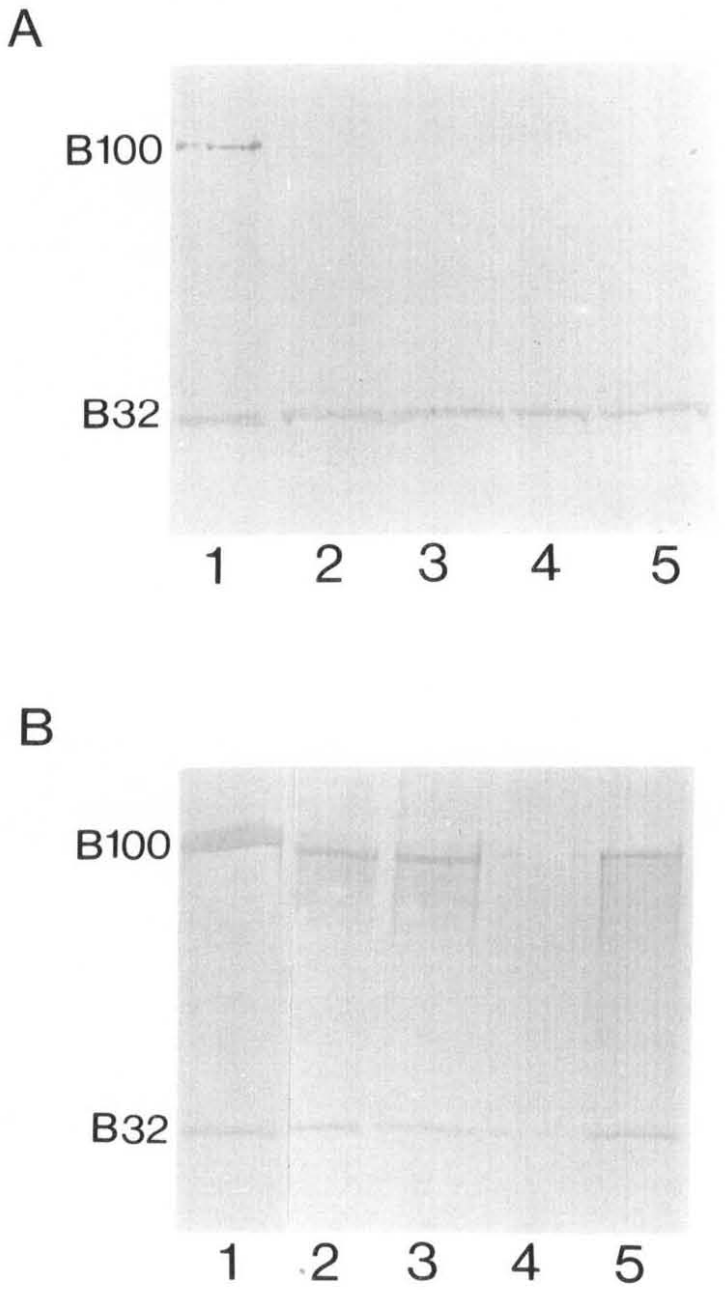


Figure 4.9 a Western blot of supernatant after precipitation of lipoproteins from patient plasma. Lane 1, 5μL unprecipitated plasma; lanes 2–5, plasma supernatant after precipitation with sodium phosphotungstate, polyethylene glycol, heparin manganese and dextran sulphate respectively. Loadings are equivalent to 5μl plasma.

b Western blot of redissolved precipitants after precipitation of lipoproteins from patient plasma. Lane 1, 5μL unprecipitated plasma; lanes 2–5, redissolved precipitants after precipitation with sodium phosphotungstate, polyethylene glycol, heparin manganese and dextran sulphate respectively. Loadings are equivalent to 5μl plasma.

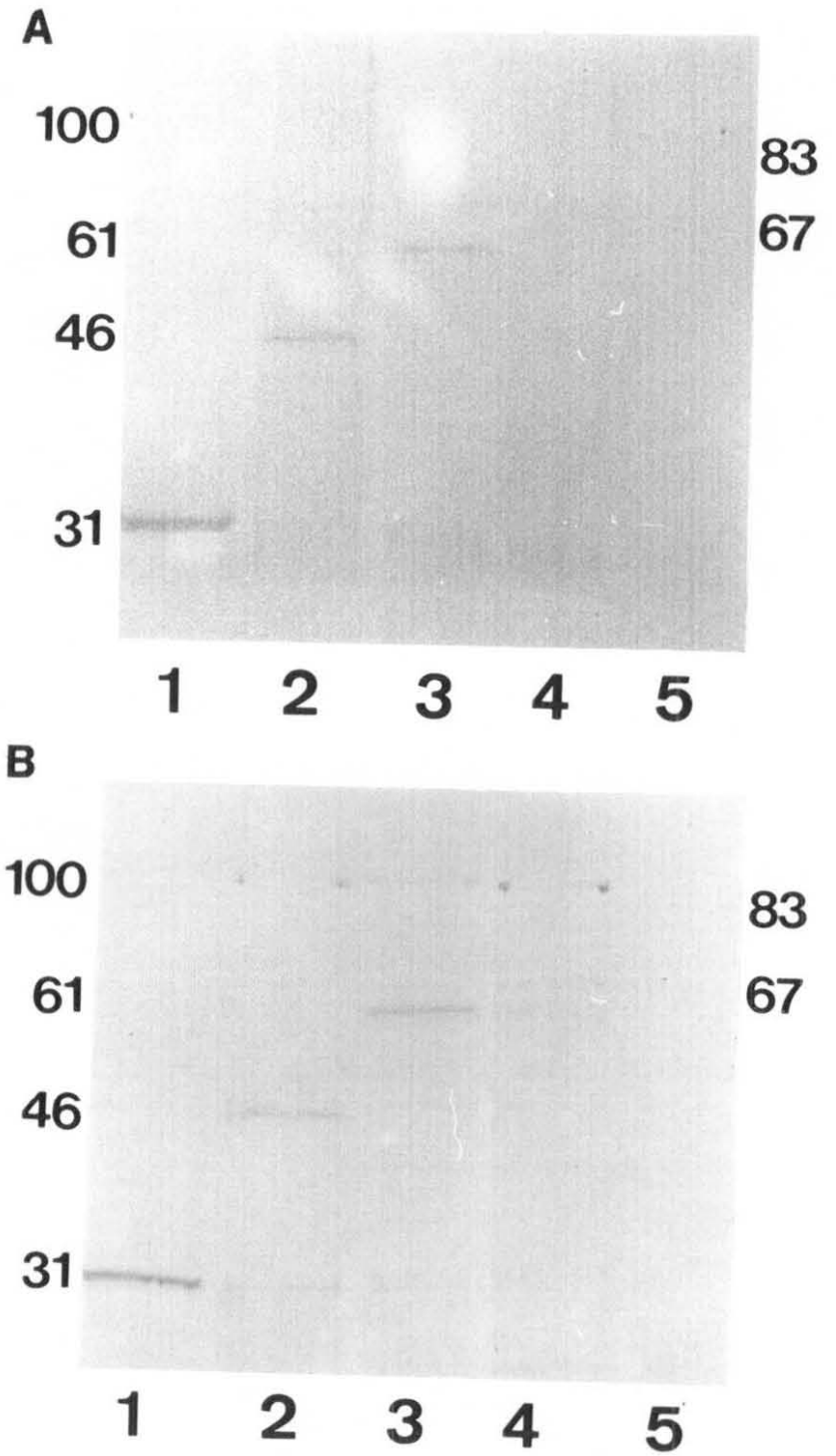


Figure 4.10 a Western blot of supernatants after heparin manganese precipitation of lipoproteins from subjects with truncated apo B variants. Lane 1, apo B-31; lane 2, apo B-46; lane 3, apo B-61; lane 4, apo B-67; lane 5, apo B-83. Loadings are equivalent to 5 μL of plasma.

b Western blot of supernatants after dextran sulphate/$MgCl_2$ precipitation of lipoproteins from subjects with truncated apo B variants. Lane 1, apo B-31; lane 2, apo B-46; lane 3, apo B-61; lane 4, apo B-67; lane 5, apo B-83. Loadings are equivalent to 5 μL of plasma.

4.11 PCR amplification of an apo B gene fragment

Based on the molecular weight estimation of the abnormal apo B, a fragment of the apo B gene (apo B cDNA nucleotides 4369-5127) was selected and amplified using PCR. Analysis of the PCR reaction on agarose electrophoresis (figure 4.11) showed a single band of about 759 bp in the patient DNA. The same band was evident in a control DNA sample (not shown).

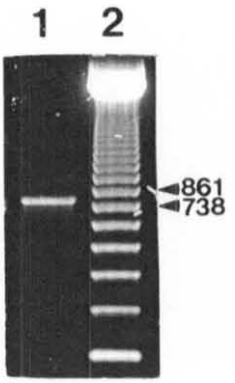


Figure 4.11 Agarose gel electrophoresis of PCR amplified DNA encoding a 759 bp fragment of apo B cDNA. Lane 1, PCR amplified DNA from the apo B-32 subject; lane 2, 1 kb DNA molecular weight markers.

4.12 DNA sequencing of the apo B PCR fragment

Direct DNA sequencing was performed on the 759 bp apo B gene PCR product from the subject and a normal control. Sequencing revealed a single C→T transition of nucleotide 4548 of the coding sequence (figure 4.12). This mutation changes the CAG codon for glutamine 1450 to an in-frame stop codon (TAG). As a result, a truncated apo B species (apo B-32) is generated which is predicted to contain the amino-terminal 1449 amino acids of apo B-100. Apo B-32 has a calculated molecular weight of 164 160.

