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SEQUENCE VARIATION IN THE LDL RECEPTOR GENE, AND ITS EFFECT ON PLASMA LIPID LEVELS.

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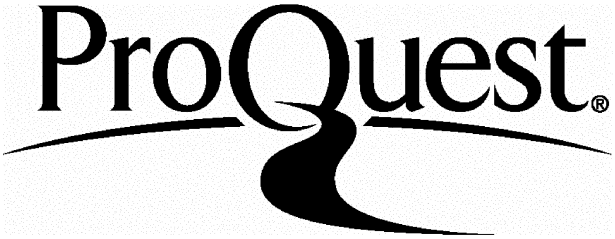

"...því að það er mælt, að
fjórðungi bregði til fósturs."

"...the saying goes that one-
fourth comes from the nurture."

Njáls Saga, 12th century

ABSTRACT:

Two hundred patients with familial hypercholesterolaemia (FH) were examined for known mutations in the 3′part of exon 4 of the LDL receptor gene by electrophoresis of a DdeI digested PCR amplified fragment. Fifteen individuals (7.5%) were identified with 4 different mutations (of which 2 were novel) in this region of the gene. DNA from 311 patients with FH were then subjected to analysis of the 3′part of exon 4 by single strand conformation polymorphism which had been specifically adapted for sensitive detection of sequence variation in this region of the LDL receptor gene. A total of 29 patients or 9.3% (including the previous 15 patients) were identified, with a total of 6 mutations (additional two novel ones) in a region of 50 bases in the 3′part of exon 4. To examine the impact of the mutations on plasma cholesterol concentration, patients were grouped according to the localisation and nature of the mutations they carried. Four groups were compared, those with a defective protein and a mutation in repeat 5 of the ligand binding domain, those with a defective protein due to mutations elsewhere in the gene, those with a null mutation producing no protein at all and those with no detected mutation. Those individuals with null mutations had the highest plasma cholesterol concentration (11.31 mmol/l) which was similar to that in patients with any mutation in repeat 5 producing defective protein (11.23 mmol/l). These levels were both statistically significantly higher than in patients with mutations elsewhere in the gene producing a defective protein (9.64 mmol/l), or in the whole group (9.37 mmol/l). This suggests that the nature of the mutation in the LDL receptor gene is to some extent determining the phenotype of patients with FH.

In addition to mutations in the LDL receptor gene that cause FH there may be mutations affecting plasma cholesterol concentration without causing FH; one such mutation is the common

amino acid change A370T. The effect of this amino acid change on the uptake and degradation of LDL was investigated by analysis in CHO cells transfected with the human LDL receptor cDNA with either the T370 or the A370 allele. There was no detectable difference in receptor activity between the two alleles within the limits of the sensitivity of these assays. The effect of this polymorphism on plasma cholesterol concentration was also examined in three samples. In an Icelandic sample of 318 men and women the T370 allele was associated with 8.9% higher mean plasma cholesterol concentration in the men which was statistically significant. In contrast, women carrying the T370 allele, had 6% lower mean plasma cholesterol concentration. In a sample of offspring of men that had had a myocardial infarction before 55 years of age, the same trend was seen. Female offspring carrying the T370 allele had 8.8% lower, and male offspring carrying the T370 allele had 5.6% higher mean plasma cholesterol concentration, though this difference only reached statistical significance in the female. In addition, the frequency of the rare T370 allele was statistically significantly higher in the offspring of cases than in the controls, 0.06 vs 0.042 respectively ($p<0.05$). In the third sample examined, which consisted of 706 British healthy men that were followed for 4 years, the T370 allele was associated with 7.7% higher mean plasma cholesterol concentration at baseline. When only those for whom data were available at all visits were examined, those with the T370 allele had 2.8% lower mean plasma cholesterol concentration. Those individuals carrying the T370 allele who were lost on follow up had 0.5 mmol/l higher mean plasma cholesterol concentration than those with an A370 allele only. These results suggest that the A370T polymorphism may have a small impact on the development of hypercholesterolaemia and the risk of coronary artery disease, but the mechanisms of these effect is yet to be determined.

ACKNOWLEDGEMENTS:

I would like to thank my supervisor, Professor Steve Humphries for his continuing encouragement and support both during the time I was carrying out the work described in this thesis and during the writing up. The fruitful discussions we had and his useful suggestions are very much appreciated.

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Thanks to Dr. Rachel Fisher, Dr. Sandra O'Dell and Ms Manjeet Bolla for critical proofreading of the manuscript. I would like to thank: Mrs. Ros Whittall for help with running some of the SSCP gels in the search for more mutations. Sister Sue McCarthy for her help with the collection of the data on the FH patients and her commitment to the studies we have been carrying out. Dr. Mary Seed, Dr. John Betteridge and Dr. Gilbert R. Thompson for access to their patients. I would like to thank Dr. Viviane Nicaud and Dr. George Miller for carrying out the statistical analysis in the EARS and the NPHSII groups, respectively.

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ABBREVIATIONS:

apoB	apolipoprotein B
apoE	apolipoprotein E
apoAI	apolipoprotein AI
apoAII	apolipoprotein AII
apoC	apolipoprotein C
ASO	allele specific oligonucleotide
CCM	chemical cleavage of mismatch
CAD	coronary artery disease
CHD	coronary heart disease
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
ds	double stranded DNA
DTT	ditheiotreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene-bis (oxy-ethylenenitriol) tetraacetic acid
FCR	fractional catabolic rate
FCS	foetal calf serum
FH	familial hypercholesterolaemia
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
LMP	low melting point

LPDS	lipoprotein deficient serum
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
ss	single stranded DNA
SSCP	single strand conformation polymorphism
Taq polymerase	*Thermus Aquaticus* DNA polymerase
UV	ultraviolet
VLDL	very low density lipoprotein

The following abbreviations for the nucleotide bases of DNA are used:

A	adenine
C	cytosine
G	guanine
T	thymine

The following three letter abbreviations and one letter codes are used for the amino acids:

Ala	Alanine	A
Val	Valine	V
Leu	Leucine	L

Ile	Isoleucine	I
Pro	Proline	P
Phe	Phenylalanine	F
Trp	Tryptophan	W
Met	Methionine	M
Gly	Glycine	G
Ser	Serine	S
Thr	Threonine	T
Cys	Cysteine	C
Tyr	Tyrosine	Y
Asn	Aspargine	N
Gln	Glutamine	Q
Asp	Aspartic acid	D
Glu	Glutamic acid	E
Lys	Lysine	K
Arg	Arginine	R
His	Histidine	H

For description of mutations the suggested nominaclature of Beaudet and Tsui (1993) is used. For example an amino acid substitution of an Aspartic acid for Glutamic acid at codon 206 is written D206E.

DECLARATION.

The work carried out for this thesis was in many instances dependant on collaborations with a number of people, who are coauthors of the papers published in relation to this thesis. However I have carried out the bulk of the work my self and the projects were mainly my ideas and planning. I do though want to mention specifically the parts of the work that has been performed by others that is described in the thesis.

The haplotype analysis described in Chapter 3 was mostly carried out by Linda King-Underwood. Some of the SSCP analysis (less than 5%) on the FH patients were carried out by Mrs. Ros Whittall and by Dr. Y.T. Mac, according to the protocols I had developed.

The site directed mutagenesis Chapter 4. was carried out in close collaboration with Dr. Xi-Ming Sun, who performed the latter stages of the work, including the cutting out of the mutated fragment from the pALTER vector and ligating it into the pLDLR4 vector, and analysing the constructs. The transfection of the pLDLR4 in to CHO cells was carried out by Dr. Dilip Patel, who also performed the labelling of the LDL and the antibody to the LDL receptor. The rest of the cell work was carried out by me, either alone or under the expert supervision of Dr. Dilip Patel and Dr. Brian L. Knight.

The statistical analysis of the EARS samples was solely carried out by Dr. Viviane Nicaud at the EARS analysis team in Paris, using a strategy devised by myself. The statistical analysis for the NPHSII study was performed by Dr. George Miller at St Bartholomew's

Hospital in London using a strategy devised by myself.

The measurements of blood chemistry was performed by departments of chemical pathology as described in Chapter 2. A large proportion of the DNA used in the analyses described in this thesis has been isolated by various people going through Professor Steve Humphries laboratory over the last 10 years.

All clinical evaluations were carried out by Dr Mary Seed, Charing Cross Hospital, Dr. John Betteridge, UCH and Dr Gilbert R.Thompson, Hammersmit Hospital. The evaluation of the clinical criteria for the Southampton FH patients was carried out by Dr. Ian N.M. Day, Cardiovascular Genetics, UCLMS.

CHAPTER 1 -INTRODUCTION

1.1. Coronary Heart Disease

Coronary heart disease (CHD) is the leading cause of death in men and women in the developed countries. In the UK it is responsible for nearly 160 000 deaths every year, or approximately quarter of all annual deaths, and for 40% of deaths in men of working age (Durrington 1990). The morbidity from CHD is also very costly for the society both in treatment as well as in lost workdays, and is a major annual burden on the health service's resources. However, most of coronary heart disease is caused by atherosclerosis which, begins at a much earlier age. There are numerous factors contributing to atherosclerosis, and these are of both genetic and environmental origin. Modifying or reducing the environmental risk factors has resulted in a decline in CHD over the last two to three decades in industrialised populations like the UK and USA (Marmot 1988). Modifying the genetic factors is much more difficult than intervention with the environmental factors, but there are some genetic tests that have the potential to identify individuals at risk of developing CHD, and the identification and characterisation of these genetic factors is progressing and advancing the understanding of the atherosclerotic process.

In the introduction I will go through the pathogenesis and aetiology of atherosclerosis and CHD with main emphasis on the role of cholesterol. I will then describe the metabolism of cholesterol and the regulation of plasma cholesterol concentration. Finally, I will describe the role of three genes in the regulation of plasma cholesterol concentration (apolipoprotein B (apoB)-, apolipoprotein E (apoE)- and the low density lipoprotein (LDL) receptor- genes) and discuss their contribution to the plasma cholesterol concentration on a continuous scale; from a small effect to a large impact.

1.2. Atherosclerosis

1.2.1. Pathogenesis

Atherosclerosis is a disease of the arteries, which is seen as patchy lesions that are generally distributed in similar manner in all individuals (Stary 1989). The lesions are a continuum of alterations of the arterial wall with an intracellular and extracellular accumulation of lipids, fibrin deposition and connective tissue formation. The normal arterial wall consists of three layers. The first is the intima, demarcated by the endothelium and the internal elastic lamina. Normally there are no other cell types found in the intima. Next is the media, consisting solely of smooth muscle cells, in either a single layer in the smaller arteries or as multiple lamellae in the larger more elastic arteries. The smooth muscle cells are thought to be responsible for laying down the connective tissue in the arterial wall, such as collagen and elastic fibres, as well as the proteoglycans (Ross 1986). The third outermost layer, the adventitia is a loose areolar connective tissue, containing the arteries and nerves supplying the arterial wall, and divided from the media by the external elastic lamina.

The atherosclerotic process can be divided into three parts; fatty streak formation, fibrous plaque and complicated lesions (Strong and McGill 1969), with further division of each stage according to histological examination (Stary et al 1992, Stary et al 1994). In experimental atherosclerosis, induced by diet, the first event seen is the attachment of monocytes and T lymphocytes to the arterial endothelium, with subsequent subendothelial migration and accumulation of lipids, and followed by their transformation into the macrophage-foam cell (Ross 1986). These

accumulations turn into "fatty streaks" which are thought to be the initial lesions of atherosclerosis, and consist of an infiltration of macrophage foam cells, and lipid laden smooth muscle cells, and scattered extracellular lipid particles in the intima under the endothelium (Ross 1986). These lesions are seen at various parts of the arterial tree and occur in the aorta as early as at 3 years of age (Holman et al 1958), and usually after the age of 15 in the coronary arteries (Stary 1989), and seem to evolve regardless of race and gender (Strong and McGill 1969), but are somewhat dependent on the environment (McGill et al 1968). The integrity of the arterial wall is still maintained and these lesions are described as reversible (McGill 1977, Stary et al 1994). When the lesions start to show evidence of disorganisation of the arterial wall structure it is thought that irreversible changes, with respect to the atherosclerotic process, are taking place (Stary et al 1994). For CHD prevention, this stage is probably a crucial one to identify, to impliment life-style changes (Stary et al 1994). With time, these lesions continue to accumulate extracellular lipid, there is increased accumulation of smooth muscle cells, macrophages and T-lymphocytes and the laying down of collagen, forming a fibrous cap (Ross 1986). This is the advanced lesion of atherosclerosis, called the fibroatheroma, or the atherosclerotic plaque (Ross 1986). With further time this lesion starts to calcify and contains various degrees of necrosis, thrombosis and ulceration, and becomes the so called complicated lesion, giving rise to the clinical symptoms of atherosclerosis, ischaemia and infarction from thrombosis and thromboembolism (Strong and McGill 1969).

It is from these anatomical pathological pictures of the atherosclerotic arteries that the hypotheses of the pathogenesis of the disease have evolved. In the 19th century there were two major hypotheses of the pathogenesis of atherosclerosis cited in Fuster et al (1992). These were the "incrustation" hypothesis of Rokitansky, modified by Duguid, and the "lipid" hypothesis of

Virchow. The former suggested an intimal thickening from fibrin deposition and organization by fibroblasts, with secondary lipid accumulation. The latter suggested that the lipids in the arterial wall were derived from plasma lipid, which then formed complexes with acid mucopolysaccharides. The reason for the lipid accumulation in the arterial walls was suggested to be because the processes leading to the deposition of lipids predominated over those of removal. These two hypothesis form the basis for the more recent "response-to-injury" hypothesis by Ross and Glomset (1973), which in addition and upon update takes into account more experimental evidence (Ross 1986,1993). The essence of this hypothesis is that an injury to the endothelium is the initiating event in atherogenesis. The injury can be of various origins, such as oxidised LDL, homocysteine, immunologic, toxins, viruses and possibly others, in addition to mechanical injury (Ross 1993). The mechanical injury is mainly seen at places of sheer stress, such as at bifurcations of the arteries. This is very much in line with the distribution of atherosclerotic lesions in both humans and animals (Stary 1989, Stary et al 1992, Stary et al 1994). Knowledge on atherosclerosis initiation and progression in humans comes from a number of autopsy studies. These studies show that there are locations in the vascular system known for their predisposition to develop clinical lesions, the so called "progression-prone" or "advanced lesion-prone" regions of arteries. For example, atherosclerosis starts first in the abdominal part of the aorta (Holman et al 1958, Strong and McGill 1969, Stary 1989), and the coronary arteries and the cerebral arteries are only affected later (Stary 1989, Stary et al 1992, Stary et al 1994).

Coronary atherosclerosis is of great importance, as it accounts for most of the deaths from atherosclerosis. CHD, on most occasions is due to a narrowing of the coronary arteries by an atherosclerotic plaque. The atherosclerosis can be distributed widely along the coronary arteries reducing the blood supply to the heart muscle. The clinical symptoms of CHD are both chronic

and acute and result from various determinants in addition to the narrowing of the lumen and the location of the lesion. The acute symptoms, like unstable angina pectoris and myocardial infarction, result most often from a fissuring of a plaque (Fuster et al 1992). The rupture is most frequently at the shoulder of the lesion on the junction between the normal and diseased tissue (Davies 1989). Certain types of the fibro-fatty lesions are more prone to rupture, specifically the lipid rich plaques (Davies and Thomas 1985). These unstable lesions tend to be relatively small (Richardson et al 1989) and not detected by coronary angiography as significant stenosis. It has been speculated that these lesions are dependent on plasma lipid levels and can be stabilised by reducing the plasma cholesterol concentration (Levine et al 1995).