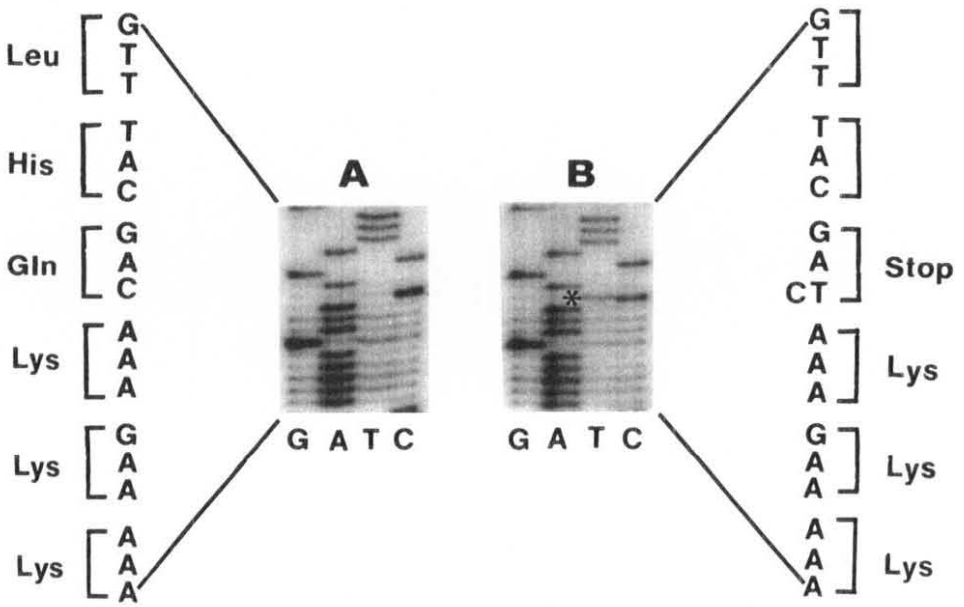


Figure 4.12 DNA sequencing gels showing the mutation responsible for apo B-32. The relevant region of the apo B gene in a normal control (A) and the apo B-32 subject (B) was enzymatically amplified and subjected to direct DNA sequencing. Both a C and T (marked by an asterisk) can be seen at apo B DNA nucleotide 4548 in the subject's DNA. In the abnormal allele, a C→T transition converts a codon for glutamine (CAG) into a premature stop codon (TAG).

4.13 Cloning of the PCR product and Southern blot analysis

The PCR amplified apo B gene fragment shown in figure 4.11 was cloned into the pUC19 plasmid. Figure 4.13 shows the presence of both the apo B insert (750 bp) and pUC 19 (2.7 kb) after PstI/XbaI digestion in 9 positive clones (lanes 2-10) identified by the disruption of β-glucosidase activity. A negative (blue colony) clone contained only the pUC19 plasmid (lane 11). The same agarose gel was blotted and probed with allele specific probes for the wild type and mutant alleles. The resultant Southern blot is also shown in figure 4.13. Six out of the nine clones carried the mutant B-32 allele, while three carried the normal apo B-100 allele. Two of each were sequenced to confirm this finding. The sequence of a negative clone (A) carrying the normal allele and a positive clone (B) containing the mutant apo B-32 allele is shown in figure 4.14.

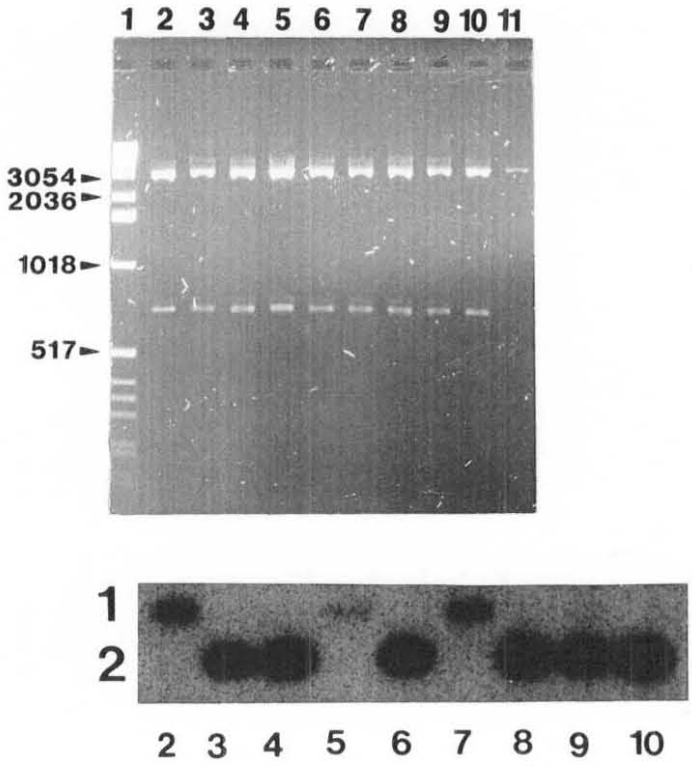


Figure 4.13 Agarose gel electrophoresis of the cloned apo B PCR fragment in the pUC19 vector. Lane 1, ϕX174/HaeIII DNA molecular weight markers; lanes 2-10, PstI/XbaI digested DNA from 9 white colony clones; lane 11, PstI/XbaI digested from a blue colony clone containing pUC19 only. Below is an autoradiograph of the same clones analysed by allele specific hybridisation to B32-1 (1), a probe with the normal sequence at the B-32 position and B32-2 (2), a probe with the mutant C→T transition at the B-32 position. Six out of the nine clones carried the mutant apo B-32 allele.

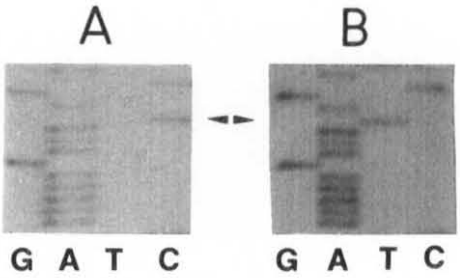


Figure 4.14 DNA sequencing gels of the cloned apo B PCR fragment. Panel A shows the sequence of a normal clone with the normal CAG codon while panel B shows the sequence of a mutant clone carrying the mutant TAG stop codon.

4.14 Allele specific probing

In order to confirm the DNA sequence, PCR amplified DNA from the subject and controls was hybridized to allele-specific probes (figure 4.15). The results showed that the subject's DNA hybridized to both the normal apo B-32-1 probe and the mutant apo B-32-2 probe confirming that he was heterozygous for the apo B-32 mutation. The amplified DNA was also probed with allele-specific oligonucleotide probes for the normal and mutant apo B-31 sequences (probes B-31-3 and B-31-4).This confirmed the specificity of these probes for the mutant sequences.

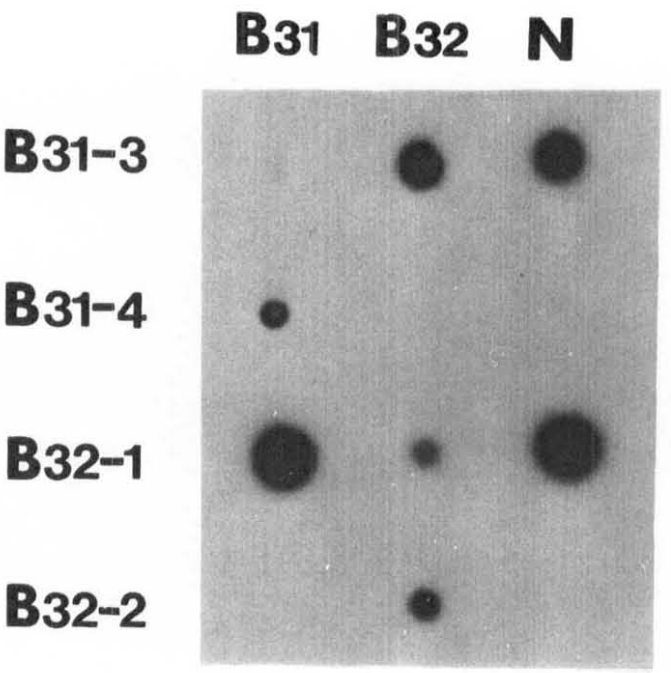


Figure 4.15 Binding of allele-specific oligonucleotide probes for the apo B-31 and apo B-32 mutations to amplified DNA. B31, B32 and N are DNA amplified from an apo B-31 expression vector, the apo B-32 subject and a normal control respectively. Probes B 31-3 and B 31-4 are allele-specific oligonucleotides for the apo B-31 normal and apo B-31 mutant sequences. Probes B 32-1 and B 32-2 are allele-specific oligonucleotides for the apo B-32 normal and apo B-32 mutant sequences.

4.15 Production of an apo B-32 expression vector

Figure 4.16 shows the HpaI digests of the pB42 plasmid DNA of seven clones from the B-32 mutagenesis reaction. Five of the seven clones showed uncut plasmid DNA while DNA from two clones (lanes 2 and 4) appeared to have been digested by HpaI as indicated by the presence of a single band of around 11 kb.

The same uncut plasmid DNA from five clones was sequenced to check for the presence of the C→T transitions in the B-32 mutation. Figure 4.17 represents sequencing data showing only the C and T dinucleotides. Three out of five uncut pB42 plasmids from B-32 mutagenesis had the B-32 mutation. Sequencing of a further five plasmids showed four more mutants indicating an efficiency of the USE mutagenesis method of around 60-80%. The

mutant plasmid was designated pB32. Figure 4.18 shows the full sequence of the pB32 plasmid alongside the original pB42 vector on which mutagenesis was performed.