1.2.2. Aetiology

Most information on, and evidence for, the aetiological factors involved in the development of atherosclerosis comes from the long term study of the epidemiology of atherosclerosis, mainly as determined by cardiovascular events, such as acute myocardial infarction, the development of angina pectoris, or sudden death. In the epidemiological studies a number of factors or characteristics have been identified as being statistically associated with the development of CHD (Kannel et al 1971, Martin et al 1986, Meade et al 1986). These factors, habits and traits are known as "risk factors" and include both modifiable and unmodifiable factors, with most having both genetic and non-genetic components.

Table 1.2.1. shows some of the most commonly cited risk factors for the development of atherosclerosis and CHD. The first three risk factors listed in the table; age, male sex and family history of premature CHD are referred to as non reversible. With increasing age atherosclerosis

Table 1.2.1. Some common risk factors for coronary heart disease (CHD)

Age
Male sex
Family history of premature CHD

Hyperlipidaemia:
a) Raised total plasma cholesterol
b) Raised plasma LDL cholesterol
c) Raised plasma triglycerides
Low plasma HDL concentration
Raised plasma concentration of Lp(a)
High intake of cholesterol and/or saturated fat

Cigarette smoking

Hypertension

Hyperinsulinaemia or impaired glucose intolerance
Diabetes mellitus
Abdominal obesity

Raised plasma levels of fibrinogen

Lack of exercise
Type A personality

is seen to some extent in all people, although a large number do not get clinically significant disease. Male sex is a well known non-reversible risk factor, and men get CHD on average 10 years earlier than women. This might be related to sex hormones, as the incidence of CHD rises in women after the menopause. Genetic traits such as positive family history for CHD is in it self non-reversible, though in some cases the intermediate traits associated with the genetic components can be interfered with like cholesterol levels (discussed below), but not in others, such as Lp(a) (Dahlén 1994). A risk factor like hyperlipidaemia is potentially or possibly a treatable risk factor and is a clear combination of genetic and environmental factors. Hypercholesterolaemia is one of the earliest identified risk factors for CHD (Kannel et al 1971). This is the risk factor that is most widely believed to be causal for atherosclerosis and CHD (The

International Task Force for prevention of coronary heart disease 1992). The importance of plasma cholesterol concentration in this respect is discussed below. With more and more drugs available for reduction of plasma cholesterol concentration, prevention of CHD and other complications of atherosclerosis is a reality (Scandinavian Simvastatin Survival Study Group 1994). Other plasma lipid traits like high triglycerides have been associated with the risk of CHD, though not as strongly as that of cholesterol and mainly when accompanied with low HDL cholesterol (Castelli 1986 and 1992). Low plasma concentration of HDL cholesterol has been associated very strongly with the risk of CHD (Castelli 1986). This is, in some studies, reflected in the association of CHD with the plasma concentration of apoAI (Sigurdsson et al 1992a), which is the main protein component of HDL. The effect of diet is clear on plasma lipids (Mann 1993), but both the dietary intake of cholesterol and that of saturated fats have been shown to be independent risk factors for CHD (Stamler 1982).

Cigarette smoking is a well recognised risk factor and is one of the strongest for CHD (Castelli 1984, Kannel et al 1986, Reid et al 1976, Shaper et al 1985). Though reversible as such, irreversible damage may have occurred already upon cessation of smoking.

Another reversible risk factor is hypertension, which definitely has some genetic components (Castelli 1984, Kannel et al 1986, Reid et al 1976, Shaper et al 1985), but in most cases is treatable.

Truncal obesity is still another reversible risk factor, though increased body mass is inevitable for most individuals as they grow older (Donahue et al 1987, Reichley et al 1987). In addition, hyperinsulinaemia has been associated with CHD risk (Welborn and Wearne 1979). This is

thought to reflect insulin resistance, particularly in the muscle tissue and occurs with impaired glucose tolerance and may thus be an antecedent of diabetes mellitus, which is associated with an excess risk of CHD (Pyörälä et al 1987).

Recently, fibrinogen has been identified as an independent risk factor for CHD (Wilhelmsen et al 1984, Meade et al 1986). Plasma fibrinogen levels are very much affected by smoking (Wilhelmsen et al 1984, Meade et al 1987), but there is some evidence for genetic effects on plasma fibrinogen concentration (Humphries et al 1987). Whether the effect of fibrinogen is through the impact on the procoagulant stage and thus the increased risk of thrombosis or whether the effect is on atherosclerosis itself remains to be elucidated. In addition to this, lack of exercise has in some studies been associated with increased risk of CHD (Stamler 1982) as has the competitive, aggressive, type A personality and stress (Haynes et al 1980).

It is though evident from this list of risk factors that most of them are the results of a combination of genetic and environmental factors. It is also important to notice that when more than one risk factor is present, the combined risk is markedly increased, so two or more risk factors multiply rather than simply adding the effect (The International Task Force for prevention of coronary heart disease 1992). This is of particular importance when trying to assess the individual's risk of developing CHD through atherosclerosis. As plasma cholesterol concentration is, in epidemiological studies, one of the strongest predictors of CHD, enormous work has been put into estimating the effects of various components on this trait. There is conclusive evidence that the association between cholesterol concentration and CHD is one of cause and effect (The International Task Force for prevention of coronary heart disease 1992).

1.2.3. The role of Cholesterol in atherosclerosis

One of the first risk factors for CHD to be determined was elevated plasma cholesterol, which more than 50 years ago was identified as being associated with premature CHD in humans (Müller 1939, Tannhauser and Magedantz 1938). The evidence for raised plasma cholesterol causing atherosclerosis comes from three sources:

1) From epidemiological studies,
2) from genetic studies, and
3) from experimental animal studies

On a population scale, elevated plasma cholesterol was identified in the Framingham Heart Study as a major factor associated with CHD (Kannel et al 1971). Soon after, the pooling project (Pooling Project Research Group 1978) confirmed the findings of the Framingham study, and the Israeli Prospective Study (Goldbourt et al 1985) added further to it. Grundy (1986) pooled the data from these three studies and produced a graph showing that the CHD risk relationship with blood cholesterol is essentially curvilinear (see Figure 1.2.1.).

Very similar results were obtained from the MRFIT (Multiple Risk Factor Intervention Trial) (Martin et al 1986, Stamler et al 1986), where 361.662 men aged 35 to 57 years were followed-up after 6 years and the number of deaths caused by CHD and in total were recorded. Since then a number of studies have confirmed these initial results. The relative size of the association is though uncertain (Law et al 1994a) but is believed by some, to be underestimated (Davis et al

1990, Law et al 1994b) because of the regression dilution effect when cholesterol levels are estimated by a single measurement.

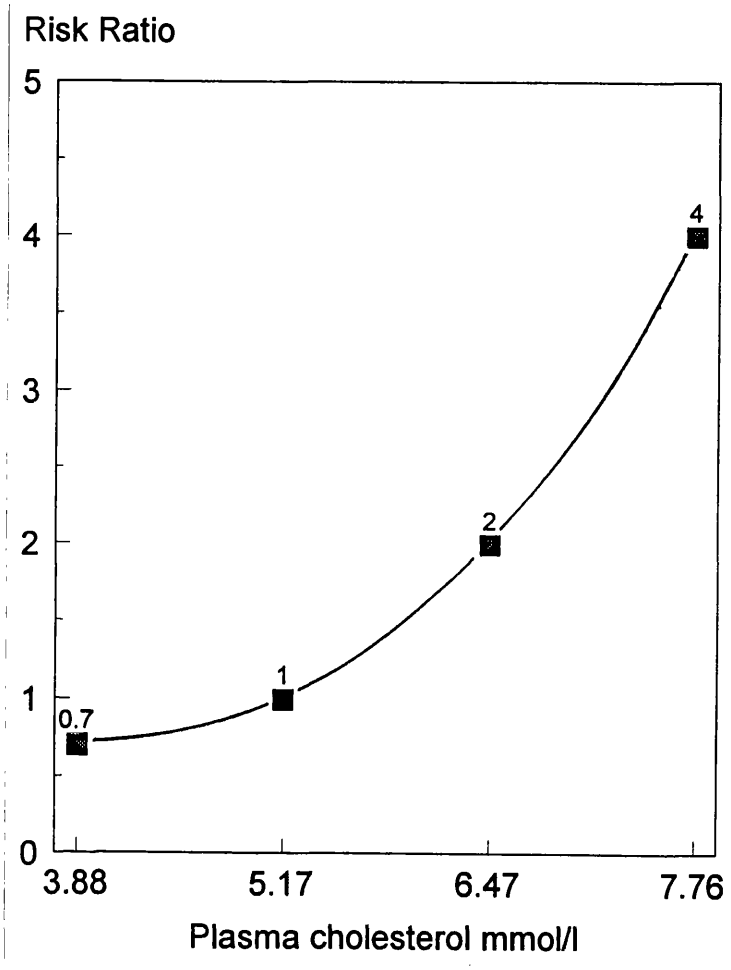

Figure 1.2.1. Coronary mortality expressed as risk ratios (from Grundy 1986). Risk ratio of 1 is set at plasma cholesterol concentration of 5.17 mmol/l.

Proof of the effect of elevated plasma cholesterol concentration on atherosclerosis and CHD comes from sources other than association studies. The best example is familial hypercholesterolaemia (FH) (discussed in detail below). FH, an autosomal dominant disease is found in two forms, as heterozygous individuals who have two to fourfold increase in the concentration of plasma cholesterol and as homozygous individuals, (or compound

heterozygotes), with more than fivefold elevation in cholesterol (Goldstein and Brown 1989). In the homozygote state there is severely accelerated general atherosclerosis with disease developing early in the coronary arteries and myocardial infarction occurring as early as in an eighteen months old individual (Fredrickson and Levy 1972) though most often they suffer MI in the second decade of their lives (Goldstein and Brown 1989). The heterozygotes very frequently have premature CHD and 60% of the affected males have had an MI before the age of 55 years (Goldstein and Brown 1989).

Yet another line of evidence for the importance of blood cholesterol in the development of atherosclerosis comes from animal studies. As early as at the beginning of this century it was shown that by feeding rabbits a high cholesterol diet, they developed atherosclerotic plaques, similar to that seen in humans (Anitsckow 1912, cited in Roberts 1988). Since then a number of studies on animal models of atherosclerosis have been undertaken, further indicating the importance of blood cholesterol in the atherosclerotic process, one of the most well known being the LDL receptor deficient Watanabe heritable hyperlipidaemic rabbit (Watanabe 1980). More recently, both knockout and transgenic mice have been used to demonstrate the role of over or under expression of certain genes on cholesterol metabolism and subsequent atherosclerosis (Reviewed by Maeda 1993), further underlining the importance of blood cholesterol in the initiation and progression of CHD.

There is also considerable evidence for the influence of plasma cholesterol on the progression of the atherosclerotic lesion in humans. Histopathology studies have shown that atherosclerosis is a continuous process from early childhood to adulthood (Holman et al 1958, Strong and McGill 1969, Stary 1989), with an increasing proportion of the arterial surface occupied with

advanced atherosclerotic lesions with rising age (Holman et al 1958, McNamara et al 1971, Strong et al 1984, Stary 1989). The extent and severity of the lesions have been examined in autopsy studies of children and young adults and shown to correlate with the level of plasma cholesterol (Freedman et al 1988, PDAY Research Group 1990 and 1993, Newman et al 1991). The results from these studies have been used to argue the case for a low fat diet in children in the industrialised world since diet is thought to play an important role, both in the initiation as well as the progression of atherosclerosis (Kays 1980). In populations, such as Japan and China the incidence of CHD is low (Kays 1980, Chen et al 1991, Tao et al 1992), and this has been directly linked to the low fat diet that is consumed in these countries (Kays 1980) which results in a low plasma cholesterol concentration in the general population. Even individuals heterozygous for a defect in the LDL receptor gene in China (Sun et al 1994) have a much lower plasma cholesterol concentration than FH patients in the Western countries, though they have higher blood cholesterol than other individuals in their country. This is reflected in a much lower incidence of CHD in Chinese heterozygous FH patients compared with heterozygous FH patients in the industrialised countries, though those with homozygous FH are equally badly affected with atherosclerosis and CHD (Cai et al 1985, Sun et al 1994).

There is considerable evidence for the importance of reduction of plasma cholesterol to prevent CHD, from both primary prevention trials (Lipid Research Clinics Program 1984, Manninen et al 1988) as well as from secondary prevention trials (for meta-analysis see Yusuf et al 1988, Law et al 1994a). In addition, a number of studies have addressed the impact of lowering plasma cholesterol concentration on the regression of the atherosclerotic plaque by using coronary angiography (Brensike et al 1984, Brown et al 1990, Kane et al 1990, Watts et al 1992, Blankenhorn et al 1993, Waters et al 1994). Both the National Institutes of Health (Consensus

conference 1985) and the European Atherosclerosis Society (Study Group EAS 1987, The International Task Force for prevention of coronary heart disease 1992) have concluded that there is sufficient evidence to demonstrate a causal relation between hypercholesterolaemia and CHD and that cholesterol reduction is warranted in patients with hypercholesterolaemia.

1.3. Cholesterol metabolism

1.3.1. The regulation of plasma cholesterol concentration

Plasma cholesterol concentration is determined by the availability of cholesterol from external sources and from the synthesis of cholesterol by the liver. The availability to the cell of cholesterol from external sources is dependent on the individual's intake of fat. Figure 1.3.1. shows the pathway and formation of LDL, which is the principal cholesterol carrying particle in the plasma.