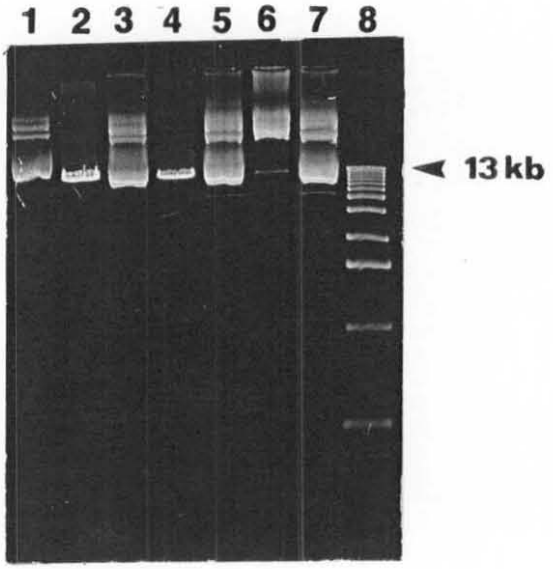


Figure 4.16 Agarose gel electrophoresis of pB42 DNA after USE mutagenesis to introduce the B-32 mutation. Lanes 1-7, DNA from seven clones after 16 hr digestion with HpaI; lane 8, 1kb DNA molecular weight markers. Five of the seven clones appear to posses uncut DNA while the pB42 DNA from two clones (lanes 2 and 4) appears linear.

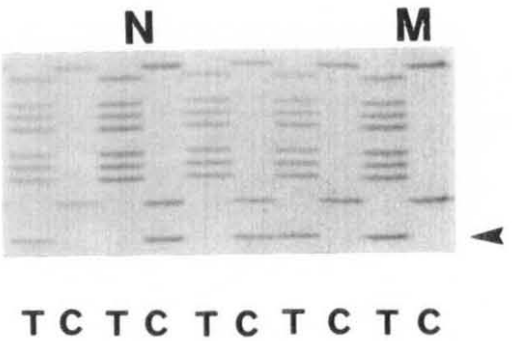


Figure 4.17 Sequencing gels (C and T lanes only) of clones yielding uncut DNA after mutagenesis. The arrow indicates the base at which the C→T transition occurs in the B-32 mutation. Three out of the five clones possessed the B-32 mutation. N indicates a clone with the normal sequence at the B-32 position. M indicates a mutant clone with the B-32 mutation.

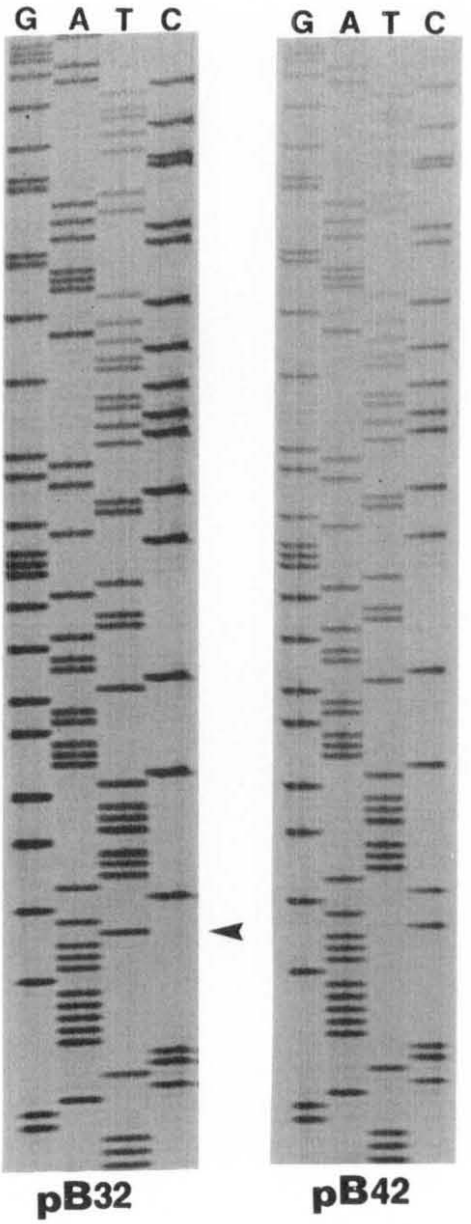


Figure 4.18 DNA sequencing gel of the mutant pB32 apo B expression vector. The sequence of the original pB42 expression vector on which B-32 mutagenesis was performed is also shown. The arrows indicate the position of the C to T transition which occurs in pB32.

4.16 Western blot analysis of expressed lipoproteins

4.16.1 Identification of McA-RH7777 cell line clones expressing apo B-32 and apo B-42

A rat hepatoma cell line McA-RH7777 was transfected with the pB32 and pB42 apo B expression vectors. The media from the cells was analysed by Western blotting to identify those clones expressing the human apo B-32 and apo B-42 proteins. Figure 4.19a shows a Western blot of the media from three pB32 transfected (lanes 2–4) and four pB42 transfected cell lines (lanes 5–8). The blot was incubated with a polyclonal anti-human apo B antibody which also reacts with the endogenous rat apo B. Both the rat apo B-100 and B-48 proteins are present as well as some breakdown products from rat apo B-100. The expressed apo B-32 from the cell line in lane 2 is the same size as the apo B-32 in the plasma of the apo B-32 subject (lane 1). The expressed apo B42 (lanes 5 and 7) was also of the correct size. Probing of the same blot with the monoclonal anti-human apo B antibody 1D1 (figure 4.19b), which does not cross react with rat apo B, confirmed the identity of the expressed human apo B proteins. The growth of the cell lines shown in lanes 2 and 5 were scaled up and the media subjected to density distribution analysis to determine the density of the expressed apo B-32 and apo B-42 lipoproteins.

4.16.2 Density distribution of the expressed apo B-32 and apo B-42 lipoproteins

Lipoproteins were fractionated from the media of both an apo B-32 expressing and apo B-42 expressing cell line by density ultracentrifugation to obtain the four fractions, VLDL ($d<1.006$), LDL ($d=1.006–1.063$), HDL ($d=1.063–1.21$) and $d>1.21$. Western blot analysis of these fractions (figures 4.20a and 4.20b) showed the expressed apo B-32 to be exclusively in the $d>1.21$ fraction while the apo B-42 was found in both the HDL and $d>1.21$ fraction, the majority being in the $d>1.21$ fraction.

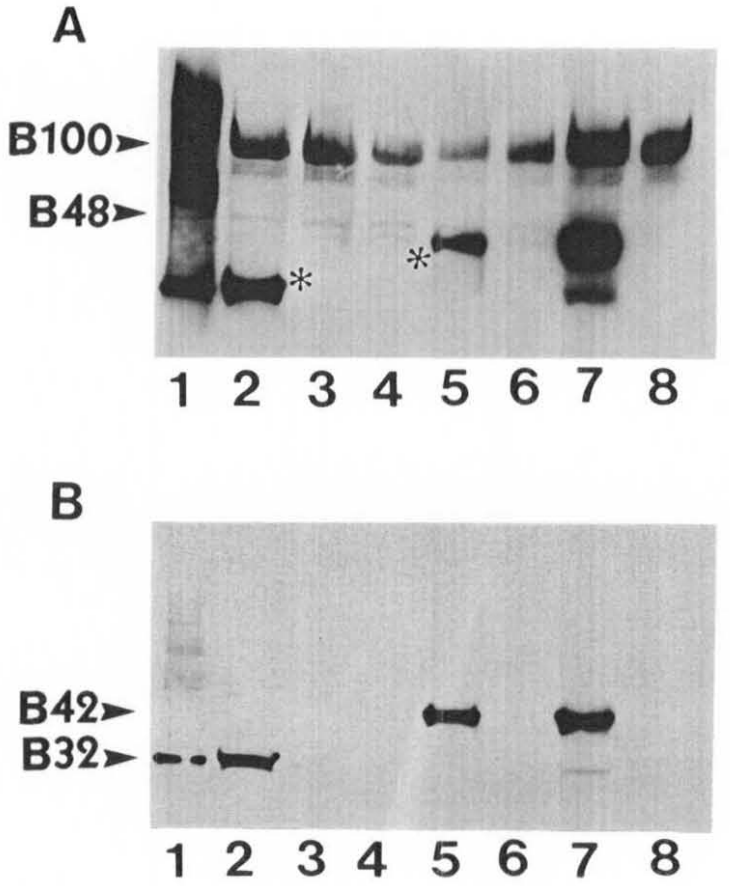


Figure 4.19a Western blot analysis of McA-RH7777 cell media with a polyclonal anti-apo B antibody. Lipoproteins in 1mL of media from pB32 and pB42 transfected cells were absorbed with fumed silica then subjected to SDS PAGE and immunoblotting. Lane 1 is a plasma sample from the subject with the apo B-32 variant; lanes 2–4, media from three pB32 transfected cell clones; lanes 5–8, media from four pB42 transfected cell clones. The blot was incubated with a polyclonal anti-human apo B antibody which also reacts with the endogenous rat apo B-100 and apo B-48.

b Western blot analysis of McA-RH7777 cell media with a monoclonal anti-apo B antibody. The same blot described in figure 4.19a was incubated with the monoclonal anti-apo B antibody 1D1 which reacts with human apo B only.

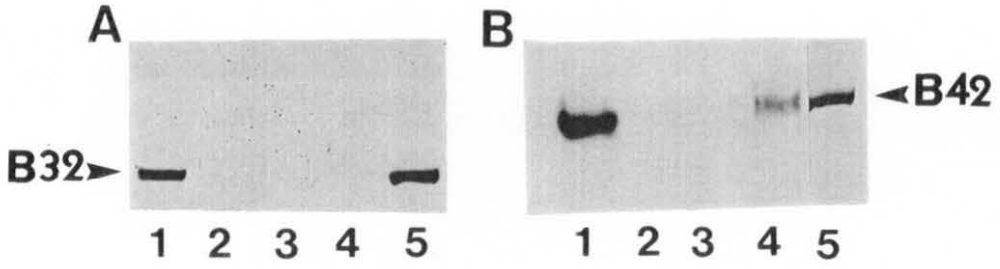


Figure 4.20a Density distribution of expressed apo B-32. The various lipoprotein classes were fractioned from the media of an apo B-32 expressing cell line by density ultracentrifugation. Lipoproteins were absorbed with fumed silica and subjected to SDS PAGE and immunoblot analysis. Lane 1, whole media; lane 2, VLDL (d<1.006); lane 3, LDL (d=1.006–1.063); lane 4, HDL (d=1.063–1.21); lane 5, d>1.21. The blot was incubated with the monoclonal anti-human apo B antibody 1D1 which only reacts with human apo B.

b Density distribution of expressed apo B-42. The various lipoprotein classes were fractioned from the media of an apo B-42 expressing cell line by density ultracentrifugation. Lipoproteins were absorbed with fumed silica and subjected to SDS PAGE and immunoblot analysis. Lane 1, whole media; lane 2, VLDL (d<1.006); lane 3, LDL (d=1.006–1.063); lane 4, HDL (d=1.063–1.21); lane 5, d>1.21. The blot was incubated with the monoclonal anti-human apo B antibody 1D1.

4.16.3 The effect of oleic acid supplementation on density distribution of expressed lipoproteins

Supplementation of the media with 1mM oleic acid did not have any effect on the density distribution of the apo B-32 protein which remained in the d>1.21 fraction (not shown). It did however increase the proportion of apo B-42 in the HDL compared to the d>1.21 fraction. Figure 4.21a shows the density distribution of apo B-42 before (1) and after (2) supplementation of the media with 1mM oleic acid. The density distribution of the endogenous rat apo B-100 was also altered after supplementation with oleic acid. Figures 4.21b shows the density distribution of the rat apo B-100 before (1) and after (2) supplementation with oleic acid in a cell line expressing apo B-32. In both this and the apo B-42 expressing cell line (not shown) the proportion of rat apo B-100 in the VLDL compared to the LDL fraction was increased by oleic acid supplementation.

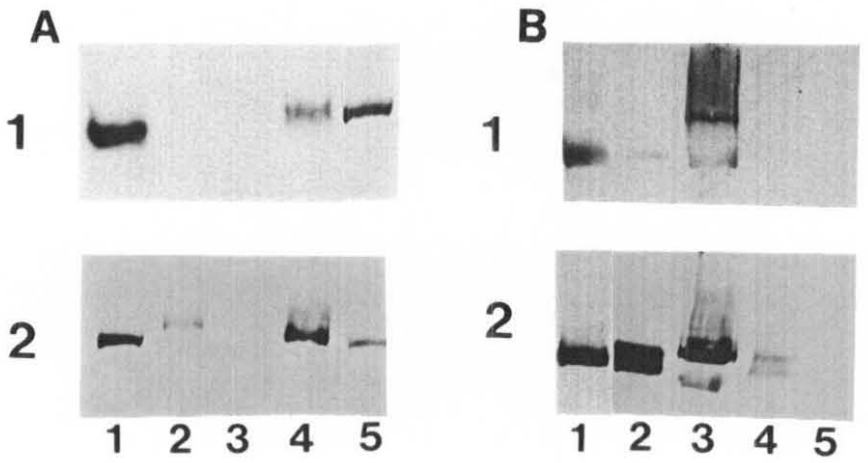


Figure 4.21a The effect of oleic acid supplementation on the density distribution of expressed apo B-42. The various lipoprotein classes were fractioned by density ultracentrifugation from the media of an apo B-42 expressing cell line before (1) and after (2) supplementation of the media with 1mM oleic acid . Lipoproteins were absorbed with fumed silica and subjected to SDS PAGE and immunoblot analysis. Lane 1, whole media; lane 2, VLDL (d<1.006); lane 3, LDL (d=1.006–1.063); lane 4, HDL (d=1.063–1.21); lane 5, d>1.21. The blots were incubated with the monoclonal anti-human apo B antibody 1D1.

b The effect of oleic acid supplementation on the density distribution of rat apo B-100. The various lipoprotein classes were fractioned by density ultracentrifugation from the media of an apo B-32 expressing cell line before (1) and after (2) supplementation of the media with 1mM oleic acid. Lipoproteins were absorbed with fumed silica and subjected to SDS PAGE and immunoblot analysis. Lane 1, whole media; lane 2, VLDL (d<1.006); lane 3, LDL (d=1.006–1.063); lane 4, HDL (d=1.063–1.21); lane 5, d>1.21. The blots were incubated with a polyclonal anti-human apo B antibody which reacts with endogenous rat apo B-100.

CHAPTER 5 DISCUSSION

5.1 Reiteration of aims

The initial aim of this thesis was to focus on subjects with low LDL cholesterol levels in order to investigate any linkage between the hypobetalipoproteinaemia and the apo B gene. Five subjects that had low LDL cholesterol and apo B levels were initially identified. From these it was demonstrated that one of the cases of hypobetalipoproteinaemia was due to a defect in the apo B gene. This was established when immunoblot analysis of the subject's plasma revealed a truncated species of apo B approximately one third the normal length of apo B-100. The focus was then on characterisation of the defective protein and the exact defect causing the truncation. Once this was established the role of the defective apo B (apo B-32) in the formation of lipoproteins was investigated by characterisation of the lipoproteins formed with the apo B-32 variant.

This type of investigation was directed towards providing more information about the structural properties of the large apo B protein since naturally occurring variants of apo B (and other proteins) have in the past provided important structural information. Although a number of truncated apo B variants have now been reported, information is lacking on the type of lipoproteins formed with each variant and their metabolism. It was expected that characterisation of the apo B-32 lipoproteins would give more insight as to the possible metabolism of lipoproteins made with shorter variants of apo B.

5.2 Subjects studied

The subject on which this thesis was based was originally investigated because he had very low levels of plasma apo B and LDL cholesterol (20 and 31mg/dL respectively). These concentrations of apo B and LDL cholesterol were similar to those seen in heterozygous hypobetalipoproteinaemia patients (Farese *et al,* 1992). The LDL cholesterol and apo B levels in the only available relatives to the subject were relatively normal hence the familial

nature of the hypobetalipoproteinaemia in this case could not be demonstrated. This was not surprising since the number of relatives was small and the relationship distant. There were no clinical signs of fat malabsorption in the subject and serum levels of the fat-soluble vitamins were normal. It was noted that the subject had a high HDL cholesterol level in association with a high level of apo A1. High HDL levels have previously been reported in hypobetalipoproteinaemia heterozygotes (Young *et al*, 1989; Young *et al*, 1990; Welty *et al* 1991). The mechanism behind this association has not been established.

The other four subjects studied all had low total and LDL cholesterol levels indicative of heterozygous hypobetalipoproteinaemia. Although no clinical signs of fat malabsorption were observed in these individuals, their low vitamin A and E status in some cases suggested impaired intestinal absorption of the fat soluble vitamins. Western blot analysis of these patients however did not reveal any truncated apo B. Other possibilities for the cause of the hypobetalipoproteinaemia observed in these subjects include:

1. A truncated apo B of less than 30% the amino-terminal length of apo B-100 which would not be observed in plasma since apo B-29 and smaller variants are not secreted into plasma (Collins *et al*, 1988; Huang *et al*, 1989).
2. A defect in a non-coding region of the apo B gene controlling expression which reduces the amount of full length apo B-100.
3. The hypobetalipoproteinaemia is not linked to the apo B gene and may be due to a defect in another protein involved in the lipoprotein metabolism pathway. There have been reports of hypobetalipoproteinaemia within families where the low cholesterol levels have not cosegregated with polymorphic markers on the apo B gene (Hobbs *et al*, 1989; Fazio *et al*, 1991).
4. The hypobetalipoproteinaemia is from a non genetic cause such as liver malfunction. This was certainly a possibility for one of the subjects who was in hospital for liver function tests at the time his hypobetalipoproteinaemia was diagnosed.

Detailed family studies would have to be performed on these individuals and family members in order to investigate the above possibilities.

5.3 Identification and characterisation of apo B-32

During this study a new truncated apo B species (apo B-32) in an individual with heterozygous hypobetalipoproteinaemia was identified. The truncated apo B was initially detected by immunoblot analysis of whole plasma which showed an abnormal apo B of slightly higher molecular weight than apo B-31. The molecular weight estimate of 165 kDa corresponded to the truncated apo B being about 31.9% the length of normal apo B-100 hence the truncated apo B was named apo B-32.

5.3.1 Reaction with three monoclonal anti-apo B antibodies

The apo B-32 protein was characterised further using monoclonal antibodies which bind to different epitopes of the apo B-100 protein. The reaction of apo B-32 with the MB3 antibody and not the MB44 antibody demonstrated that the amino-terminus and not the carboxy-terminus was intact. Immunoblot analysis also showed that the 2D8 antibody, which is thought to bind around apo B-100 amino acids 1403 to 1480 (Pease *et al*, 1990), failed to react with apo B-32. The apo B-31 variant (Young *et al*, 1990) did not bind to 2D8 either, suggesting that the binding epitope for 2D8 was beyond amino acid 1425 of apo B-100. Thus the lack of 2D8 reaction with apo B-32 suggests that the binding epitope for 2D8 is on the carboxy-terminal side of amino acid 1449 of apo B-100 . The apo B-32 protein has therefore been useful in more narrowly defining the binding epitope for the 2D8 monoclonal anti apo-B antibody.

5.3.2 Density distribution.

Lipoprotein fractionation from plasma showed the apo B-32 protein to be largely in the HDL fraction (d=1.063–1.21 g/mL). Since apo B-100 is only present in the more buoyant VLDL and LDL fractions this suggested that an abnormally dense particle was being made. A small amount of the apo B-32 protein was also detected in both the LDL (d=1.006–1.063) and d>1.21 fractions. The density distribution of apo B-32 is unique since it is the shortest known apo B molecule to be found in the LDL density range. The presence of apo B-32 in LDL was checked by double ultracentrifugation of the d=1.006–1.063 fraction to reduce the possibility that the apo B-32 detected on Western blotting of LDL did not originate from contaminating HDL.