Dietary fat is taken up by the intestinal mucosal cell and secreted into the lymphatic system as chylomicron particles, rich in triglycerides and containing apoB48, apoA apoE, and apoC proteins. Lymph chylomicrons lose apoA but gain apoC and apoE when they enter plasma. Chylomicron triglyceride is lysed by lipoprotein lipase (LPL) to produce free fatty acids and glycerol and a much smaller remnant particle, which is taken up by the liver by the apoE receptor (Redgrave 1970), which probably is the LDL receptor related protein (LRP) (discussed in Soutar 1989). Inside the liver the remnant particle is metabolized and very low density lipoprotein (VLDL) is synthesized. This is secreted into the circulation to distribute cholesterol and triglycerides to the peripheral tissues. These particles are rich in triglycerides and contain apoE

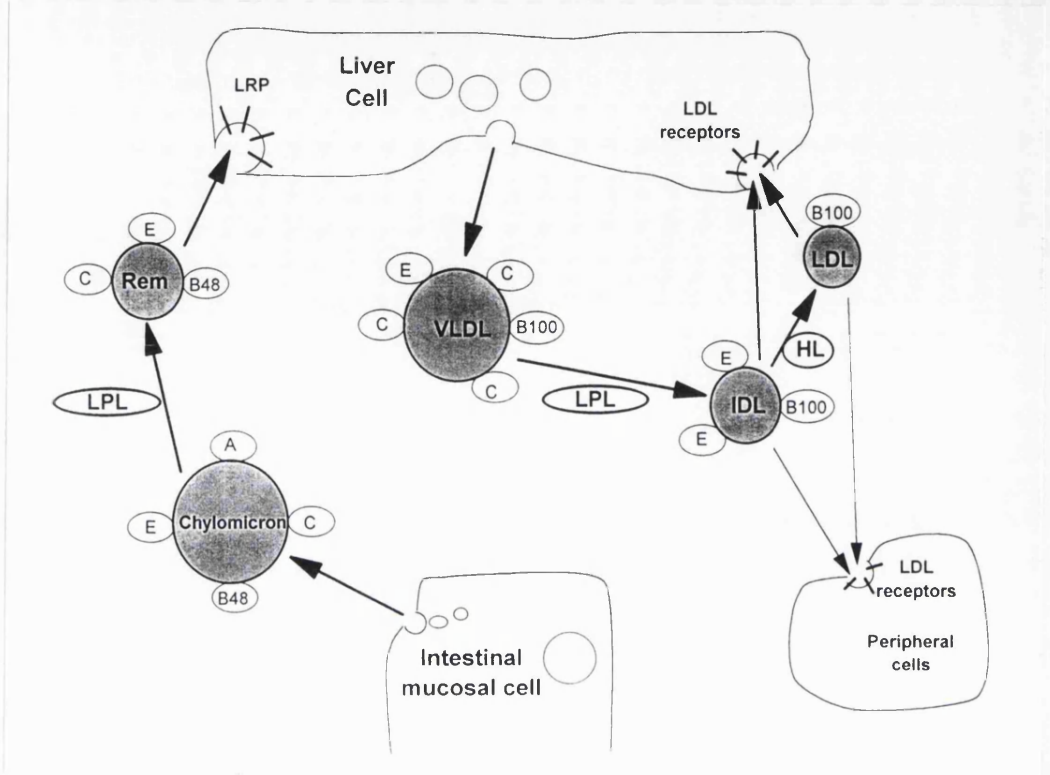

Figure 1.3.1. Schematic drawing of the fate of absorbed cholesterol and the pathway of LDL cholesterol. The encircled letters denote the apolipoproteins. Lipoprotein lipase (LPL) is bound to the vascular endothelium and hepatic lipase (HL) is located on the luminal surface of the hepatic endothelial cells. The chylomicron remnants (Rem) are taken up by the remnant receptor, LRP.

and apoC in addition to apoB100. They are acted on by LPL on endothelial cells in muscle and adipose tissue to release fatty acids. The resultant smaller intermediate density lipoprotein (IDL) particles lose apoC but are apoE rich, whilst always having one molecule of apoB100 per particle. IDL has a very short life span in the circulation, and it binds to the LDL receptor via apoE and is taken up by the cells containing those receptors, mainly liver cells. The intracellular fate of IDL-cholesterol can be storage, recycling (into VLDL) or destruction to bile acids; IDL-proteins are catabolized. Those particles not taken up by the liver remain in the circulation where hepatic lipase removes residual triglycerides, the apoE dissociates from it, and it becomes LDL.

The lifespan of LDL in the circulation is on average two and a half days, with the majority being taken up by the LDL receptor (Bilheimer et al 1978); this half-life is longer in FH patients where the LDL receptor does not function properly (described in detail below). Changes in LDL receptor functional activity will thus raise plasma LDL cholesterol level, and this is the reason for the hypercholesterolaemia in patients with FH. The pathological consequence of raised LDL is increased deposition (particularly of modified LDL particles) in the arterial wall.

Alterations in many parts of the lipoprotein pathway can act to raise plasma cholesterol and thus cause an acceleration of atherosclerosis and coronary heart disease. An example of a defect at a different site in the pathway is familial defective apoB100 (FDB) (described in more detail below) (Innerarity et al 1987; Soria et al 1989; Tybjærg-Hansen et al 1990) where a mutation in the apoB gene produces a protein that does not bind to the LDL receptor and thus is not metabolized through that pathway, and in many patients causes raised levels of plasma cholesterol and often a clinical picture indistinguishable from FH.

The LDL receptor is a membrane protein of 839 amino acids which is responsible for cholesterol uptake into cells via receptor-mediated endocytosis of cholesterol-rich lipoproteins secreted by the liver (Goldstein and Brown 1979). The LDL receptor binds two different ligands: apoB and apoE (Goldstein et al 1985; Mahley and Innerarity 1983). ApoB is the sole protein in LDL whereas apoE is a component of several lipoproteins and lipoprotein remnants, including β-migrating very low density lipoproteins (β-VLDL), where it is found in multiple copies (Innerarity and Mahley 1978; Mahley and Innerarity 1983). It has been demonstrated that lipoproteins containing multiple copies of apoE bind to the LDL receptor with up to 20-fold higher affinity than LDL, which contains only one copy of apoB (Innerarity and Mahley 1978).

Once the LDL receptor has bound a ligand it clusters in coated pits, where it is taken up by the cell, via endocytosis (Goldstein et al 1979). The ligand is released from the receptor in the lysosome and the receptor is then recycled to the cell surface where it can bind a ligand again (Goldstein et al 1985). The unesterified cholesterol derived from the hydrolysis of LDL cholesteryl esters in the lysosomes, mediates a sophisticated system of feedback control, which maintains homeostasis of intracellular cholesterol concentrations (Goldstein and Brown 1977). The cholesterol mediates its effects at three places: first, it suppresses the activity of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), which is the rate controlling enzyme in cholesterol biosynthesis in the cell (Brown et al 1974). Secondly, it activates a cholesterol esterifying enzyme, acyl CoA:cholesterol acyltransferase (ACAT), allowing excess cholesterol to be stored as cholesteryl esters, in storage droplets (Goldstein et al 1974). Last, and most significant, the cholesterol turns off the synthesis of the LDL receptor, which prevents further entry of cholesterol into the cell, and therefore protects the cell against an over-accumulation of cholesterol (Brown and Goldstein 1975). When LDL is available, cells preferentially use the receptor to take up LDL and suppress their own cholesterol synthesis, which they can turn on when external sources are not available (Brown and Goldstein 1975; Goldstein and Brown 1977).

In addition to the regulation of the cholesterol homeostasis by the supply of cholesterol to the cell described above, the liver cell has a mechanism for the removal of cholesterol. The major mechanism for the removal of cholesterol is via the biliary excretory pathway, either as free cholesterol or after conversion to bile acids (Turley and Dietschy 1981). There are a number of enzymes involved in the biosynthesis of bile acid, and the enzyme cholesterol 7α-hydroxylase catalyses the rate limiting step (reviewed in Russell and Setchell 1992). In humans only 30 - 40%

of the cholesterol is converted to bile acids (Quintao et al 1971, Grundy et al 1971). As a consequence, relatively large quantities of cholesterol are secreted into bile in humans (Grundy et al 1972, Mok et al 1979, Mok et al 1980). This means that the enterohepatic circulation is enriched with cholesterol, suppressing LDL receptor synthesis (Goldstein and Brown 1977, Dawson et al 1988).

It is evident from this brief review of cholesterol metabolism that the regulation of these pathways potentially have a large genetic component. Genetic control is possible at practically every stage, where there are one or more proteins participating in the pathways. A large number of studies have focused on the quantitatively measurable plasma proteins involved in the transport and clearance of cholesterol from the circulation, such as the apolipoproteins like apoB and apoE. Other studies have focused on the proteins involved in the supply of cholesterol to the cell, such as the LDL receptor (reviewed in Hobbs et al 1990). These studies have provided considerable insight into the components, regulation and genetics of cholesterol supply, and will be reviewed in more detail.

1.4. Apolipoprotein B

The human apolipoprotein B gene is 43 kb long (Blackhart et al 1986) and is localised on the short arm of chromosome 2 (Barni et al 1986, Deeb et al 1986, Huang et al 1985, Knott et al 1985, Law et al 1985). It consists of 29 exons and 28 introns and codes for a mature protein of 4536 amino acids (Cladaras et al 1986, Knott et al 1986, Law et al 1986, Yang et al 1986) and 550.000 daltons, called apoB-100. This makes the apoB protein one of the largest proteins in the human body and the largest of the apolipoproteins. ApoB is the sole protein component of

LDL, but is also found in VLDL and Lp(a), all implicated in the development and progression of CHD (Goldstein and Brown 1989, Dahlén et al 1972). A number of genetic DNA and protein polymorphisms have been described for apoB-100 (Allison and Blumberg 1961, Humphries and Talmud 1995) and a number of studies have found an allelic association of some of these polymorphisms with plasma apoB and LDL cholesterol concentration (Berg et al 1976, Berg 1986, Talmud et al 1987) as well as with the development of CHD (Hegele et al 1986, Rajput-Williams et al 1988, Myant et al 1989). Although raised levels of apoB and LDL cholesterol are very commonly observed in the survivors of MI the apoB gene has not been identified as "the gene" responsible for these raised levels. A major effect of a mutation in the apoB gene itself on the function of the apoB protein has only been observed in the case of familial defective apoB-100 (FDB), which was initially identified in a subset of moderately hypercholesterolaemic individuals who had a decreased fractional catabolic rate (FCR) of autologous LDL but normal FCR when LDL from others were used (Vega and Grundy 1986). Innerarity et al (1987) showed that LDL from one of these individuals only bound to LDL receptors with 30% of the binding activity of normal LDL. Soria et al (1988) then showed that in this disorder a single base substitution in exon 26 of a nucleotide in position 10708 occurs, that changes the codon CGG to CAG, resulting in the substitution of an arginine at residue 3500 to a glutamine.

Having severely reduced binding to the LDL receptor, this disorder could theoretically have the clinical phenotype of FH. The moderately hypercholesterolaemic individuals in which this disorder was initially identified did not have a family history of CHD (Vega and Grundy 1986), but it was shown that those individuals in the families that had inherited the gene coding for 3500Q had raised plasma cholesterol concentration (Soria et al 1988). When patients from London with the diagnosis of FH were screened for this mutation about 3% of them turned out

to be carriers (Tybjærg-Hansen et al 1990). This was subsequently confirmed in other studies of patients with the clinical diagnosis of FH (Schuster et al 1990a). One study observed tendon xanthomata in 26% of 54 FDB patients identified and premature CHD in 22% (Rauh et al 1992), which is similar to that observed in FH (Goldstein and Brown 1989). It is clear that FDB can cause hypercholesterolaemia with tendon xanthomata and premature CHD and thus can be indistinguishable from FH (reviewed in Tybjærg-Hansen and Humphries 1992), but it is equally clear that a number of FDB individuals have much milder phenotype. In particular, the identified homozygotes for FDB are much less severely affected than FH homozygotes (Myant 1993). This is because a normally functioning LDL receptor can clear apoE containing particles like VLDL and IDL.

There are number of estimates of the frequency of the FDB 3500Q in various populations (Tybjaerg- Hansen et al 1990, Schuster et al 1991) derived from the frequency observed in FH patients. The estimates are between 1 in 600 to 1 in 1000 (Tybjærg-Hansen and Humphries 1992), and whether or not this is accurate, the FDB 3500Q mutation is the most frequent single mutation disorder causing hypercholesterolaemia, known today. In addition to FDB 3500Q, another mutation R3531C has been described, which has reduced binding activity to the LDL receptor (Pullinger et al 1993). It is not inconceivable that other mutations in the apoB gene with an effect on the function of the apoB protein, leading to raised plasma cholesterol will be identified.

1.5. Apolipoprotein E

The human apoE gene is 3.7 kb long and is localized on chromosome 19q13 in a cluster with

apoCII and apoCI (Das et al 1985, Paik et al 1985). The gene has four exons and three introns and it codes for an apoE polypeptide of 299 amino acids in the mature protein (Rall et al 1982a). There are well established genetic polymorphisms in the apoE gene with three common alleles found in all populations. These are the E2, E3 and the E4 alleles, coding for the three isoforms of E2, E3 and E4 respectively. Table 1.5.1. shows the frequency of the different alleles in some populations. The frequency for the E4 allele varies from 0.065 in Japan to 0.227 in Finland. However, the difference between the western countries is much smaller.

Table 1.5.1. Frequency of the common apoE alleles in various populations.

Country	N	E2	E3	E4	Reference
Finland	615	0.041	0.733	0.227	Ehnholm et al 1986
Iceland	316	0.052	0.752	0.196	Gudnason et al 1993b
Scotland	400	0.083	0.770	0.148	Cumming & Robertson 1984
Holland	2018	0.082	0.750	0.167	Smit et al 1988
Germany	1557	0.082	0.782	0.136	Assmann et al 1984
Canada	102	0.078	0.770	0.152	Sing & Davignon 1985
Japan	100	0.090	0.845	0.065	Yamamura et al 1984

The isoforms show differences in cysteine and arginine amino acid residues at two sites, residues 112 and 158 (Weisgraber et al 1981). The most common isoform is E3, which has a cysteine at residue 112 and an arginine at residue 158. The E4 isoform has an arginine at both sites and the E2 has cysteine at both sites. These differences are reflected in a charge difference detectable by iso electric focusing by which the polymorphism was first described, and by a different affinity for binding to the LDL receptor (and the remnant receptor), with the E2 isoform having only 1-2% of the binding activity compared to the E3 isoform (Schneider et al 1981). The effect of the

different alleles on plasma cholesterol concentrations in the general population is well established (Davignon et al 1988), and those with an E2 allele have lower plasma cholesterol concentration, and those with an E4 allele higher plasma cholesterol concentration than individuals with only E3 alleles (Dallongeville et al 1992). This can be explained by the experimental observations for the different alleles. Having an E2 phenotype results in lipoproteins with lower affinity and binding to the apoE remnant receptor and thus less uptake of the chylomicron remnants into the cells, the intracellular concentration of cholesterol leads to enhanced expression of LDL receptors on the cell surface of liver cells enhancing the clearance of LDL from the circulation (Weintraub et al 1987). In addition, there is some *in vitro* evidence for apoE to be necessary for the efficient conversion of IDL to LDL, with apoE3 functioning better than apoE2 in this regard (Enholm et al 1984). If this holds *in vivo* then individuals with the E2 allele may be slower to form LDL from IDL, leading to lower levels of LDL in plasma. This possibility is supported by the fact that individuals lacking lipoprotein lipase have extremely low levels of LDL, due to the lack of ability to convert VLDL to LDL (Nikkilä 1983). This effect is so pronounced that in an individual with both a mutation in the LDL receptor gene (that normally causes FH) and mutations causing LPL deficiency, the LDL levels are very low (Zambon et al 1993) further supporting the possible role of E2 in the conversion of IDL as a participating mechanism for lower plasma LDL concentration in individuals with E2 alleles.

ApoE4 has been shown to be associated with an increased clearance of remnant particles (Weintraub et al 1987). In the case of the E4 isoform there is no difference in the receptor binding activity compared with apoE3 isoform (Weisgraber et al 1982). However, as apoE4 does not contain free cysteine it is unable to form complexes with apoAII as do the other apoE isoforms (Weisgraber et al 1981). As these complexes block the binding of apoE containing

lipoproteins to the LDL receptor (Innerarity et al 1978), it is likely that when they are unable to form there is an enhanced uptake of remnant particles due to increased availability, with increased concentration of intracellular cholesterol with the consequent down-regulation of the LDL receptor (Goldstein and Brown 1989).

This effect on plasma cholesterol concentration is reflected in the risk of atherosclerosis and CHD. In the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study, involving a study of males between 15 and 34 years of age who died of external causes (accidents, homicides or suicides), a clear effect of the apoE genotypes on the involvement of the aorta with atherosclerosis was shown (Hixson 1991). Those who were E2/E3 heterozygotes had the least involvement by atherosclerotic lesions of both the thoracic and the abdominal aorta, with a tendency of the E4 allele to be associated with increased lesions (Hixson 1991). This is also reflected in some studies of individuals suffering from MI where there was an overrepresentation of the E4 allele (Cumming and Robertson 1984, Luc et al 1994). In studies of both octogenarians (Davignon et al 1987) and centenarians (Schachter et al 1994) there was an overrepresentation of E3/E2 heterozygotes, indicating a protective effect of that genotype.

The E2 allele has also been implicated in type III dyslipaemia, with severely raised cholesterol, but mainly triglycerides in a subfraction of the individuals homozygous for the E2 allele (Utermann et al 1977, Utermann et al 1979), and with substantially increased risk of CHD (Morganroth et al 1975). This disorder is though clearly dependant on other genetic or environmental factors for full expression (Utermann et al 1979). Similar effects have also been seen for variations in the apoE gene causing abolished binding of apoE to its receptors (Rall et al 1982b, Rall et al 1983, Wardell et al 1987) and at least one variation has been shown to cause

a dominant form of type III hyperlipidaemia in carriers (Havekes et al 1986) with increased risk of CHD.

Studies of the role and importance of apoE in lipid metabolism and atherosclerosis have gained important information from experiments carried out in gene knockout experiments in mice. Those mice, when fed a cholesterol rich diet have extremely high cholesterol concentrations and severely accelerated atherosclerosis (Piedrahita et al 1992, Plump et al 1992, Zhang et al 1992). In these studies, the essential role of apoE in the clearance of plasma cholesterol and thus in the prevention of atherosclerosis has been demonstrated clearly (Plump et al 1992, Piedrahita et al 1992, Zhang et al 1992), adding not only to the knowledge of the role and importance of apoE in atherosclerosis, but supporting the mechanisms as to how the effect of apoE on atherosclerosis in defective apoE in humans is mediated.

From all this it is evident that apoE has an effect on plasma lipid traits and on atherosclerosis, exerting both large and small effects on a continuous scale.

1.6. The LDL receptor and familial hypercholesterolaemia

1.6.1. Familial Hypercholesterolaemia (FH)

Familial hypercholesterolaemia is characterised clinically by the elevation of LDL cholesterol in plasma with deposition of LDL derived cholesterol in tendons and skin as well as in arteries, causing premature CHD and by the autosomal dominant inheritance with a gene dosage effect on the trait (Goldstein and Brown 1989).

Historically the association between tendon xanthomata and atheromas was described as early as in the eighteenth century (see references in Goldstein and Brown 1989). It was though not until the 1930's that Müller (1939) and Tannhauser et al (1938) recognised the clustering of xanthomata, hypercholesterolaemia and premature CHD in families. Over the next two decades their suggestion of a genetic basis for hypercholesterolaemia in these patients were substantiated by a number of studies (see references in Goldstein and Brown 1989). The first unequivocal evidence for a single gene inheritance of this disorders came from studies by Khachadurian (1964) in Lebanon where he demonstrated the difference between heterozygotes and homozygotes. Studies in the 1950s showed a selective elevation of LDL in FH (see references in Goldstein and Brown 1989). The studies of Brown and Goldstein in the 1970s and 1980s then disclosed the existence of the cell surface LDL receptor and demonstrated that mutatations in the LDL receptor gene caused FH (Goldstein and Brown 1989).

The frequency of FH in most populations, calculated from the number of homozygotes is 1:500 (Goldstein and Brown 1989). In certain populations such as in Lebanon (Slack 1979) and in South Africa (Jenkins et al 1980) the frequency was calculated much higher, or about 1 in 170 and 1 in 100 respectively for heterozygotes. Heterozygotes for FH have two to three-fold elevation of plasma cholesterol concentration whereas homozygotes have severe hypercholesterolaemia of five times that in normal individuals (Goldstein and Brown 1989). This high plasma cholesterol concentration in homozygous FH is reflected in early xanthomatosis and CHD, which has been described as a fatal MI in as young as an eighteen months old individual (fredrickson and Levy 1972, Goldstein and Brown 1989). The progression of atherosclerosis and onset of CHD varies and is thought to depend on the residual receptor activity in the homozygotes (Sprecher et al 1985).

1.6.2. The LDL receptor

1.6.2.1. Functional classes of LDL receptor defects

Five classes of mutations at the LDL receptor locus have been identified on the basis of the phenotypic behaviour of the mutant protein (Goldstein and Brown 1989). Class 1 mutations fail to produce any immunoprecipitable protein (null alleles). Class 2 mutations encode proteins that do not fold properly after synthesis and are blocked, either partially or completely, in transport between the endoplasmic reticulum and the Golgi complex (transport defective alleles). Class 3 mutations encode proteins that are synthesized and transported to the cell surface, but fail to bind LDL normally (binding defective alleles). Class 4 mutations encode proteins that move to the cell surface and bind LDL normally, but are unable to cluster in clathrin-coated pits and thus do not internalize LDL (internalization-defective alleles). Class 5 mutations encode receptors that bind and internalize ligand in the coated pits, but fail to discharge the ligand in the endosome and fail to recycle to the cell surface (recycling-defective alleles). From these classes it can be seen that mutations can lead to nonfunctional proteins, partially functional protein or possibly even proteins that can hinder the function of the normal receptor in a patient heterozygous for a receptor defect. Such variation in the mutant receptor could thus be responsible for differences in plasma lipid levels and therefore contribute to the variability of the clinical phenotype of different patients. This variation in mutant cell phenotypes also predicts that there would be a number of different mutations at the gene level.

1.6.2.2. Structure of the LDL receptor gene and the protein

The human LDL receptor gene was cloned in 1982 (Russell et al 1983; Yamamoto et al 1984) and characterized shortly thereafter (Südhof et al 1985) (Figure 1.6.1.).

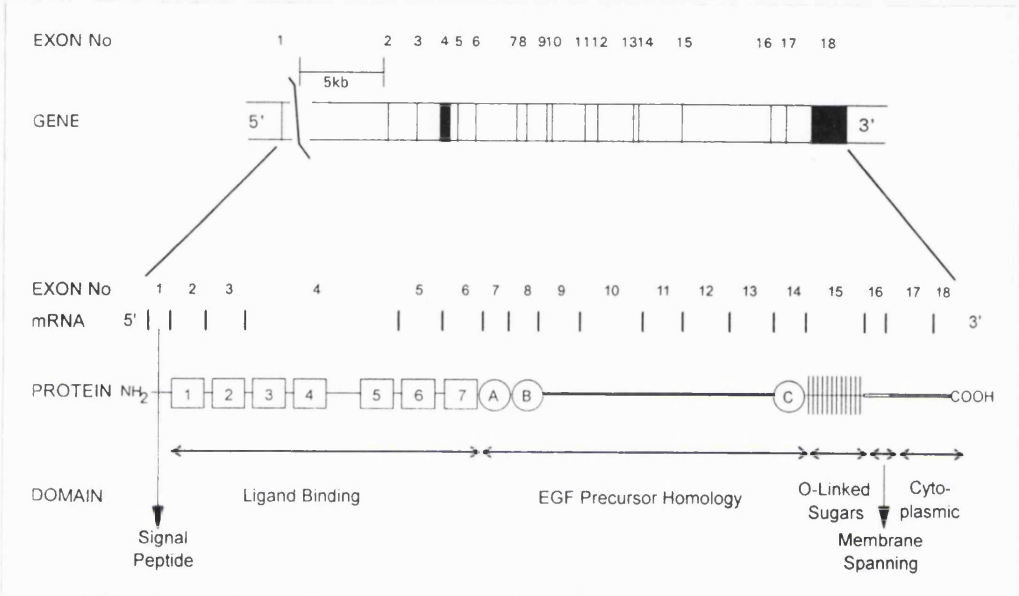

Figure 1.6.1. From gene to protein. A cognate map of the functional domains of the LDL receptor protein, its mRNA and the gene. Vertical bars in the mRNA denotes exon-exon junctions. The 3' untranslated region is not shown. Numbered boxes and circles in the protein map denote the cysteine-rich repeats.

The LDL receptor gene is located on the short arm of chromosome 19 (p13.1-p13.3). It spans 45 kilobases (kb) and comprises 18 exons and 17 introns. The mRNA is 5.3 kb long and about half of it codes for a protein of 860 amino acids. The 2.6 kb 3' half is untranslated and contains three copies of repetitive DNA from the *Alu* family (Yamamoto et al 1984). The LDL receptor gene is a mosaic of exons shared with other genes (Südhof et al 1985) and the exon-intron organization correlates remarkably well with the functional domains of the receptor protein (Südhof et al 1985). Exon 1 encodes a short untranslated 5' region as well as 21 hydrophobic

amino acids that comprise the signal peptide. The 839 amino acid mature protein consists of five domains (Figure 1.6.1.). The ligand binding domain is made of seven cysteine rich repeats of approximately 40 amino acids each. These repeats are coded for by exons 2-6, one exon coding for each repeat, except exon 4 which codes for repeats 3, 4 and 5. Exons 7-14 encode a region that has a sequence homology to the epidermal growth factor (EGF) precursor gene (Südhof et al 1985). This domain includes three cysteine rich repeats of 40 amino acids each that are designated A,B and C and differ from the ligand binding repeats. The first two, A and B (encoded by exons 7 and 8) are separated from the third, repeat C (encoded by exon 14) by five repeats of 40-60 amino acids (encoded by exons 9-13). Each of these repeats has a conserved motif, Tyr-Trp-Thr-Asp (YWTD) (Davis et al 1987a). This domain has been suggested to play a role in the recycling of the receptor (Davis et al 1987a). Exon 15 encodes a sugar binding domain of 58 amino acids rich in serine and threonine residues, many of which are the attachment sites for O-linked carbohydrate chains. Exon 16 and the 5' end of exon 17 encode 22 hydrophobic amino acids of the membrane spanning domain. The remainder of exon 17 and the 5' end of exon 18 encode the 50 amino acids of the cytoplasmic tail. The cytoplasmic tail contains a tetrameric signal sequence; Asn-Pro-Val-Tyr (NPVY) that targets the LDL receptor protein to the coated pit, and is conserved in LDL receptors from six animal species (Chen et al 1990).

The 5' flanking region of the LDL receptor gene probably contains all the *cis*-acting DNA sequences that are responsible for the sterol regulated expression of this gene in animal cells (Goldstein and Brown 1990; Smith et al 1990). Within 200 bp of the initiator methionine codon there are three imperfect direct repeats, each 16 bp long, two TATA boxes and a cluster of mRNA initiation sites, all functioning in transcription (Smith et al 1990).

1.6.2.3. Structure and function of the LDL receptor

A number of studies on both naturally occurring and introduced mutations have contributed to the analysis of the function of various parts of the LDL receptor. The receptor consists of five domains, as described above. The first domain, the binding domain has been subject to a number of studies by the introduction of mutations. Esser et al (1988) and Russell et al (1989) described a number of mutant cDNAs expressed in a monkey COS cell line in which endogenous receptors were fully repressed, examining the ability of the transfected cells to bind both LDL and ßVLDL. These studies examined both single base substitutions as well as deletions of whole cysteine rich repeats. Repeat one is not required for ligand binding according to the results of experiments where it was completely deleted (van Driel et al 1987). A naturally occurring mutation, where amino acids 26 and 27 are deleted does however cause FH but that is probably related to the severely delayed processing of the receptor that results from the deletion (Leitersdorf et al 1988).

A very important observation in the studies by Esser et al (1988) and Russell et al (1989) was the identification of repeat five, coded for by the 3' part of exon 4, as the sole repeat essential for the binding of the apoE containing ßVLDL. This is supported by both naturally occurring mutations in this repeat (D206E) (Leitersdorf et al 1989b) and by introduced mutations (D206N and D206T) (Esser et al 1988). The ability of a mutant LDL receptor to bind ßVLDL despite having lost the ability to bind LDL has been documented on a number of occasions (Esser et al 1988). One of the first naturally occurring mutations, a deletion of the whole of repeat six, coded for by exon 5, bound ßVLDL normally (Hobbs et al 1986) as did a naturally occurring single base substitution; S156L (Hobbs et al 1990).

These studies by mutational analysis of the cysteine rich repeats of the LDL receptor have suggested that the binding site comprises repeats 2-7 arranged with a two-fold symmetry, with the short stretch of poorly conserved amino acids located between repeats three and four acting as a link between the two halves (Russell et al 1989).

Although, the EGF precursor domain has been shown to be important for the binding of LDL to a certain extent (Esser et al 1988), this domain is believed to be essential for the recycling of the receptor as well as for the acid dependent ligand dissociation (Davis et al 1987a). In the study by Davis et al (1987a) the whole of the EGF precursor homology domain was deleted resulting in a loss of the ability to bind LDL, but the protein still retained the ability to bind and internalise ßVLDL. A naturally occurring deletion mutant which results in the production of a similar, if not identical mutant LDL receptor has been reported (Miyake et al 1987). This mutant appears to have the same properties as the one described by Davis et al (1987a). In addition, a total of 11 naturally occurring mutations in this domain have been described that result in a recycling defective phenotype (Hobbs et al 1992). Other naturally occurring mutations in this domain that have been described result in the production of a receptor in which the transport to the cell surface is affected (Hobbs et al 1992), and of the nine mutations described that result in a complete block of the transfer of the receptor to the cell surface, eight are in this region (Hobbs et al 1992).

Mutational analysis on the third domain, the sugar binding domain, have shown that when the domain has been deleted (Davis et al 1986a) this does not result in any detectable loss of function of the receptor. However, a naturally occurring mutation (deleting exon 15) in an FH homozygous Japanese individual has been described (Kajinami et al 1988). Heterozygote

individuals with this mutation seem to be very mildly affected, which could reflect the generally lower plasma cholesterol concentration due to dietary effects in this population described above (Section 1.2.3.), though a study of 40 Japanese FH heterozygotes did not show much difference from other FH heterozygotes with regard to plasma cholesterol concentration (Hirobe et al 1982). Interestingly though, a recently described deletion of exon 15 coding for this domain has been described in Finland designated "FH Espoo" (Koivisto et al 1993). In this population it was described as a mild mutation, further supporting the experimental observations (Davis et al 1986a).

Only one mutation has been described in the transmembrane domain of the LDL receptor (Hobbs et al 1992). This mutation results in the production of a protein that has a delayed processing. The cytoplasmic tail has been shown by site directed mutagenesis to be essential for the assembly of the receptors in the coated pits and for its internalisation (Davis et al 1987b), which has been confirmed when naturally occurring mutants have been analyzed (Brown and Goldstein 1976, Lehrman et al 1985, Davis et al 1986b, Davis et al 1987b, Chen et al 1990, Loux et al 1991, Hobbs et al 1992).

From the structure-function analysis of the LDL receptor it is evident, that a number of different phenotypes of FH can be expected because of the variability of the underlying mutations. It is also conceivable that additional mutations in the LDL receptor gene will be identified that will be of milder effect and will not cause FH.