5.3.3 Lipid binding properties

Together with the information provided by the apo B-31 variant (1425 amino acids, Young *et al*, 1990), the finding of apo B-32 amongst the lipoprotein fractions suggests that an amino-terminal sequence of approximately 1425 amino acids provides the critical length of apo B required for the formation and secretion of apo B-containing lipoproteins. In contrast, the apo B-29 species found by Collins *et al* (1988) was not found in plasma suggesting that the apo B-29 protein is shorter than the critical length required for lipoprotein formation. Alternatively it was suggested that the absence of apo B-29 from plasma may have been due to either impaired secretion or rapid clearance of any lipoprotein particle formed. The finding of apo B-32 on a lipoprotein particle adds weight to the fact that the amino-terminus of apo B may be important in the formation of lipoprotein particles. A study by Herscovitz *et al* (1991) suggests that even the amino-terminal 17% of apo B is important for the formation of lipoproteins. A major structural feature of the amino-terminus of apo B is the high content of cysteine residues which are associated with a high degree of cross linkage. It may be that this structural feature is important in the formation and/or stabilisation of lipoprotein particles. It is likely however that other regions of apo B such as the mid-region or the carboxy-terminal region identified by Xiong *et al* (1991) are important in lipoprotein formation also.

5.3.4 Role in the formation of LDL

Since apo B-31 (1425 amino acids) was not detected in LDL, apo B-32 (1449 amino acids) is the shortest known apo B molecule capable of forming particles in the LDL density range. Although it is hard to visualise why the addition of an extra 24 amino acids of apo B allows the formation of these particles, it is possible that the region around amino acid 1449 is critical for the formation of a lipid binding domain. Yang *et al* (1989a) have analysed the structure of apo B-100 by tryptic digestion and subsequent separation of trypsin-releasable and trypsin-nonreleasable peptides. Amino acid 1449 is at the end of a large trypsin-releasable peptide which does not appear to be directly associated with lipid. However this trypsin-releasable peptide follows a trypsin-nonreleasable peptide which is thought to be lipid associated. In view of the findings of this study it seems possible that the extra amino acids are required to give the appropriate conformation for lipid binding and lipoprotein particle formation. Thus apo B-31 which terminates immediately after this putative lipid associated peptide, may be unable to form LDL even though apo B-32 , which contains only a further 24 amino acids forms at least some LDL.

Recent work by Xiong *et al* (1991) suggests that it is a region in the carboxy-terminus that is important for the formation of LDL. Expression of a carboxy-terminal region of apo B-100 in transgenic mice and the finding of the expressed protein exclusively in the LDL density fraction suggested that this region of apo B (amino acids 2878–3925) must be important for LDL formation. However there was no evidence that the expressed apo B protein was forming its own discreet particles which leaves the possibility that it could have been associating with pre-existing mouse LDL.

In summary, it seems the apo B-32 protein has defined an important lipid binding region of the apo B molecule. Hopefully, the critical length of apo B required for the formation of LDL particles will be further defined by studies of the lipoproteins in other patients with different mutations in this region.

5.3.5 Heparin binding characteristics of apo B-32: comparisons with apo E

The apo B-32 protein does bind heparin as demonstrated by its isolation by heparin-Sepharose chromatography. It was also demonstrated that the apo B-32 protein has a greater affinity for heparin than does the apo E protein. The apo E-containing HDL particles in the apo B-32 subject were only just retarded by the heparin column whereas the apo B-32 containing lipoproteins were eluted by NaCl at a concentration of 0.1M using a NaCl gradient. The apo E protein has two heparin binding sites, only one of which reacts with heparin when the apo E is associated with lipid (Weisgraber and Rall, 1987). The apo B-32 protein contains three of the seven heparin binding sites in apo B-100 predicted by Weisgraber and Rall (1987). The difference in heparin affinity between apo B-32 and apo E is probably due to the two extra binding sites in apo B-32. This study indicates that the first three predicted heparin binding sites from the amino-terminus in apo B-100 are active in binding heparin.

5.4 The apo B-32 mutation

DNA sequencing showed that the mutation involved was a C→T transition in exon 26 at apo B cDNA nucleotide 4548. This mutation was confirmed when PCR amplified DNA from the subject was shown to hybridise to an allele-specific oligonucleotide (B32-2) containing the C→T transition. This mutation introduces a stop codon which causes premature termination of mRNA translation and the generation of a truncated apo B protein. Apo B-32 is predicted to be 1449 amino acids in length, with a calculated molecular weight of 164 160. This corresponds with the molecular weight estimate of apo B-32 derived from SDS-PAGE.

The latest review on apo B gene mutations by Farese *et al* (1992) records 21 mutations, including the apo B-32 mutation, that result in truncated apo B proteins. The majority of these mutations (12) are deletions of one or more nucleotides leading to an out of frame stop codon. The nine remaining mutations are due to single base changes to stop codons, the majority of which are C→T transitions at CGA codons. There are 12 CGA codons in the apo B mRNA, 5 of which have been associated with C→T transitions causing truncated apo B

variants. The C→T transition that occurs in the apo B-32 mutant occurs at a CAG codon as does the mutation in the apo B-50 variant (Hardman *et al*, 1991). There are many more CAG than CGA codons in the apo B mRNA, 121 in total. This suggests that the CGA codon is far more susceptible to mutation than the CAG codon. In humans 35% of single base substitutions in coding sequences which cause genetic disorders are due to C→T or G→A transitions within CpG dinucleotides (Cooper and Youssofian, 1988). It has been demonstrated that the cytosine in CpG dinucleotides is methylated at the 5'position and that this modification makes the cytosine very susceptible to deamination to form a thymidine hence explaining the high frequency of C→T transitions in CGA codons (Cooper and Youssofian, 1988). The remaining 7 CGA and 119 CAG codons still provide ample opportunity for single base mutations to form stop codons within the coding sequence of the apo B gene. In time more of these mutations causing truncated apo B variants should be discovered.

5.5 Characterisation of apo B-32 lipoproteins

Apart from the apo B-37 lipoproteins characterised by Young *et al* (1987a) the majority of lipoproteins formed from truncated apo B variants have only been characterised on their size and density. Information about the electrophoretic mobility and chemical composition of these lipoproteins is lacking. In addition there has been no investigation of the precipitation characteristics of these lipoproteins to date.

5.5.1 Density distribution

As discussed in section 5.3.2 the majority of the apo B-32 lipoproteins had a density similar to that of normal HDL (d=1.063–1.21). The presence of apo B-32 in the LDL (d=1.006–1.063) and d>1.21 fraction indicated that the apo B-32 lipoproteins had a wide density distribution.

5.5.2 Electrophoretic mobility

Agarose gel electrophoresis of the apo B-32 lipoproteins in plasma showed the particles to have a pre-β mobility similar to that of normal VLDL. This is in keeping with the lipoproteins made with the similar sized apo B-31 and apo B-37 variants (Young *et al*, 1987a; Young *et al* ,1990). The apo B-32 lipoproteins isolated by heparin-Sepharose chromatography had the same pre-β mobility.

5.5.3 Particle size

The average particle size of the apo B-32 lipoproteins isolated from HDL were approximately 2.3 nm bigger than that of the normal HDL particles. Young *et al* (1987a) found the apo B-37 lipoproteins to be 3.5 nm bigger than normal HDL particles. Results from non-denaturing gel electrophoresis of the apo B-31, B-37 and B-46 variants (Young *et al*, 1990) demonstrated that the bigger the truncated apo B variant, the bigger the lipoprotein particles formed with the variant. The presence of a larger than normal lipoprotein particle in the patients HDL, although at low concentration, would be expected to increase the particle size heterogeneity of HDL. This was confirmed by the abnormally wide distribution of particle sizes seen in the HDL of the apo B-32 subject compared with a normal HDL sample.

5.5.4 Chemical composition

The chemical composition of the apo B-32 lipoproteins was similar to normal HDL being high in protein and phospholipids and low in triglyceride and cholesterol. The apo B-32 lipoproteins did however contain slightly more lipid and less protein than normal HDL. The low lipid to protein ratio of the apo B-32 particles would explain their presence in such a high density class. The composition of apo B-32 particles was similar to that of the apo B-37 particles characterised by Young *et al*, (1987a) which also had a chemical composition similar to that of HDL.

The low lipid to protein ratio of the apo B-32 particles would also explain the lack of full precipitation by the apo B-32 particles when treated with the polyanion/cation reagents which normally precipitate apo B containing particles. The propensity of a lipoprotein particle to precipitate is governed by its lipid to protein ratio (Burstein and Scholnick, 1973). Particles with low lipid to protein ratios, such as HDL particles, are not precipitated by the polyanion/cation reagents. That is, the greater the lipid to protein ratio the greater the propensity of the lipoprotein particle to precipitate. This was demonstrated by the precipitation of plasma lipoproteins from plasma samples of subjects with apo B variants of varying length. Generally, those apo B variants nearer to full size such as apo B-67 and apo B-83 behaved as apo B-100 in that they were fully precipitated by the polyanion/cation reagents. Shorter variants such as apo B-31 and apo B-32 were only partially precipitated. The apo B-46 variant was almost completely precipitated. This variant was found in the more buoyant LDL and VLDL fractions in plasma (Young *et al,* 1989) suggesting that it binds more lipid than the apo B-31 and apo B-32 variants which were found in the denser HDL and d>1.21 g/mL plasma fractions. It seems that the amino-terminal half of apo B binds enough lipid to form particles capable of being partially precipitated by the reagents used to precipitate the normal apo B-100 containing LDL and VLDL particles. In general as the apo B molecules increase in length, the propensity of the lipoproteins formed from these molecules to precipitate increases, suggesting that with increasing length of apo B lipid binding capacity is increased. This is in keeping with results from studies that show that the apo B-100 has lipid binding regions throughout its structure (Yang *et al,* 1989a) .