1.6.3. Variation in the LDL receptor gene not causing FH

1.6.3.1. PvuII polymorphism in the LDL receptor gene and plasma cholesterol concentration

It is quite possible that, in addition to mutations that cause FH, there are sequence changes in the gene that have only a small effect on the function of the receptor and as a consequence, on LDL cholesterol concentration. If such sequence changes were common, they might make an important contribution in determining lipid levels within the normal population. Support of this hypothesis comes from studies of the heritability of the LDL receptor activity in human blood mononuclear cells from normolipidaemic male twins (Weight et al 1982). This study showed five times greater within pair variance for the dizygotic twins than for the monozygotic twins, although there was no difference in the average LDL receptor activity between mono- and dizygotic twin cells. This suggests that the maximal LDL receptor activity of peripheral cells in normolipidaemic subjects is largely genetically determined (Weight et al 1982), though it is by no means a proof of this genetic effect being due to the LDL receptor gene. Another study on fibroblasts from mono- and dizygotic twins demonstrated a large difference in intrapair LDL receptor activity (Magnus et al 1981). Furthermore, studies by Maartmann-Moe et al (1981a and 1981b) on fibroblasts from both non-hypercholesterolaemic individuals and individuals heterozygous for FH showed some evidence for differences in LDL receptor activity attributable to the non-FH or "normal" alleles.

In addition to this work, associations between plasma cholesterol concentration and a *Pvu*II restriction fragment length polymorphism (RFLP) in intron 15 in the LDL-receptor gene have

been shown in a number of studies (Pedersen and Berg 1988, Schuster et al 1990b, Humphries et al 1991). In those studies, individuals having one or more alleles with the cutting site for *Pvu*II present had significantly lower plasma cholesterol concentration than those lacking the restriction site. The *Pvu*II polymorphism has also been shown to mediate some of the effects attributable to the apolipoprotein E (apoE) polymorphism (Pedersen & Berg 1989, Pedersen & Berg 1990). A common RFLP in the LDL-receptor gene of the baboon has also been found to be associated with differences in plasma cholesterol concentration (Hixson et al 1989). All this taken together suggests that variation in the LDL receptor gene as well as the apoB and the apoE genes exerts both a large and a small effect on plasma cholesterol concentration on a continuous scale.

1.7. The aim of the thesis.

The aim of the thesis is to examine the influence of sequence differences in the LDL receptor gene on variability in plasma cholesterol concentration in:

1) FH patients with a major defect in this gene, and

2) individuals from the general population with "normal" LDL receptor activity.

This will be done, firstly by identifying (rare) mutations in a theoretically important part of the LDL receptor (coded for by exon 4) in FH patients, and then by examining their impact on differences in plasma lipid levels.

Secondly the impact of a previously identified common mutation in exon 8 that causes an amino acid variation (alanine to threonine in codon 370) but that does not cause FH, will be examined for its effect on plasma lipid levels in healthy individuals in the general population.

CHAPTER 2 - METHODS, SUBJECTS AND MATERIALS

2.1. DNA extraction from blood

DNA was normally extracted from 5 ml of whole blood using EDTA as an anticoagulant. The blood was thawed out on ice or at 4°C in the fridge.

2.1.1 Phenol Chloroform extraction

The phenol chloroform extraction method is based on that of Kunkel et al (1977).

Lysing the cells

1. Thaw out the blood and transfer to a 30 ml centrifuge tube.

2. Add 25 ml of ice cold sucrose lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100).

2. Spin at 10.000 rpm for 10 minutes at 4°C (Sorvall RC5C centrifuge using rotor SA-600).

3. Discard the supernatant and resuspend the pellet in 4.5 ml of 0.075 M NaCl, 0.024 M EDTA pH 8.0.

4. Add 250 μl 10% SDS, 100 μl proteinase K (Sigma, UK) (10 mg/ml), and 150 μl ddH_2O.

5. Incubate O/N at 37°C or for 3 hours at 56°C.

Phenol chloroform extraction

1. Mix with 5 ml equilibrated phenol, by inverting the tube gently 20 - 30 times. Centrifuge at 20°C for 5 minutes at 10.000 rpm. Remove the top layer (the aqueous phase) into a 15 ml tube using a hooked pasteur pipette. Discard the rest.

2. Add 2.5 ml of phenol and 2.5 ml of chloroform:isoamylalcohol (24:1) and mix by inverting the tube several times. Centrifuge at 20°C for 5 minutes at 10.000 rpm. Transfer the upper phase to a new tube.

3. Re-extract the upper phase with 5 ml of chloroform:isoamylalcohol (24:1) and remove the upper phase to a new 30 ml tube.

DNA precipitation

Add 500 μl of 3 M NaOAc pH 5.0 and 11 ml of 100% ethanol at room temperature. Gently invert the tube several times. The DNA should precipitate into a visible clot. Hook the DNA out with a hooked pasteur pipette and transfer into a Bijoux bottle. The DNA can be spun down as an alternative.

Dissolving the DNA

Dissolve the DNA in 0.5 ml of TE buffer in the Bijoux by rotating on a rotator at 4°C until dissolved.

Determining DNA concentration

Dilute 20 μl of DNA solution into 1 ml of TE and measure absorbance at 260 nm (DNA) and 280 nm (protein).

2.1.2. Salting out method

The DNA extraction by the salting out method is based on that described by Miller et al (1988).

Lysing the cells

1. Thaw out the blood and transfer to a 30 ml centrifuge tube.

2. Add 25 ml of ice cold sucrose lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100).

2. Spin at 10.000 rpm for 10 minutes at 4°C (Sorvall RC5C centrifuge using rotor SA-600).

3. Discard the supernatant and resuspend the pellet in ice cold sucrose lysis buffer and spin again. Repeat if pellet is very brown.

4. Pour off supernatant. Add 3 ml nucleic lysis buffer (10 mM Tris-HCl pH 8.2, 400 mM NaCl, 2 mM EDTA). Vortex to resuspend.

5. Add 200 μl 10% SDS and 150 μl proteinase K (Sigma, UK) (10 mg/ml).

6. Incubate O/N at 37°C or for 3 hours at 56°C.

DNA precipitation

1. Add 1.5 ml saturated (approximately 6 M) NaCl and shake vigorously for 15 seconds.

2. Centrifuge at 10.000 rpm for 8 minutes.

3. Using sterile long pasteur pipettes, carefully remove the supernatant to another 30 ml tube. Discard the pellet.

4. Add 5 ml of 100% ethanol to the supernatant and mix gently. The DNA should precipitate into a visible clot. Hook the DNA out with a hooked pasteur pipette and transfer into a Bijoux bottle containing 70% ethanol and then into an eppendorf containing 200-400 μl H_2O.

Dissolving the DNA

Incubate at 37°C (in water bath) to dissolve the DNA. Alternatively rotate at 4°C.

Determining DNA concentration

Dilute 20 μl of DNA solution into 1 ml of TE and measure absorbance at 260 nm (DNA) and 280 nm (protein).

2.2. Polymerase Chain Reaction (PCR)

PCR is based on the method described by Saiki et al (1981).

All the amplifications were done in the standard PCR buffer with 1.5 mM $MgCl_2$ with 1 pmol/μl of each of the primers, 50 ng of genomic DNA and 0.02 U of *Thermus aquaticus* (Taq) DNA polymerase (Gibco, UK)/10 μl of reaction volume. The standard 1 x PCR buffer is:

50 mM	KCl
10 mM	Tris (pH 8.3)
0.001%	gelatin (Sigma, UK)
0.2 mM	each dNTP (Pharmacia, Sweden)

The amplifications were performed in, either 0.5 ml eppendorf tubes or in a 96 well plates, in the described volume for each reaction, with the equal volume of paraffin overlay.

2.2.1. PCR of exon 4 of the LDL receptor gene

Exon 4 of the LDL receptor gene was amplified in six reactions for mutation detection by SSCP, restriction enzyme digestion and for sequencing. Table 2.2.1. shows the sequences of the primers used and the conditions for the PCR for each fragment amplified. Figure 2.2.1. shows the interrelations of the primers, the fragments and the exon.

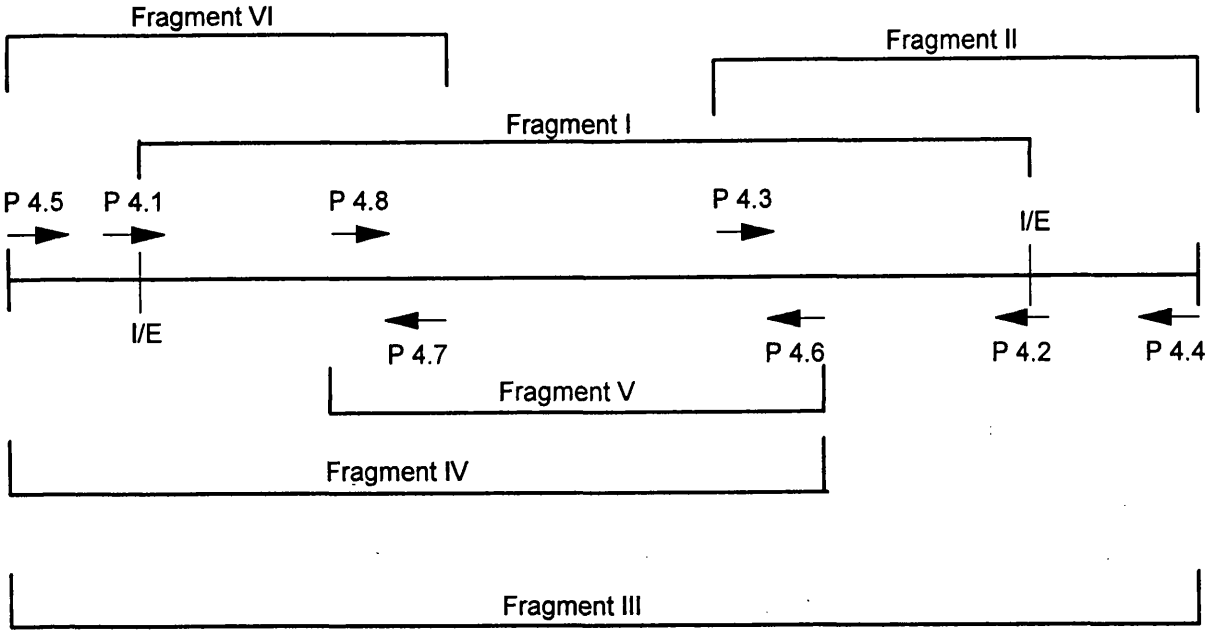

Figure 2.2.1. Schematic figure of exon 4 of the LDL receptor gene, with the primers and the fragment positions.

Table 2.2.1. Exon 4 PCR primers and conditions

Exon 4 PCR information				
	oligonucleotide primers	Reference	Fragment number and size	PCR conditions*
P 4.1.	5'CATCCATCCCTGCAGCCC3' Intron 3 - Exon 4 boundary	Gudnason et al 1993a	Fragment I 405 bp	1)
P 4.2.	5'CGCCCATACCGCAGTTTTCC3' Exon 4 - Intron 4 boundary	Gudnason et al 1993a		
P 4.3.	5'CGACTGCGAAGATGGCTCGGA3' bp 528 to bp 547	Gudnason et al 1993a	Fragment II 234 bp	1)
P 4.4.	5'GGGACCCAGGGACAGGTGATAGGAC3' Intron 4	Kotze et al 1990		
P 4.5.	5'AAAGTCGCGGTCTCGGCCATCCATCCCTG3' Intron 3	Gudnason et al 1994	Fragment III 450 bp	1)
P 4.4.	5'GGGACCCAGGGACAGGTGATAGGAC3' Intron 4	Kotze et al 1990		
P 4.5.	5'AAAGTCGCGGTCTCGGCCATCCATCCCTG3' Intron 3	Gudnason et al 1994	Fragment IV 340 bp	1)
P 4.6.	5'GAGCAGGGGCTACTGTCC3' bp 606 to bp 588			
p4.8.	5'AGACGAGGCCTCCTGCCCGGT3' bp 426 to bp 447		Fragment V 192 bp	2)
P 4.6.	5'GAGCAGGGGCTACTGTCC3' bp 606 to bp 588			
P 4.5.	5'AAAGTCGCGGTCTCGGCCATCCATCCCTG3' Intron 3	Gudnason et al 1994	Fragment VI 180 bp	2)
p4.7.	5'CTGTTGCACTGGAAGCTGGCG3' bp 484 to bp 463			

*The standard volume of PCR was 50 μl, The amplifications were performed in an automated thermal cycler, (Cambio, Cambridge, UK). Two conditions were used:

1) 95°C for 5 min, 68°C for 6 min once and 95°C for 1 min 68°C for 6 min for 30 cycles

2) 95°C for 5 min, 57°C for 2 min and 72°C for 3 min once and 95°C for 1 min, 57°C for 1 min and 72°C for 1:30 min for 30 cycles.

2.2.2. PCR of exon 8 of the LDL receptor gene

Exon 8 of the LDL receptor gene was amplified in two reactions for the detection of the sequence change. Table 2.2.2. shows the sequences of the primers used and the conditions for the PCR for each fragment amplified. Figure 2.2.2. shows the interrelations of the primers, the fragments and the exon.

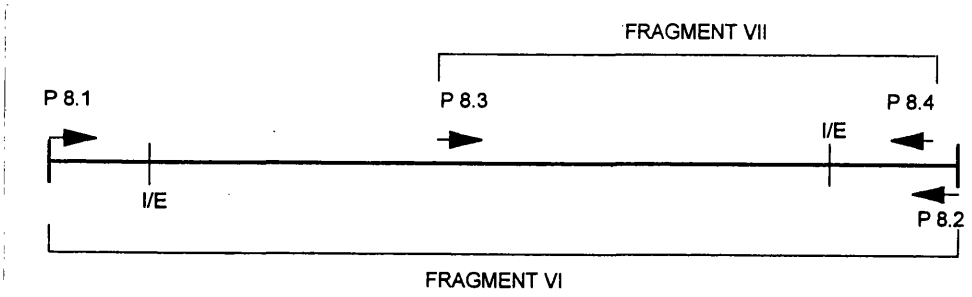

Figure 2.2.2. Schematic figure of exon 8 of the LDL receptor gene, with the primers and the fragment positions.

Table 2.2.2. Exon 8 PCR primers and conditions

Exon 8 PCR information				
	oligonucleotide primers	Reference	Fragment number and size	PCR conditions
P 8.1.	5'AATGTCGACCAAGCCTCTTTCTCTCTCTTC3'	Gudnason et al 1995a	196 bp fragment VI	**
P 8.2.	5'AAGTCGACCCACCCGCCGCCTTCCCGTGC3'	Gudnason et al 1995a		
P 8.3.	5'CCTGGAGGGTGGCTACAAGTGCCAG3'		109 bp fragment VII	**
P 8.4.	5'CCACCCGCCGCCTTCCCGTGCTCAC3'	Hobbs et al 1992		

**The standard volume of PCR was 10 μl. The reactions were performed on a Hybaid Omnigene Intelligent Heating Block (Hybaid,UK).
One condition was used: 95°C for 5 min, 57°C for 2 min, 72°C for 3 min, once and 95°C for 1 min, 57°C for 1 min, 72°C for 2 min for 30 cycles

2.3. Gel electrophoresis

2.3.1. Agarose gel electrophoresis

Agarose (Ultrapure Agarose, BRL, USA) was melted in a microwave oven to a homogenous solution of the appropriate concentration (usually 1 - 2%) in 1 x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). The melted agarose was poured into the gel trays at 50°C and the combs put in place. Three sizes of trays were used: 1) Mini (5 cm x 7 cm), 2) midi (10 cm x 14 cm), and 3) large (20 cm x 24 cm), depending on the experiment. The gels were run submerged in 1 x TAE at 25 - 100 V, dependent on the experiment.