5.5.5 Apolipoprotein composition

The apo B-32 lipoproteins seemed to contain a small amount of the A1 and E apolipoproteins identified by SDS PAGE and subsequent staining of the isolated apo B-32 preparation with Coomassie blue. This result was confirmed by Western blot analysis with both the apo A1 and E antibodies. Agarose gel electrophoresis and western blotting with the apo A1 and E antibodies confirmed that the presence of the A1 and E apolipoproteins was not a

consequence of contaminating HDL. Small amounts of the A1 and E apolipoproteins were also present on the apo B-37 lipoproteins characterised by Young *et al,* (1987a). Both the A1 and E apoproteins are readily exchanged between lipoprotein particles in circulation. Whether the apo A1 and apo E are secreted on the apo B-32 lipoproteins or whether they are added once in circulation is not known.

5.6 Metabolism of apo B-32 and other truncated apo B lipoproteins

Apo B-32 was found in very low concentrations in plasma. This was first evident on protein staining of the subject's HDL fraction and was subsequently apparent during isolation of apo B-32 lipoproteins from plasma, and protein concentration analysis. The extremely low concentration of apo B-32 is in keeping with observations made on other truncated variants (Young *et al,* 1990). The basis for the low plasma levels of truncated apo B variants has not yet been fully investigated. Expression of truncated variants in cell culture has shown the secretion rates of these variants to be normal (Yao *et al,* 1991). This suggests that either the synthesis or catabolism of lipoproteins made with the truncated apo B is abnormal.

It has been well documented that the near full length truncated apo B variants, which have the LDL receptor binding region, compete with apo B-100 for the LDL receptor and are catabolised by this pathway (Gabelli *et al,* 1989; Parhofer *et al,* 1990; Parhofer *et al,* 1992). As yet no one has identified any catabolic pathway for lipoproteins made from the shorter apo B variants without LDL receptor binding characteristics. It has been demonstrated that shorter variants do not compete with apo B-100 for the LDL receptor (Young *et al,* 1987b). Either the lipoproteins made with the truncated apo B species remain in plasma or are catabolised by an alternate pathway. Our characterisation of the apo B-32 particles has shown the presence of both A1 and E apolipoproteins on the particles. The remnant clearance pathway, which metabolises chylomicron and VLDL remnants by the reaction of apo E with its receptor, may therefore be a possible pathway for the metabolism of apo B-32 lipoproteins. The HDL clearance pathway, which involves metabolism of plasma HDL by

interaction of apo A1 with the HDL receptor on liver cells, is also a possible clearance pathway for apo B-32 lipoproteins. This study did not directly address the question of apo B-32 lipoprotein metabolism but it is hoped that a further study as outlined in chapter 6 will provide more information.

5.7 Expression of apo B-32 in cell culture

After introduction of the apo B-32 mutation into an apo B expression vector (pB42), the resultant vector (pB32) was used to transfect a rat hepatoma cell line McA-RH7777. This particular cell line has been used previously in studies expressing truncated apo B variants. It was originally chosen over the human hepatoma cell line HepG2 because McA-RH7777 cells have a greater capacity to secrete triglyceride-rich VLDL particles (Tanabe *et al*, 1989). HepG2 cells secrete particles that are LDL-like in density, size and composition (Bostrom *et al*, 1988). Consequently it was thought that a wider range of particles could be made from various lengths of apo B in McA-RH7777 cells since the apo B-100 containing lipoproteins secreted by these cells seemed more akin to those in rat and human circulation.

The apo B-32 protein expressed by the McA-RH7777 cells was identical in size to that found in plasma, indicating that the protein had been processed correctly by the cell line. Apo B-32 has four of the apo B-100 glycosylation sites (Yang *et al,* 1989a), all of which are utilised. If the glycosylation of apo B-32 in McA-RH7777 cells had been impaired, a difference in the molecular weight between expressed apo B-32 and plasma apo B-32 would have been observed. The expressed apo B-42 protein was also of the correct size. The reaction of apo B-32 and apo B-42 proteins with the monoclonal anti-human apo B antibody 1D1, which binds to an epitope between apo B-100 amino acids 474 and 539 (Pease *et al,* 1990), confirmed the identity of both proteins.

5.7.1 Density distribution of the expressed apo B-32 and apo B-42

The density distributions of apo B-32 and apo B-42 did not parallel that seen in the circulation of subjects with the same or similar length apo B species. Whereas apo B-32 was found mainly in the HDL of the apo B-32 subject, the apo B-32 when expressed in McA-RH7777 remained largely in the d>1.21 fraction. Similarly, although an apo B-40 variant was found mainly in the VLDL and LDL fractions, when expressed in McA-RH7777 cells, the larger apo B-42 protein was found mostly in the denser HDL fraction. The fact that both apo B species exhibited their own characteristic density suggested that each was forming their own characteristic lipoprotein particle rather than associating with any pre-existing lipoprotein in the media. Since apo B-32 was in the d>1.21 fraction and apo B-42 in the HDL it was apparent that the apo B-42 protein bound significantly more lipid than apo B-32. Expression of apo B-32 and apo B-42 and subsequent density distribution studies revealed that as the length of apo B increased, the density of the lipoproteins formed decreases. This is in agreement with the results from other expression studies (Yao *et al,* 1991; Graham *et al,* 1991; Spring *et al,* 1992). That is, the longer the variant the more buoyant the lipoprotein made with the variant.

It is apparent from this and other studies that the lipoprotein particles assembled and secreted from cultured McA-RH7777 cells are denser than those found in the circulation of affected individuals. Yao *et al* (1991) expressed a range of truncated apo B species of varying length in McA-RH7777 cells including the naturally occurring apo B-37 variant. Although found in the VLDL, LDL and HDL of the affected subject, apo B-37 when expressed in McA-RH7777 cells was only found in the HDL and d>1.21 fraction. Even the endogenous rat apo B-48 was found in the HDL density range in this study. In summary it was not possible to reproduce the density distribution of apo B-32 seen in plasma by expression studies to confirm the importance of apo B-32 in forming particles in the LDL density range. This was largely due to the fact that the rat hepatoma cell line used in this study was not very efficient at assembling buoyant lipoproteins from shorter forms of apo B. The reason for this is unclear.

5.7.2 Effect of oleic acid supplementation

Since initially, a significant proportion of the apo B-42 protein was located in the d>1.21 fraction, it was suspected that the cells may have been deficient in fatty acids. The lack of endogenous rat apo B-100 in the VLDL fraction also suggested an impairment in the secretion of the more triglyceride-rich particles. Supplementation of the media with 1mM oleic acid did significantly increased the amount of rat apo B-100 appearing in VLDL. The increase in the lipid content of the cell after supplementation was apparent by the presence of large lipid droplets in the cytoplasm of McA-RH7777 cells. Oleic acid supplementation also increased the proportion of apo B-42 in the HDL fraction leaving only a small amount of apo B-42 associated with the d>1.21 fraction. Similar results were obtained by White *et al* (1992) in McA-RH7777 cells expressing apo B-36 and apo B-48. A shift to a lower density range was shown by both expressed proteins, after supplementation of the media with 0.8mM oleate. Supplementation with oleic acid had no effect on the density distribution of apo B-32 which remained in the d>1.21 fraction. This is not surprising given that the apo B-32 lipoproteins characterised in this thesis were triglyceride-poor. That is, it would be expected that supplementation with oleic acid would only have an effect on apo B lipoproteins containing significant amounts of triglyceride.

5.8 Conclusions

A new truncated apo B variant was identified during this investigation. The defect causing the new variant was found to be a C→T transition changing the CAG codon coding for amino acid 1450 to a stop codon. At the time of discovery of apo B-32 there were seven reported mutations in the apo B gene causing truncations of various lengths of apo B. However, an increased focus on individuals with low cholesterol and apo B, the majority of which exhibit no clinical symptoms, has led investigators to identify a number of mutations causing truncated apo B variants over the past 2–3 years. These mutations are therefore more common than was first believed.

Some of the reported truncated variants have enhanced our knowledge on the structure of the large apo B-100 protein, mainly in terms of lipid-binding and LDL receptor binding characteristics. Apo B-32 has joined the ranks of these informative variants by identifying an important lipid binding region as well as confirming three of the proposed heparin binding sites in apo B-100.

The lipoprotein particles formed with apo B-32 differ from those formed with normal apo B-100. Apo B-32 cannot support the formation of the triglyceride-rich VLDL particles and forms particles which are like HDL in their density, size and composition. The apo B-32 lipoproteins possess other surface apolipoproteins (apo A1 and apo E) which provides the possibility that these particles may be metabolised through an apo A1 or apo E mediated pathway, since the apo B-32 protein does not possess the LDL receptor binding region.

Expression studies of apo B-32 in the rat hepatoma cell line McA-RH7777 showed that the apo B-32 protein was secreted on a denser class of lipoprotein than those characterised from human circulation. This study, along with similar studies by other investigators, has established that the lipoproteins made from truncated apo B variants in cell culture are different from those found in circulation. Thus the expression system used in this study is not an ideal system for reproducing the subtle differences in lipoprotein density that occurs in the circulating lipoproteins of subjects with truncated apo B variants.

The results obtained from this work, as is often the case in scientific investigation, raise questions which call for further investigation. These questions are outlined in chapter 6.

CHAPTER 6 FUTURE WORK

This thesis has focused on hypobetalipoproteinaemia due to truncated apo B variants. Cases of hypobetalipoproteinaemia have been reported that lack any link with the apo B gene (Hobbs *et al,* 1989; Fazio *et al,* 1991). Four subjects were identified during the course of this investigation all of whom had no evidence of a truncated apo B in their plasma. Truncated apo B variants <30% the length of apo B-100 are not secreted into plasma. Since it would be an extremely large undertaking to sequence the region of the large apo B gene coding for the amino-terminal 30% of apo B, a link between the hypobetalipoproteinaemia seen in these individuals and the apo B gene would first have to be established. Boerwinkle *et al (*1989) have established a linkage analysis study of apo B using PCR of a hypervariable region of the apo B gene comprising at least 12 different alleles. This could be applied to the above subjects and their family members to establish if the apo B gene is involved in any of the remaining as yet uncharacterised hypobetalipoproteinaemic cases.