2.3.1.1. Detection of DNA on agarose gels.

The DNA was stained with ethidium bromide, either by mixing it into the gel (0.25 mg/ml final concentration) or by soaking the gel in a solution of 1 x TAE and ethidium bromide (2.5 mg/ml) for 15 - 30 minutes. The gels were viewed on a UV transilluminator and photographed.

2.3.2. Polyacrylamide gel electrophoresis (PAGE)

For DNA analysis, the gels were cast using a 30% stock solution of acrylamide (Severn Biotec Ltd., Kidderminster, UK), diluted to the final concentration needed for each experiment, and bisacrylamide to acrylamide ratio as appropriate, usually 1:49 for the SSCP and 1:19 in other experiments. The gels were made in 1 x TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) with or without glycerol, using 1 μl/ml of NNN'N'-Tetramethylethylenediamine (TEMED) and 1 μl/ml of 25% ammonium persulphate (APS). The electrophoresis was performed in 1 x TBE (pH 8.3).

SDS-PAGE was used for the analysis of the LDL receptor protein. A gel consisting of two different compositions was cast, one laid on the top of the other. The lower gel occupied approximately 75% of the total volume of the gel, in the final concentration of 7.5% acrylamide and 0.05% bisacrylamide (the ratio 1:150), 375 mM Tris-HCl (pH 8.9), 0.35% SDS, 0.3 μl/ml TEMED, 8,25 μl/ml 10% APS. The stacking gel consisted of 5% polyacrylamide and 0.13% bisacrylamide (the ratio 1:37.5), 160 mM Tris-HCl (pH 6.7), 0.1% SDS, 0.3 μl/ml TEMED, 8.25 μl/ml 10% APS. The electrophoresis was performed in a 25 mM Tris - 50 mM glycine buffer (pH 8.6), 0.1% SDS.

The gels were poured between two glass plates, that had been washed thoroughly in a detergent and water, rinsed in distilled water and then rinsed with ethanol and wiped dry. The plates used for protein electrophoresis were additionally soaked for 12 hours in 1% sulphuric acid. Three sizes of gels were used: 1) Mini gels (6 cm x 12 cm x 0.8 mm). For these, two types of apparatus were used; the BioRad apparatus (BioRad, USA) and the Mighty Small apparatus (Hoefer, USA). Both apparatus come with specific casting trays for 1 to 10 gels. 2) Midi gels (13 cm x 20 cm x 1.0 mm (1.5 mm for protein gel electrophoresis)). 3) Large gels (30 cm x 40 cm x 0.4 mm).

2.3.2.1. Detection of DNA on polyacrylamide gels

2.3.2.1.1. Ethidium bromide

Gels are placed in distilled water with ethidium bromide (2.5 mg/ml final concentration) for 15 minutes and then put on a UV transilluminator and photographed.

2.3.2.1.2. Silver staining

The silver staining is based on that of Merril (1990).

1. Gels were placed in 10% ethanol solution for 5 minutes and shaken gently (the ethanol was then poured off).

2. Gels were then oxidised in 1% nitric acid for 5 minutes and shaken gently (the nitric acid was then poured off).

3. The gel was rinsed in distilled water for 30 seconds

4. The gel was placed in 0.012 M silver nitrate solution for 20 minutes

5. The silver solution was poured off (it can be used several times) and the gel rinsed in distilled water for 2 - 5 minutes (dependent on the thickness of the gel, the thicker the gel the longer the rinsing time).

6. The gel was then put into a solution of 0.28 M sodium carbonate (anhydrous) and 0.019% formalin to reveal the DNA. The solution may need to be changed several times during this development stage.

7. When the desired image had developed the process was stopped with 10% glacial acetic acid for 5 minutes and then rinsed in distilled water for other 5 minutes.

8. Finally the gel was transferred onto a 3MM Whatman paper and dried in a slab gel drier and stored permanently as the record for evaluation or marking.

2.3.2.1.3. Radioactivity

(see Sections on SSCP (2.4.4.2.1.) and Sequencing (2.4.5.3)).

2.4. Detection of sequence variation

2.4.1. Haplotype analysis.

Genotypes for six LDL receptor gene RFLPs were determined either by restriction digestion of genomic DNA followed by Southern blotting as described for *Pvu*II (Taylor et al 1988), or by PCR amplification using oligonucleotides flanking each of the variable restriction sites for *Taq*I, *Stu*I (Leitersdorf et al 1989a), *Hinc*II (Leitersdorf and Hobbs 1988), *Ava*II and *Nco*I (King-Underwood et al 1991, Humphries et al 1993). Alleles were designated as "+" or "-" indicating the presence or absence of the cutting site respectively. Most of the haplotype analysis on this sample was done by Linda King-Underwood, who is a joint author of the publication reporting this study, described later (Gudnason et al 1993a) see Section 3.1. in Chapter 3.

2.4.2. PCR and digestion with restriction endonucleases

2.4.2.1. *Mbo*II, *Dde*I and *Msp*I digestion of the PCR

The D154N mutation results in a loss of restriction site for *Mbo*II (Boehringer Mannheim, Germany). To detect this, 20 μl of PCR fragment I (see Table 2.1.1. in Section 2.2.1.) were digested with 10 units *Mbo*II (Anglian Biotec Ltd, UK) for 16 hours in a total volume of 30 μl. The digestion mixture was then analyzed by electrophoresis for 2 hours at 50 V through 1.2% agarose gel in 1 x TAE with ethidium bromide (0.25 mg/ml) and illuminated on a transilluminator for photographing (see Figure 3.1.1.a) in Chapter 3 Section 3.1.3.).

To detect the D206E mutation, PCR fragment II (see Table 2.1.1. in Section 2.2.1.) (20 μl) was digested with 12 U of *Dde*I (Anglian Biotec Ltd, UK) for 16 hours in a volume of 30 μl in the buffer supplied. The

digested fragments were then analyzed by electrophoresis on a 7.5% polyacrylamide gel for 1.5 hours in 1 x TBE buffer. DNA bands were visualised by UV transillumination of ethidium bromide stained gels and photographed. (see Figure 3.1.2. in Chapter 3 Section 3.1.3.).

To detect the D200G mutation, PCR fragment I (see Table 2.1.1. in Section 2.2.1.) was incubated with 10 U of *Msp*I in the buffer recommended by the manufacturer (Boehringer Mannheim, Germany) for 16 hours at 37°C. The digested DNA was loaded onto 1.8% agarose gel in 1 x TAE buffer and the fragments were separated by electrophoresis, stained with ethidium bromide and photographed on a transilluminator (see Figure 3.2.7. in Chapter 3 Section 3.2.3.3.).

2.4.2.2. *Stu*I digestion of the PCR

After analysis of PCR Fragment VI (see Table 2.2.2. in Section 2.2.2.) (2 μl of the reaction mix) by agarose gel electrophoresis, samples (1 μl of the reaction mix) were pooled in groups of 5 and digested overnight with 5 U of the restriction enzyme *Stu*I (Boehringer Mannheim, Germany) in a total volume of 10 μl of buffer recommended by the supplier. The DNA fragments were separated by non-denaturing polyacrylamide gel (7.5 %) electrophoresis and stained with silver (see Figure 4.2.2. in Chapter 4 Section 4.2.2.). Where the presence of larger bands suggested the presence of an uncut sample in a pool, 1.5 μl of the individual PCR products were digested with 2 U of *Stu*I as described above. The analysis of fragment VII (see Table 2.2.2. in Section 2.2.2.) by *Stu*I digestion was done by digesting 5 μl of the 102 bp long PCR for 1 hour with 5 U of the enzyme in a total volume of 10 μl. The DNA fragments were separated by non-denaturing polyacrylamide gel (10 %) electrophoresis and stained with silver, showing fragments of 49 and 53 bp (data not shown).

2.4.3. PCR and allele specific oligomelting

The differential hybridisation with ASOs is based on the described by Wood et al (1985)

2.4.3.1. Allele Specific Oligonucleotides (ASO)

The general concept of the oligonucleotides used for the oligomelting is that 1) the size should be between 12 and 20-mer. 2) The calculated melting point $T_d = 4 \times (G+C) + 2 \times (A+T)$ and 3) the mismatch should be central, that is the most unstable.

The pairs of allele specific oligonucleotides (ASO) were used are described in Table 2.4.3.1.:

Table 2.4.3.1. The sequence of the allele specific oligonucleotides used.

Aso No	Allele Specific Oligonucleotides	Allele	Reference
	Exon 4		
ASO 1	5'AAGATGGCTTGGATGAGTG 3'	S156	Hobbs et al 1989
ASO 2	5'AAGATGGCTCGGATGAGTG 3'	L156	Hobbs et al 1989
ASO 3	5'CAAATCTGACAAGGAAAACT 3	E207	Leitersdorf et al 1990
ASO 4	5'CAAATCTGACGAGGAAAACT 3'	K207	Leitersdorf et al 1990
ASO 5	5'TGTGATGGCCCC 3'	G197	Gudnason et al 1993a
ASO 6	5'GTGATGGTGGCC 3'	ΔG197	Gudnason et al 1993a
	Exon 8		
ASO 7	5'ACACGAAGGCCTGCAAG3'	A370	Gudnason et al 1995a
ASO 8	5'ACACGAAGACCTGCAAG3'	T370	Gudnason et al 1995a

2.4.3.2. 5' end labelling with γ-^{32}P ATP

The 5' end labelling is as described by Maniatis et al (1982)

All the ASO were labelled at the 5' end with T4 polynucleotide kinase (BRL, Paisley, UK) and [τ-^{32}P] ATP (Amersham, UK) to a specific activity of approximately 0.1 μCi/pmol.

1. For each oligonucleotide probe the reaction was done in the total volume of 50μl, in an 1.5 ml screwcapped eppendorf tube.

5.0 μl	10 x PNK buffer	(10 x: 0.5 M Tris-HCl pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT,1 mM Spermidine (Sigma, UK), 1 mM EDTA)
1.0 μl	oligonucleotide probe	(30 pmol of the appropriate probe)
1.0 μl	T4 Kinase (10 U/ml)	
2.5 μl	γ-^{32}P ATP (Amersham, UK)	
40.5 μl H_2O		

50.0 μl Total volume

2. Each tube containing the above mixture was then incubated at 37°C in a waterbath for 1 hour.

3. Sephadex G25 spun columns (Maniatis et al 1982) were prepared: The plunger from a 1 ml plastic syringe was removed and the end plugged with a very small amount of glass or nylon wool. The syringe barrel was filled with Sephadex G25 (Pharmacia, Sweden) in TE to the 1 ml mark. An empty screwcap eppendorf tube was put in the bottom of a 15 ml Falcon tube. The Sephadex column was put in the

Falcon tube with the end in the eppendorf to collect the liquid. The column was then spun in the centrifuge at 1.500 rpm for 3 minutes (using a benchtop centrifuge; Sorvall T6000B).

4. The eppendorf tube was replaced with a fresh one, and the column put in the Falcon tube again. Fifty μl of H_2O was then added to the reaction mixture, and the 100 μl transferred to the Sephadex column and centrifuged at 1.500 rpm for 3 minutes collecting the ^{32}P oligonucleotide probe.

5. Two μl of the labelled oligonucleotide probe were transferred into another screwcapped eppendorf and counted in a β-counter.

2.4.3.3. Synthesis of positive controls for Allele Specific Oligomelting

The synthesis of positive controls for Allele Specific Oligomelting was done as described by Gudnason & Humphries (1991)

Positive controls for the S156L and the E207K mutations were synthesised by PCR amplification from a preamplified PCR (fragment I of exon 4, see Table 2.1.1. in Section 2.2.1.), using the mutant ASO to introduce the specific mutation. Fragment I was amplified and electrophoresed on 1% agarose gel, excised from the gel, and frozen at -20°C. After thawing, 1 μl was taken and diluted 100 fold in sterile distilled water, and 1 μl of that was taken to amplify the mutant fragment using the mutant ASO as the 5' PCR primer, keeping the 3' primer the same. The PCR conditions were one cycle of 95°C for 5 minutes, 45°C for 3 minutes, and 72°C for 3 minutes; followed by 30 cycles of 95°C for 1 minute, 45°C for 1 minute, and 72°C for 2 minutes, and finally 72°C for 10 minutes. The PCR reaction was then treated in the same way as the samples from the patients.

2.4.3.4. Transfer of the PCR fragments to a membrane

For transfer of the PCR amplified fragment to nylon membranes, two methods were used:

1) the PCR fragment was fractionated by electrophoresis for 3 hours on a 1% agarose gel, which was then denatured in 1.5 M NaCl, 0.5 M NaOH for 30 minutes at room temperature and then "double blotted" (10 μl) between two pieces of nylon membrane (Hybond-N+, Amersham, UK), using the denaturing solution as the transfer buffer. The filters were put on a UV transilluminator for 1 minute.

2) a 5 μl aliquot of the PCR product were mixed with 3 μl of 2 M NaOH, 2 M NaCl solution and kept at room temperature for 10 minutes. 3 μl of the mix were then blotted (in duplicate) onto a Hybond N+ membrane (Amersham, UK) in the same format as in the 96 well plate, using an eight channel multipipette for the transfers. One positive control (for a homozygous T370 allele in the experiments described in this thesis) was included in each filter, and a negative control (absence of DNA) was included in one well of each plate.

2.4.3.5. Hybridization and washing of the filters

The filters were prehybridized in 5 x SSPE (1 x SSPE is 0.9% NaCl, 50 mM sodium phosphate and 5 mM EDTA), 5 x Denhardt's solution (Ficoll 1% polyvinylpyrrolidone 1%, BSA 1%), and 0.5% SDS for 15 minutes. The filters were then hybridised with ^{32}PγATP end labelled oligonucleotide probes (see Section 2.5.2.) in 5 x SSPE, 0.5% SDS, 5 x Denhardt's solution at 5 to 10°C lower than the calculated melting point of the oligonucleotide for from 1 hour to O/N depending on convenience.

For detection of the mutations in exon 4 the filters were washed for 3 minutes at room temperature in 2 x SSPE and 0.2% SDS for all ASOs, and subsequently for 10 minutes in 0.2 x SSPE and 0.1% SDS at

individually adjusted temperature for each ASO probe. The temperatures were: 47°C for S156L and E207K, and 39°C for the ΔG197 mutation. They were exposed on film (Konica, Japan) with intensifying screens at -70°C for 16 hours and then developed for typing (See Figures 3.1.7., 3.1.8. and 3.1.9. in Chapter 3 Section 3.1.3. for the results).

For detection of the A370 and the T370 alleles in exon 8, the filters were rinsed at room temperature for 10 minutes in 2 x SSPE, 0.1% SDS and exposed to a film (Hyperfilm MP, Amersham, UK) for 2 hours with intensifying screens at - 70°C and then developed to check presence of PCR products. The filters were then washed for 10 minutes in 0.2 x SSPE, 0.1% SDS at 49°C for the T370 allele and at 52°C for the A370 allele. They were exposed on film (Konica, Japan) with intensifying screens at -70°C for 16 hours and then developed for genotyping (see Figure 4.4.1. in Chapter 4 Section 4.4.2.).

2.4.4. Single Strand Conformation Polymorphism (SSCP)

2.4.4.1. Conditions of the SSCP

SSCP analysis on PCR fragments from exon 4 was performed using the fragments described in Table 2.4.4.1. and the various conditions referred to in the table and described below.

Table 2.4.4.1. SSCP fragments and conditions of exon 4

Exon 4 SSCP		
Fragment id (as in table 2.2.1.)	Size (bp)	SSCP conditions*
Fragment II	234	1), 2), 3), 4)
Fragment IV	340	5), 6)
Fragment V	192	1)
Fragment VI	180	1)

* The following SSCP conditions were used

1) 6% acrylamide, bisacrylamide:acrylamide(1:49), 10% glycerol, electrophoresed at 5 mA for 16 hours at room temperature on a 30 cm x 40 cm x 0.4 mm gel.

2) 7% acrylamide, bisacrylamide:acrylamide(1:49), 10% glycerol, electrophoresed at 10 mA for 5 hours at room temperature on a 6 cm x 12 cm x 0.8 mm gel.

3) 7% acrylamide, bisacrylamide:acrylamide(1:49), 10% glycerol, electrophoresed at 30 mA for 9 hours at room temperature on a 13 cm x 20 cm x 1.0 mm gel.

4) 6% acrylamide, bisacrylamide:acrylamide(1:49), electrophoresed at 45 mA for 3 hours at 4 °C on a 30 cm x 40 cm x 0.4 mm gel.

5) 6% acrylamide, bisacrylamide:acrylamide(1:49), 10% glycerol, electrophoresed at 8 mA for 20 hours at room temperature on a 30 cm x 40 cm x 0.4 mm gel.

6) 12% acrylamide, bisacrylamide:acrylamide(1:49), electrophoresed at 45 mA for 20 hours at 4 °C on a 30 cm x 40 cm x 0.4 mm gel.

2.4.4.2. PCR and detection of the single strands

2.4.4.2.1. Radioactive labelling of the PCR

The fragment was labelled by PCR amplification using the conditions described in Section 2.2.1., with

the addition of 0.1 μl per reaction mixture of [α-^{32}P] 2' deoxycytosine triphosphate (800 Ci/mmol, 10 μCi/μl; Amersham, UK). A quantity of 5 μl of the PCR mixture was diluted with 25 μl of 0.1% SDS and 10 mM EDTA. A 5 μl aliquot of this dilution was mixed with 5 μl formamide dye (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The PCR DNA was denatured by boiling at 100° C for 3 minutes and then put straight on ice. Samples (4 μl) were loaded onto the gels, electrophoresed as described (Section 2.3.2.) and then transferred onto Whatman 3MM chromatographic paper, dried and exposed to hyperfilm β max (Amersham, UK) for 12-24 hours at - 70°C before developing.

2.4.4.2.2. Silver staining of the SSCP

For detection by silver staining the PCR conditions were as in Sections 2.2.1. and 2.2.2. without the addition of the radioactive isotope. A quantity of 3 μl of the PCR product was mixed with 5 μl formamide dye and denatured by boiling at 100° C for 3 minutes and then put straight on ice. Samples (7 μl) were loaded onto non-denaturing polyacrylamide gel (ratio of acrylamide to bisacrylamide 49:1) in 1 x TBE buffer, with 10% glycerol. Silver staining was performed in all cases on the two smaller sized gels 6 cm x 12 cm and 13 cm x 20 cm and as a test on the larger format gel, 30 cm x 40 cm. The gels were stained according to the protocol described in Section 2.3.2.1.2. For the large gel, electrophoresis tape was wound around the glass plate to form a tray to carry out the silver staining. The gels were then transferred onto Whatman 3MM chromatographic paper and dried.

2.4.5. Direct sequencing

The method used is a chain termination (Sanger et al 1977) and relies on the ability of dideoxynucleotides, which lack the 3'-hydroxyl group critical to phosphoribosyl chain extension, to terminate a strand synthesis from a specific oligonucleotide priming site by a DNA polymerase. The template must be single

stranded DNA, and using suitable mixtures of dideoxyN (where N is one of the bases A, C, G or T) and a deoxynucleotide mix, a range of sequence specific size fragments is generated which can be resolved by electrophoresis to determine the sequence of the synthesized strand. Chain terminations for a given sized fragment should only occur in one gel track (A,C,G or T) in accord with the specific base-pairing of the dideoxynucleotides with the template single strand. Improvements of the original technique have centred upon methods to obtain isolated single strands, polymerases with different characteristics, and modified nucleotides with different characteristics. At least in part, this reflects the problems of sequencing: if the strand complementary to the template is present during the sequencing reaction, it will tend to interfere by reannealing; if the template adopts secondary structure or the reaction is somehow suboptimal, nonspecific terminations will occur. Two techniques were used for obtaining templates ; first a double stranded PCR fragment was used and a single strand obtained by boiling the template, and secondly a biotinylated oligonucleotide primer was used to obtain a single stranded DNA template.

2.4.5.1. Purification of the PCR amplified template

2.4.5.1.1. Purification from low melting point agarose after gel electrophoresis, for sequencing from a double stranded PCR product

For checking of the PCR product 10% of the reaction volume was run on a 1-1.5% agarose gel and stained in ethidium bromide solution and examined on a transilluminator. When the expected fragment was identified, the rest of the PCR reaction (usually 45 μl) was loaded into a single well on a 1.5% LMP agarose (Nusieve, FMC Bioproducts, USA) and electrophoresed in 1 x TAE with ethidium bromide in a cold room (4-6°C) for the time necessary to separate the fragment clearly from the oligonucleotides. The gel was then transferred on to a transilluminator and the band excised and transferred into an 1.5 ml eppendorf tube.

For the extraction of the DNA from the excised gel

1. The tube was placed in a heating block at 68°C until melted (for 15 minutes).

2. Then prewarmed TE solution (37°C) was added to a final volume of 0.5 ml in the tube.

3. Immediately 0.5 ml of prewarmed water saturated phenol was added, vortexed for 15 seconds and spun at 14.000 rpm in an Eppendorf microcentrifuge at room temperature for 5 minutes.

4. The upper phase was then transferred to a new clean eppendorf tube (care taken not to disturb the white intermediate layer) and spun for 30 seconds and transferred into a new tube.

5. Then 0.5 ml of chloroform:isoamylalcohol (24:1) was added, the mix vortexed for 15 seconds and spun at 14.000 rpm for 1 minute.

6. The upper phase was transferred into a new tube and 50 μl of 3 M NaOAc and 1 ml of ethanol added, mixed by inversion and stored at -20°C for 12 hours minimum.

7. The tubes were then spun at 4°C for 30 minutes and the supernatant poured off.

8. The pellet was washed in 70% ethanol and the supernatant poured off and the pellet dried for few minutes and dissolved in 14 μl of ddH_2O and used for sequencing.

2.4.5.1.2. Purification by magnetic streptavidin beads (Dynabeads), using biotinylated oligonucleotide primers, for sequencing from a single stranded PCR product

Dynabeads (Dynal, Norway) are a magnetic solid phase for capturing the DNA strand generated in a PCR reaction by use of one biotinylated and on non biotinylated oligonucleotide primer in the reaction. The double stranded DNA product is captured by binding of the biotinylated strand to the streptavidin coat on iron beads, using a magnet to immobilise the PCR product. The double strand is melted using NaOH, releasing the non-biotinylated strand. Either strand can then be used as a template for sequencing. The PCR purification is according to the manufactures protocol.

Preparing the Dynabeads M-280 streptavidin

The beads need washing before use. For each PCR product 20 μl of Dynabeads (10 mg/ml) were used and they can be washed in bulk.

Two types of magnet separators were used; The Dynal MCP for single eppendorf tubes, each having places for up to six tubes, and the Dynal MCP-96 for handling up to 96 samples in a microtitre array format 8 x 12 wells.

1. The correct volume of unwashed Dynabeads to be used were placed in the Dynal MCP in a 1.5 ml eppendorf tube. The beads are drawn towards the magnet to the tube's side and the supernatant carefully removed with a pipette and discarded.

2. The Dynabeads are resuspended in the same volume of 1 x binding and washing buffer (B & W). (2 x B & W is 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 2.0 mM NaCl). They were mixed gently by agitating in the pipette tip. The supernatant was then taken off after the beads had been separated in the

Dynal MCP apparatus.

3. The Dynabeads were then separated in double the initial volume of 2 x B & W.

Immobilising the PCR product

Can be done either in a microtitre plate or individual eppendorf tubes, depending on the numbers of PCR's purified.

1. 40 μl of each PCR product were used and 40 μl prewashed beads added.

2. The PCR product and beads were incubated together for 15 minutes at room temperature, keeping the beads suspended by gently tipping the plate. The incubation allows the biotinylated DNA strand to attach to the streptavidin on the beads.

Melting the DNA duplex

1. The tube or plate containing the PCR product immobilised on the beads were placed in the appropriate Dynal apparatus and the supernatant is carefully removed and discarded.

2. The beads were then washed by adding 40 μl of 1 x B&W to the tube or well, agitated in the pipette tip. The supernatant was then taken off in the Dynal apparatus, and discarded. (The immobilised PCR product can also be stored at this stage at 4°C for several weeks).

3. Washed with 1 x B & W as in (2).

4. Dynabeads were resuspended in 8 μl of freshly prepared 0.1 M NaOH, and incubate at room temperature for 10 minutes.

Separating the DNA strands

1. The tubes or the plate were put in the Dynal apparatus and 8 μl of the supernatant taken off and transferred into 4 μl of 0.2 M HCl and 1 μl of 1 M Tris-HCl added to each sample. This contained the complimentary non-biotinylated DNA single strand which can be used for sequencing (it is essential to use the same pipette for pipetting the NaOH and the HCl so as to neutralise the alkali). This strand can be stored at -20°C.

2. The remaining beads with the immobilised biotinylated single strand DNA was then washed; once with 50 μl 0.1 M NaOH, once with 40 μl B & W buffer and once with 50 μl TE buffer.

3. The washed bound single strand DNA was then resuspended in an appropriate amount of water for sequencing.

2.4.5.2. PCR fragments and sequencing primers

For sequencing of exon 4, biotinylated PCR fragment III (Section 2.2.1.) was used. The oligonucleotides used for sequencing are described in Table 2.4.5.1.

Table 2.4.5.1. Sequencing primers

Sequencing primers		
Primer	Sequence	orientation
Sp 1	5'GAGCAGGGGCTACTGTCC3' bp 606 to 588	antisense
Sp 2	5'AAGATGGCTCGGATGAGTG3' bp 534 to 552,	sense
Sp 3	5'CGCCCATACCGCAGTTTTCC3' exon/intron boundary	antisense
Sp 4	5'CAAATCTGACGAGGAAAACT3' bp 685 to 704.	sense

2.4.5.3. Sequencing protocols

The sequencing is based on the protocol from the Sequenase 2.0 kit (Amersham, UK) The main differences are in the annealing of the primer to the template. The primers used were either the PCR oligonucleotide primers or primers internal to the PCR fragment.

2.4.5.3.1. Sequencing from a double stranded PCR product

The template was obtained as described in Section 2.4.5.1.1. The sequencing primers were used in the concentration of 50 - 200 pmol/reaction. All reagents aliquoted before the reactions were started so everything was done in a continuous way. All the reactions were performed in an eppendorf tube.

Annealing of the primer to the template.

1. 50 - 200 pmol primer were added to 6.5 μl of template and 0.5 μl of 0.1% NP-40 and 2 μl 5 x reaction buffer with ddH_2O to a final volume of 10 μl, in an eppendorf tube and gently mixed (NP-40 and 5 x reaction buffer were made up in the quantity needed for the whole experiment, and then aliquoted into separate tubes).

2. The tube was then placed in a boiling waterbath for 3 minutes.

3. The tube was then taken out of the boiling water and snap freezed in a dry ice and ethanol bath.

The sequencing reaction

1. The tube was then taken and thawed between the fingers and 6.5 μl of labelling reaction mix (1 μl 0.1 M DTT, 2 μl dGTP labelling mix (1:5 dilution in water), 2 μl Sequenase 2.0 (1:8 dilution in 1 x Sequenase dilution buffer), 0.5 μl [α^{35} S] dATP, 1 μl H_2O) added to it and gently mixed in the pipette tip.

2. Then, 3.5 μl were dispensed onto the inside wall of 4 eppendorf tubes containing 2.5 μl of one of the ddNTP's; ddATP, ddCTP, ddGTP and ddTTP. They were spun down in an benchtop centrifuge for 5 seconds and transferred to a waterbath, set at from 37 - 42°C, depending on the template.

Stopping the reaction

1. After 5 minutes in the waterbath, 4 μl of STOP solution (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) were dispensed into the inside wall of each of the tubes, the tubes removed from the waterbath and spun for 10 seconds in the benchtop centrifuge, to stop the reaction.

2. The reaction products were then either used immediately or kept frozen at -20°C until electrophoresed.

2.4.5.3.2. Sequencing from a single stranded PCR product

The template was obtained as described in 2.4.5.1.2. The sequencing primers were used in the concentration of 50 - 200 pmol/reaction. All reagents were aliquoted before the reactions were started so everything was done in a continuous way. All the reactions were performed in 96 well microtitre plates.

Annealing of the primer to the template.

1. 50 - 200 pmol/ pmol primer were added to 6 μl of template and 2 μl 5 x reaction buffer with added ddH_2O to a final volume of 10 μl in multiples of 8 where possible.

2. The plate was then placed in an Hybaid Omnigene intelligent heating block (Hybaid, UK) or MJ Research Inc. programmable thermal controller (Genetic Research Instrumentation Ltd, UK), depending on the type of microtitre plates used), and heated at 65°C for 2 minutes.

3. The plate was then taken out of the heating block and put onto ice.

The sequencing reaction

1. 6.5 μl of labelling reaction mix (1 μl 0.1 M DTT, 2 μl dGTP labelling mix (1:5 dilution in water), 2 μl Sequenase 2.0 (1:8 dilution in Sequenase dilution buffer), 0.5 μl [α^{35} S]dATP, 1 μl H_2O) added to it and gently mixed in the pipette tip and left at room temperature for 2-5 minutes.

2. Then, 3.5 μl were dispensed onto the inside wall of the wells containing 2.5 μl of one of the ddNTP's; ddATP, ddCTP, ddGTP and ddTTP. They were spun down in an benchtop centrifuge for 5 seconds and

transferred to a waterbath, set at from 37 - 42°C, depending on the template.

Stopping the reaction

1. After 5 minutes in the waterbath, 4 μl of STOP solution (98% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) were dispensed into the inside wall of each of the wells, the microtitre plate removed from the waterbath and spun for 10 seconds in the benchtop centrifuge, to stop the reaction.

2. The reactions were then either used immediately or kept frozen at -20°C until electrophoresed.

2.5. LDL receptor measurements

2.5.1. Cell culture

Chinese hamster ovary (CHO) cells that are LDL receptor deficient (ldl-A7) (Sege et al 1986), kindly provided by Dr. M. Krieger, were used for the cell studies described in this thesis. The cells were maintained on Nutrient Mixture Ham's F-12 medium (Gibco), with 10% Foetal Calf Serum (FCS) (Gibco) with streptomycin (100 μg/ml) (Gibco), and penicillin "G" (100 units/ml) (Gibco) in a humidified incubator with 5% CO_2/95% air at 37°C. For the experiments, the medium was removed and the cells washed once in an isotonic solution of Puck's saline (Gibco). The cells were released from the culture flasks by incubating in 5 ml of trypsin - EDTA (Gibco) for 5 minutes at 37°C. The detached cells were then transferred to a 15 ml Falcon tube and spun at 1000 rpm for 5 minutes in a Sorvall 6000 bench centrifuge. The trypsin solution was removed and the cells resuspended in 1 ml of Nutrient Mixture Ham's F-12 medium, with 10% FCS. Resuspended cells were diluted, counted in a haemocytometer and the appropriate number of cells dispensed into the number of dishes or wells needed for the experiment.