Throughout this study it has been assumed that the apo B-32 lipoproteins in the circulation of the subject were of hepatic origin. A recent paper by Krul *et al* (1992) demonstrated that truncated variants less than apo B-48 were present in intestinal tissue. The intestine secretes apo B-48 in the form of the large triglyceride-rich chylomicron lipoproteins. Whether shorter variants of apo B like apo B-32 are secreted in the form of lipoproteins from the intestine remains to be investigated.

An area that needs to be actively investigated is the association between truncated apo B variants and the low LDL cholesterol levels they confer. One mechanism for this association has been identified in the lipoproteins made with near full-length variants which are more rapidly cleared from plasma than apo B-100 containing LDL due to having a greater affinity for the LDL receptor. The metabolism of the lipoproteins made from truncated apo B variants lacking the LDL-receptor binding region is unknown. It would be expected that these lipoproteins would remain in plasma and therefore supplement LDL cholesterol levels.

This is clearly not the case since subjects with these variants all have very low cholesterol levels. The presence of other apolipoproteins on the surface of these lipoproteins may be the clue to their metabolism by alternative pathways. We propose to investigate this by determining the plasma half lives of radiolabelled apo B-32 lipoproteins versus radiolabelled LDL particles in rabbits.

Finally, since the cell line used in the expression studies secreted lipoproteins different to those seen in circulation, it would be of interest to investigate the characteristics of apo B-32 lipoproteins in other cell lines. These could include the human liver cell line HepG2 and the murine cell line used by Herscovitz *et al* (1991) to secrete the amino-terminal 17% of apo B.

CHAPTER 7 APPENDICES

7.1 Vitamin A and E measurements

Plasma vitamins A and E were measured by the Free Radical Research group at the Christchurch Clinical School. Measurements were based on the HPLC method of Driskell *et al* (1982). Vitamins were extracted from 200μL of plasma with 100μL ethanol, in which was dissolved an internal retinol acetate standard, followed by 400μL hexane extraction. Three hundred μL of supernatant was then dried down and redissolved in 200μL methanol. After filtering through glass wool the sample was loaded onto a Pye Unicam 4100 HPLC pre-equilibrated in 93% methanol, 7% water. A five micron C-18 Brownlee column (10 × 0.46cm) was used with a 7 micron C-18 guard column (1.5 × 0.32). Pump parameters were:

Flow rate = 2.5mL/minute

Pressure limit = 400 bars

Run Time = 10 minutes

Concentrations of vitamin A and E were calculated in relation to the retinol acetate standard using the Pye Unicam 6000 integration system.

7.2 Apolipoprotein determinations

The concentration of apo B and apo A1 in plasma was measured using an immunoturbometric assay Walmsley *et al* (1991). Plasma samples were diluted twenty one fold in 150mM PBS (pH 7.4). For apo B, 30μL of diluted sample and 10μL of distilled H_2O was preincubated with 200μL of PBS containing 5% (w/v) Polyethylene Glycol 6000 (PEG) and 0.2% (w/v) Tween 20 for 120 seconds. Antiserum (20μL of a five fold dilution of Anti-h-lipoprotein B antiserum, Boehringer Mannheim) was added and the absorbance change at 340 nm measured after 10 minutes. The apo B concentration was then calculated by comparing the absorbance change of the sample with that of six standards (Boehringer apolipoprotien calibration serum) using the Cobas-Bio log/logit data evaluation for non-linear standard curves.

For apo A1, 5μL of diluted sample was mixed with 35μL of distilled H_2O and preincubated with 200μL of PBS containing PEG and Tween 20 for 120 seconds. Antisera (20μL of a five fold dilution of Anti-human-lipoprotein A1 antiserum, Boehringer Mannheim) was added and the absorbance change at 340 nm measured after 10 minutes. The apo A1 concentration was calculated by comparing the absorbance change of the sample with that of six standards (Boehringer apolipoprotein calibration serum) using the Cobas-Bio log/logit data evaluation for non-linear standard curves.

7.3 Lipoprotein isolations

Lipoproteins were isolated from plasma by density ultracentrifugation in a Kontron TGA-65 ultracentrifuge using a Kontron TFT 45.6 rotor and the method of Havel *et al* (1955). Four mL of plasma was overlayed with 1 mL of d=1.006 g/mL density solution (see appendix 7.4) and the VLDL (d<1.006) isolated by ultracentrifugation at 37 000 rpm at 15°C for 17 hours. Subsequently LDL (d=1.006–1.063 g/mL) and HDL (d=1.063–1.21 g/mL) were isolated by centrifugation as above. The supernatant lipoproteins were separated by tube slicing and stored at 4°C while 4mL of the remaining infranatant was overlayed with 1mL of the desired density solution and recentrifuged.

7.4 Density solutions

Density solutions were prepared and density adjustments made to plasma as described by Wardell (1986). A 1.346 g/mL solution (38.25g NaCl and 88.4g KBr made up to 250mL in H_2O with the pH adjusted to 7.0–7.5 with 1M NaOH) was diluted with a 1.006 g/mL solution (20.7g NaCl and 0.2g disodium EDTA in 2L H_2O pH adjusted to 7.0-7.5 with 1M NaOH) to gain the appropriate density according to the following formula:

$$(A \times 1.006) + (B \times 1.346) = (A + B)X$$

where A is the volume of 1.006 g/mL solution, B is the volume of 1.346 g/ml solution and X the desired density of the mixture.

The density of plasma samples and infranatants was adjusted by the addition of solid KBr according to the following formula:

$$X = \frac{V_i(d_f - d_i)}{1 - \overline{V}d_f}$$

where X was the amount (g) of solid KBr to be added, V_i is the initial volume (mL) of the solution to be adjusted, d_f was the final density (g/mL) desired, and d_i was the initial density (g/mL), and V_i was the partial specific volume (cm^3/g) of KBr (taken as 0.3 for d=1.075 and 0.31 for d=1.291).

7.5 Cholesterol and triglyceride determinations

7.5.1 Cholesterol determination

Total cholesterol in plasma and lipoprotein fractions were determined on a Cobas Bio Centrifugal analyser using an enzymatic colorimetric assay (Monotest Cholesterol CHOD-PAP, Boehringer Mannheim). This method is based on that of Siedel *et al* (1983) and measures both esterified and free cholesterol using the following series of reactions:

$$\text{cholesterol esters} + H_2O \xrightarrow{\text{cholesterol esterase}} \text{cholesterol} + \text{RCOOH}$$

$$\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + 4H_2O$$

RCOOH = free fatty acids

The first reaction involves the conversion of cholesterol esters to free cholesterol. The following reactions involve the production of a quinoneimine dye, a red coloured dye which absorbs light at 500 nm. The intensity of colour produced is directly proportional to the concentration of cholesterol in the sample

7.5.2 Triglyceride determination

Total triglycerides in plasma and lipoprotein fractions were determined on a Cobas Bio Centrifugal analyser using an enzymatic colorimetric assay (Triglycerides GPO-PAP, Boehringer Mannheim). This assay is based on a modification of the method of Wahlefeld (1974) and involves the following series of reactions:

$$\text{triglycerides} + 3H_2O \xrightarrow{lipase} \text{glycerol} + 3\text{RCOOH}$$

$$\text{glycerol} + \text{ATP} \xrightarrow[kinase]{glycerol} \text{glycerol-3-phosphate} + \text{ADP}$$

$$\text{glycerol-3-phosphate} + O_2 \xrightarrow[oxidase]{glycerophosphate} \text{dihydroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{peroxidase} 2H_2O + HCl + \text{4-(p-benzoquinone-mono-amino)-phenazone}$$

The glycerol portion of the triglyceride molecule is determined through a series of reactions which lead to the formation of a quinoneimine dye which absorbs light at 500 nm. The intensity of colour is directly proportional to the amount of triglyceride in the sample.

7.6 Calculation of LDL cholesterol

Where LDL cholesterol was not measured from the LDL isolated by ultracentrifugation, the Friedwald equation (Friedewald *et al*, 1972) was used to calculate LDL cholesterol as follows:

LDL Chol = Total Chol – HDL Chol – Trigs/5

7.7 Determination of particle size by electron microscopy

Electron microscopy of isolated lipoprotein fractions was kindly performed by Mr Tony Day. Samples of lipoprotein solutions at protein concentrations of 0.1-0.4 mg/mL were applied to carbon coated grids and negatively stained with 1% phosphotungstic acid adjusted to pH 7.0. Samples were then viewed under a **Phillips CM 12** electron microscope. The diameters of 50 random particles were measured manually from electron micrographs.

7.8 Agarose gel electrophoresis

Agarose gel electrophoresis in 1% agarose (pH 8.6) was performed as described by Peach (1991). A glass mould was formed by clamping 2 glass plates (11×21cm) separated by a 1.0mm thick U-frame. To one of the plates was attached a sheet of Gelbond (FMC Bioproducts), with the hydrophobic side next to the glass plate, although this was omitted when electrophoresis was to be taken into the second dimension via SDS PAGE. A 1% agarose (LSA/LSM 50:50;Litex) in electrophoresis buffer (38mmol/L Tris/HCl (pH8.6), 46mmol/L sodium barbitone, 16mmol/L diethylbarbituric acid) was boiled to dissolve the agarose and cooled to 60°C before pouring into the glass mould. After cooling, the mould was dismantled and a 2cm strip of the gel blotted with Whatman 1MM paper. A plastic strip with slotted wells was then placed on the blotted strip and samples loaded (10μL maximum loading). A sample of human plasma (3μL) containing bromophenol blue was always loaded to determine the length of electrophoresis. After samples had diffused into the gel the well

strip was peeled off and the gel positioned on a cooling plate. Lint wicks were used to bring the gel in contact with the tank buffer and the gel electrophoresed at 250V for 50 minutes. Gels to be stained for protein were fixed in a 4:1 (v/v) saturated solution of picric acid:glacial acetic acid for 10 minutes then pressed under Whatman 3MM paper and paper towels for 10 minutes. Gels were stained with Coomassie blue then destained as in section 3.10. Alternatively gels were pressure blotted for immunoblotting (see section 3.7) or the lanes cut out for 2-dimensional electrophoresis (see appendix 7.9).

7.9 Gels for SDS PAGE

The following stock solutions were used to make gels for SDS PAGE.

Acrylamide: 60% (w/v) acrylamide, 1.6% (w/v) bis-acrylamide was dissolved in water, filtered through glass wool and stored in the dark at room temperature

Running gel buffer: 2M Tris pH 8.8

Stacking gel buffer: 0.5M Tris pH 6.8

Tank buffer: 0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS (pH 8.3)

Polymerisation agents: NNN'N'-tetramethylethylenediamine (TEMED)
10 % (w/v) ammonium persulphate (freshly prepared)

Gradient gels 3-6% and 3-15% were prepared by placing solutions into two identical glass chambers one of which was stirred and had an outlet to the Bio-Rad mini gel glass mould. A 10mL solution of 6% (or 15%) acrylamide was prepared by mixing 1mL (2.5mL) acrylamide stock, 2.0 mL running gel buffer, 50μL 20% (w/v) SDS, 6.9 mL (5.4 mL) water, 0.8g sucrose and 15μL TEMED. At the same time a 10mL solution of 3% acrylamide was prepared by mixing 0.5 mL acrylamide stock, 2.0 mL running gel buffer, 50μL 20% (w/v) SDS, 7.4 mL water and 15μL TEMED. Fifty μL of ammonium persulphate solution was then added to the 6% (15%) solution and the 3% solution and both solutions poured into the glass chambers the 6 (15%) solution being added to the stirred chamber. The gradient was immediately poured

into the glass mould by way of a peristaltic pump connected to the stirred chamber. Water was placed over the top of the gradient gel to ensue a flat surface on polymerisation.

After polymerisation of the running gel (30 minutes) a 5mL stacking gel was prepared by mixing 0.25mL acrylamide stock, 1.25mL stacking gel buffer, 25µL 20% (w/v) SDS, 3.4 mL water, 10µL TEMED and 30µL ammonium persulphate. This was immediately poured on top of the glass mould and a plastic comb was then applied to form 10 wells. After polymerisation of the stacking gel (20 minutes) the gel mould was assembled into the Bio-Rad tank system and the upper and lower chambers filled with tank buffer. When SDS PAGE was performed in the second dimension after 1% agarose electrophoresis, the stacking gel was poured approximately one third of the usual height and the agarose gel strip placed on top of this. More stacking gel was then placed around the sides and top of the strip.

7.10 ^{32}P end-labelling of oligonucleotide primers

Primers were end-labelled with [γ-^{32}P]-ATP (New England Nuclear) using the T4 polynucleotide kinase enzyme (Gibco/BRL) and the method of Sambrook *et al* (1989). One µL of 10µM primer was placed in a 0.5mL eppendorf tube along with 1µL of kinase buffer (50 mM Tris/HCl (pH 7.5), 10mM $MgCl_2$, 5mM dithiothreitol), 3µL of [γ-P32]-ATP (2.5 µCi), 4µL of H_2O and 1µL of T4 polynucleotide kinase and the reaction placed at 37°C for 30 minutes. The reaction was diluted to 100µL with 90µl of water and labelled primer then separated from unincorporated [γ-P32]-ATP in a Sephadex G-50 spin column (appendix 7.11). The labelled primer was collected in a 0.5mL eppendorf placed inside a 10mL tube. An estimate of labelling efficiency was gained by measuring the number of counts in both the labelled primer in the eluant and the unincorporated [γ-P32]-ATP left in the column with a giegercounter. Labelled primer was vacuum dried in a speed-vac and reconstituted in 10µL of water to give a 1µM solution of labelled primer.

7.11 Sephadex G50 spin columns

Sephadex G50 spin columns were constructed according to Sambrook *et al* (1989) and used to purify labelled primers and PCR products. A one mL syringe was plugged with siliconised glass wool and filled with Sephadex G50 (Pharmacia) and placed inside a 10mL plastic tube. The column was washed with two 200μL washes of distilled water by centrifugation at 2000 rpm for 5 minutes and the water eluant discarded. Labelled primer or PCR product (100μL) was added to the column and the column centrifuged as before with the eluant collected.

7.12 Transformation procedure

Competent cells (E coli DH5α, Gibco BRL) were taken from the -80°C freezer and thawed on ice. Fifty μL of cells were used for each transformation. Cells were placed into prechilled Falcon 2059 tubes and ligation reaction (approximately 40ng DNA) added. pUC19 DNA (1 ng) was used as a control transformation. DNA was pipetted gently into the cells and the tubes mixed by gentle tapping. Tubes were incubated on ice for 30 minutes before receiving a heat shock for 20 seconds at 37°C. LB (0.95mL) was added to the transformation and cells were allowed to recover by incubation at 37°C in a shaking incubator for 1 hour.

7.13 Media used for bacterial culture

The following medium and agar plates were used to maintain growth of bacteria used in this study:

LB medium (per litre):

10g Bacto/tryptone

5g Bacto/yeast/extract

5g NaCl

Autoclave and cool before adding the appropriate antibiotic if required.

LB/agar plates containing antibiotic (per litre):

15g of agar (Davis-Germantown) was added to 1 litre of LB medium and autoclaved. After cooling to 55°C ampicillin (125μg/mL) was added and the medium poured into 85mm Nunclon petri dishes (30-35mL per dish). The agar was left to harden and the plates then stored at 4°C for up to one month until required.

7.14 Alkaline lysis mini-prep

Plasmid DNA was prepared from overnight cultures using the alkaline lysis mini-prep of Sambrook *et al* (1989). Culture was placed into an 1.5 mL eppendorf tube and cells pelleted from the culture by centrifugation in a microfuge for 30 seconds. Media was removed by aspiration and the cells resuspended in 100μL of lysis buffer (25mM Tris/HCl (pH 8.0), 10 mM EDTA, 50mM glucose) and left for five minutes at room temperature. Two hundred μL of freshly prepared 0.2 M NaOH/1% SDS (w/v) was added and the tube mixed by inversion and stored on ice for five minutes. Ice cold potassium acetate solution (150μL of 5M) was then added and the tube mixed thoroughly by inversion and stored on ice for at least five minutes. Cell debris was pelleted by centrifugation in a microfuge at 4°C for 5 minutes and the supernatant transferred to a fresh tube. An equal volume of 1:1 Tris-saturated phenol/chloroform:isoamyl alcohol (24:1 v/v) was added to the supernatant, vortexed for 1 minute and centrifuged for 2 minutes in a microfuge. The supernatant was transferred to a fresh tube and DNA precipitated with 2 volumes of ethanol on ice for at least two minutes. DNA was pelleted by centrifugation for 5 minutes at 4°C and the ethanol removed by aspirated. The DNA pellet was washed with 1mL of 70% ethanol by vortexing and the DNA repelleted by centrifugation and left to air dry. The pellet was then redissolved in 50μL of TE and 20μg/mL RNAase A (Boehringer Mannheim) added and incubated at 37°C for 15 minutes to digest RNA.

7.15 Large scale alkaline lysis

Plasmid DNA was prepared from overnight cells using the method of Sambrook *et al* (1989). A single colony was inoculated into 200mL of LB/Amp media and the culture grown overnight in a 37°C shaking incubator. The culture was transferred to a 200mL Sorval centrifuge bottle and cells pelleted by centrifugation at 4000 rpm for 10 minutes at 4°C. The cell pellet was resuspended in 10mL of lysis buffer (appendix 7.14) and incubated for 5 minutes at room temperature. Twenty mL of 0.2M NaOH/1% SDS was then added and mixed by swirling followed by 10mL of ice cold 5M potassium acetate which was mixed thoroughly and left on ice for 5-10 minutes. The lysed mixture was transferred to 50mL Sorval tubes and the cell debris was pelleted by centrifugation at 10 000 rpm for 20 minutes at 4°C. The supernatant was strained through sterile gauze into fresh tubes and an equal volume of isopropanol added and the tubes left on ice to precipitate the DNA. Precipitated DNA was pelleted by centrifugation at 10 000 rpm for 10 minutes at 4°C. After the pellet had been resuspended in 2mL of TE an equal volume of 5M LiCl was added and the tubes left on ice for at least 10 minutes to precipitate RNA. Precipitated RNA was pelleted by centrifugation at 10 000 rpm for 10 minutes at 4°C and a further isopropanol precipitation step performed as before. The DNA pellet was resuspended in 0.8mL of TE and transferred to a 1.5 mL eppendorf tube where 4µL of 10mg/mL RNAase A was added and the tube incubated for 15 minutes at 37°C. DNA was then precipitated with 0.5mL of 20% PEG/2.5M NaCl on ice for ten minutes and pelleted by a 5 minute spin in a microfuge. The PEG supernatant was removed and the pellet respun in the microfuge and all the remaining PEG removed. A phenol/chloroform extraction was performed and the DNA precipitated with 30µL of 3M sodium acetate (pH 7.4) and 1mL of ethanol for 30 minutes at -20°C. DNA to be used for transfection was given two extra phenol/chloroform and an extra chloroform extraction before precipitation. DNA was pelleted by a 5 minute spin in a microfuge, washed in 70 % ethanol and dried under vacuum for 10 minutes. DNA was resuspended in 0.5mL of H_2O.

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