The number of cells used were; for 90 mm dishes 8 x 10^5 cells/dish, for 45 mm dishes 4 x 10^5 cells/dish, for 35 mm wells 3.1 x 10^5 cells/well, and for 22.5 mm wells 2 x 10^5 cells/well.

2.5.1.1. In vitro mutagenesis

The expression vector used (pLDLR4 kindly provided by Dr David Russell, Dallas, Texas), contained the A370 allele of the human LDL receptor cDNA driven by the SV40 promoter. To construct the T370 allele site directed mutagenesis was carried out using the pALTER system, (Promega, Southampton, UK), following the suppliers' protocol. This system is based on a plasmid containing resistance genes for two antibiotics, tetracycline and ampicillin. In pALTER, the ampicillin resistance gene is non-functional because it contains a frameshift mutation, and the propagation of the plasmid is performed under tetracycline selection. Mutagenesis is achieved by annealing an oligonucleotide that repairs ampicillin resistance and an oligonucleotide that introduces the desired mutation to a single stranded form of the plasmid. After filling-in of the single stranded DNA and ligation, the double stranded DNA is transformed into a repair-minus bacterial strain, with selection for ampicillin resistance. The concentration of ampicillin repair oligomer used is one tenth that of the specific oligomer, so that ampicillin resistant plasmids are likely to contain the desired mutation. In this case, a 3.2 kb *Eco*RI-*Sac*I fragment of the LDL receptor cDNA (containing the 3' part of the LDL receptor gene including exon 8 as shown in Figure 4.3.1. in Chapter 4) cloned into the pALTER vector was used to introduce the T370 allele by site directed mutagenesis (kindly provided by Dr. Anne K. Soutar).

Single stranded (ss) phagemide DNA was prepared by co-transforming JM 109 bacterial cells with the R408 helper phage and the pALTER vector containing the 3.2 kb *Eco*RI *Sac*I fragment.

For the ssDNA preparation individual tetracycline-resistant colonies were picked from fresh M-9 plates (made as described in the pALTER protocol), and transferred into 2 ml of TYP broth (made as described

in the pALTER protocol) containing 15 μg/ml tetracycline and shaken at 37°C over night. The next day 100 μl of this was transferred into 5 ml of TYP broth and the culture infected with the R408 helperphage after 30 minutes at 37°C. This was then incubated for further 6 hours for the growth and release of the single stranded plasmid DNA into the medium.

The single stranded plasmid DNA was then harvested from the supernatant after pelleting the cells and purified as described in the Promega protocol.

Oligonucleotides were phosphorylated as described in the pALTER protocol and the ampicillin repair oligonucleotide (0.25 pmol) and the T370 oligonucleotide 5'ACACGAAGACCTGCAAAG3' (25 pmol) were annealed to 150 ng of the single stranded DNA. The mutant strand was synthesised with T4 DNA polymerase and T4 DNA ligase, as described in the pALTER protocol.

The synthesised mutant strand was then used to transform BMH 71-18 mut S competent cells. This strain is a mismatch repair minus strain of E. coli. that prevents the repair of the newly synthesized unmethylated strand. After incubation for one hour at 37 °C to allow expression of the ampicillin resistance gene, the cells were grown in the presence of ampicillin at 37 °C for 14 hours.

The cells were then harvested by centrifugation at 1000 rpm in a Sorvall 6000 bench centrifuge, and the plasmid recovered by the plasmid miniprep method. An aliquot of 0.1 g of the plasmid was the used to transform JM 109 bacterial competent cells. After recovery for one hour at 37° C, the cells were plated on LB plates containing ampicillin and grown for 14 hours at 37° C.

The resultant colonies were then picked for analysis of their plasmid DNA content. Miniprep DNA was prepared and sequenced essentially as described for the sequencing of double stranded DNA in Section 2.4.5.3.1. using an oligonucleotide comprising bases 859 to 879 of the LDL receptor cDNA.

The mutant LDL receptor cDNA inserts were then cloned into pLDLR4 as described by Webb et al (1992), using standard techniques as described by Sambrook et al (1989). The presence of the mutation in an otherwise unchanged cDNA was confirmed by sequencing of a large scale preparation of the plasmids as described by Webb et al (1992) and Sun et al (1994).

2.5.1.2. Transfection of CHO ldl-A7 cells

The transfection of the CHO ldl-A7 cells with the pLDLR4 plasmid was performed using the calcium phosphate transfection method with the co-transfection of pVSneo as described by Southern and Berg (1982). Stable transfectants were selected as Geneticin resistant colonies, as described by Davis et al (1986a).

Two solutions A and B were used to prepare the calcium phosphate complexes.

Solution A: 19 μg of the appropriate plasmid, 1 μg of the pSV2neo co-transfection plasmid, 120 μl 2 M $CaCl_2$ and ddH_2O to 1 ml. Solution B: 2 x HBS buffer: (10 x HBS buffer: (8.18% NaCl (w/v) or 1410 mM, 5.94% HEPES (Sigma, UK) (w/v) or 249 mM, 0.20% Na_2HPO_4 2 H_2O(w/v) or 11.3 mM).

Solution A (1 ml) was added dropwise to constantly agitated solution B (1 ml) in a 10 ml sterilin tube and left at room temperature for 40 minutes. Then 1 ml of this calcium phosphate DNA suspension was added to each dish of ldl-A7 cells that had been grown to confluence in 90 mm dishes. The cells were then kept at 37°C for 5 hours before the solution was removed and replaced with normal growth medium. After 24 hours the medium was replaced with growth medium containing 0.8 μg/ml Geneticin G418 (Sigma, UK). Cells were then incubated at 37°C in 5% CO_2/95% air, and examined for single colonies of cells over the next 2 weeks. Colonies were picked, seeded into separate dishes and grown for use in experiments.

2.5.2. Immunoblotting of the LDL receptor

For immunoblotting, cells were grown in 90 mm dishes in Nutrients Mixture Ham's F-12 medium with 10% FCS. In the afternoon before the experiments, cells were washed once in a medium containing 10% human lipoprotein deficient serum (LPDS) prepared by removing the lipoprotein fractions with gradients of KBr as described by Goldstein and Brown (1974), and then grown overnight in Nutrient Mixture Ham's medium with 10% LPDS, 0.1 μg/ml 25-hydroxylsterol and 10 μg/ml cholesterol to suppress any possible endogenous LDL receptor synthesis.

Harvesting cells

All harvesting and lysing was performed at 4°C. The dishes were put on ice and the medium removed. The cells were washed twice in 6 ml of buffer B (50 mM HEPES, 100 mM NaCl, pH 7.4). Buffer C (1 mM PMSF (Sigma, UK), 10 mM EDTA, 10 mM EGTA, 10 mM N-ethylmalemide, 2.2% DMSO in buffer B) was added to the dishes, 0.5 ml to each, and the cells scraped off the plastic with a rubber policeman and transferred into a 1.5 ml eppendorf tube. The cells were pelleted by spinning for 4 minutes in an Eppendorf centrifuge at 4°C at a maximum speed and the supernatant removed.

Lysing cells

The cells were lysed in 100 μl of lysis buffer (1% Triton X-100, 0.5 mM leupeptin (Sigma, UK) in buffer C) by passing them twenty times through a 1 ml syringe with a 21G needle. The lysed cells were left on ice for 10 minutes, then spun for 4 minutes in an Eppendorf centrifuge at 4°C at maximum speed. Fifty μl of the supernatant were transferred to a new tube and 20 μl of sample buffer added (35% glycerol, 1.75% SDS). Proteins were separated by SDS-PAGE (described in Section 2.3.2.) at 180 V for 4 hours.

Blotting onto nitrocellulose membrane

The gel was removed from the glass plate onto a 3MM paper pre-wet in blotting buffer (25 mM Tris-HCl - 192 mM Glycine (pH 8.3), 20% methanol). A pre-wet nitrocellulose membrane was placed onto the other side of the gel, taking care not to leave air bubbles between the gel and the filter and another sheet of wet 3MM paper put on the top of the filter. This was arranged into a specific holder and transferred into a BioRad blotting apparatus (BioRad, USA) containing blotting buffer and electroblotted onto the nitrocellulose membrane at 200 V over night at 4 °C.

Hybridisation to ^{125}I labelled antibody

The filter was removed from the electroblotting apparatus and laid inside a plastic bag and the bag sealed except for one corner. Through that hole, 25 ml of blocking buffer (50 mg/ml BSA, 0.2 v/v NP40 in stock buffer (10 mM Tris, 0.15 M NaCl) and adjusted to pH 7.4 with 5 N NaOH) was added, all air bubbles removed and the bag sealed and shaken on a rotator platform at 37°C. After 1 hour, the bag was cut open and the fluid allowed to drain away. The filter was then put into another plastic bag and treated as described above with the addition of 2 x 10^6 counts/ml of ^{125}I labelled monoclonal antibody 10A2 (Soutar and Knight 1986). The incubation with the antibody was for 2 hours at room temperature on a rocking platform, after which the bag was cut open and the fluid drained away. The filter was then rinsed three times with 100 ml of rinse buffer (10 mM Tris, 0.15 M NaCl) and then washed in 100 ml of detergent buffer (0.1% SDS, 0.2% NP40, 0.25% Na deoxycholate in stock buffer) three times for 30 minutes each time on a rocking platform. Finally the filter was rinsed three times in rinse buffer, dried with tissue paper, wrapped in Saran film and put on Kodak XAR-5 film over night at room temperature, and the film developed (for results, see Figure 4.3.2. in Chapter 4).

2.5.3. LDL receptor uptake and degradation

2.5.3.1. Isolation of LDL

LDL was prepared from plasma from an overnight fasting person. 100 ml were drawn into tubes containing heparin and spun at 3000 rpm for 20 minutes for isolation of plasma. The plasma was then used to separate LDL by number of density gradient centrifugations.

1. The volume and weight of the plasma was measured to determine its specific density.

d_i (initial density) = mg plasma / ml plasma

2. The density was then adjusted to 1.019 with a KBr solution of specific density of 1.35 according to the formula:

i) $V_{ds} = V_{plasma} (d_f - d_i) / (d_s - d_f)$, where

$_{ds}$ is the KBr density solution and d_f = the final density required.

The added solution was mixed gently but well to the plasma and transferred to a 1" x 3.5" sealable centrifuge tube. The tube was filled with KBr solution of specific density 1.019 and sealed and spun at 53000 rpm and 8°C for 18 hours in 70Ti rotor in a Beckman ultracentrifuge.

3. After centrifugation the tube was removed and sliced below the visible LDL band. The solution above the blade was aspirated into a flask, weighed and the volume measured for specific density determination.

4. Solid anhydrous KBr was then added to the above according to the formula:

ii) $KBr_{grams} = V\ (d_f - d_i) / 1-(0.312(d_f))$

to reach a density of 1.30. After gentle mixing it was transferred to a small sealable tube and spun at 50000 rpm in a vertical rotor VTi 50 for 2½ hours at 8°C.

5. The clearly visible LDL band was then aspirated with a syringe by piercing the needle just at the lower border of the LDL band. A volume of 5 ml was taken into a volumetric flask and weighed for specific density determination. KBr was then added according to formula ii) to a final specific density of 1.063 and the solution re-centrifuged at 43000 rpm for 18 hours in a 50.1SW rotor.

6. The LDL was aspirated from the top of the tube and dialysed against 1 litre of 0.15 M NaCl/1 mM EDTA with two changes.

2.5.3.2. Measuring of protein

The method used was the method of Lowry (1951)

Two solutions were made up for the protein assay. Solution A: (49 ml of 2% Na_2CO_3 in 0.1 N NaOH, 0.5 ml of 2% KNa tartrate, 0.5 ml of 1% $CuSO_4$) and solution B: (Folins reagent diluted 1:1 in dH_2O).

The standards consisted of 5 μg, 10 μg, 20 μg, 30 μg, and 40 μg of BSA in 0.5 ml H_2O. The samples were in 0.5 ml aliquots (the protein concentration should be between 5 μg and 40 μg for most accurate reading), and the blank was 0.5 ml of H_2O.

2.5 ml of solution A were added to each sample, mixed well and then left at room temperature for 10 minutes. Then 250 μl of solution B were added, mixed well and left for 30 minutes. The absorbance of

the samples was then measured at λ = 750 nm, using water as a blank.

2.5.3.3. Labelling of LDL with Na ^{125}I

For labelling of LDL, 5 mg of LDL was labelled with 5 mCi of Na ^{125}I as described by Bilheimer (1972). For the experiments labelled LDL and unlabelled LDL were mixed to a final concentration of 1 g/l and approximately 150 cpm/ng specific radioactivity.

2.5.3.4. Cell experiments

The experiment for measuring the uptake and degradation of LDL was done essentially as described by Goldstein et al (1976 and 1985).

Cells were plated in 12 well (22 mm in diameter) tissue culture trays at the density of 2 x 10^5/well, two days before the experiments. They were cultured in 0.5 ml of medium.

In the afternoon before the experiments, cells were washed once in a medium containing 10% LPDS and then grown overnight in Nutrient Mixture Ham's medium with 10% LPDS, 0.1 μg/ml 25-hydroxylsterols and 10 μg/ml cholesterol. The experiments were then carried out as described below.

2.5.3.4.1. Measurement of uptake and degradation of LDL

1. On the day of the experiments the medium was replaced with 0.5 ml of medium containing 2.5 mg/ml LPDS. The required amount of labelled LDL was added and the cells incubated for 4 hours at 37°C. Non-saturable uptake and degradation were determined in wells containing excess (1 mg/ml) of unlabelled LDL. Wells without cells were used as blanks.

2. At the end of the incubation, 400 μl of the medium was frozen at - 20 °C for later measurement of the amount of LDL degraded.

3. The rest of the medium was then removed and the cells washed 5 times with ice-cold washing buffer (0.9% NaCl, 10 mM Tris-HCl buffer pH 7.4, 2 mg/ml albumin), and once with washing buffer without albumin.

4. The cells were dissolved in 0.5 ml of 0.1 N NaOH and assayed for radioactivity and protein to give values for LDL uptake.

5. The frozen samples of medium were thawed, placed on an ice-bath and 45 μl of BSA (30 mg/ml) and 111 μl of TCA (3 M) was added. The tubes were vortexed and centrifuged at 3000 rpm for 10 minutes at 4°C.

6. 400 μl of the supernatant were then transferred to a new tube and 45 μl of 0.5 M KI and 111 μl of 0.7 M $AgNO_3$ were added and then vortexed. This was then spun at 3000 rpm for 10 minutes at 4°C.

7. 400 μl were then transferred into a new tube and assayed for radioactivity to give values for labelled LDL degraded to TCA-soluble products.

2.6. Plasma lipid measurements

The London samples of FH patients

Cholesterol, HDL cholesterol and triglyceride concentrations were measured by the chemical pathology departments of each of the hospitals, which with the lipid clinics were affiliated. All the laboratories were participating in national quality control schemes (NEQAS). Lp(a) was measured as described in Seed et al (1990). All measurements were performed on plasma samples after 12 hours fasting. LDL cholesterol was estimated with Friedewalds formula (Friedewald et al 1972).

The Southampton sample of FH patients

Cholesterol, HDL cholesterol and triglyceride concentration were measured in the laboratories of the department of chemical pathology Southampton Hospital, which is participating in national quality control schemes (NEQAS). Serum triglycerides were measured on samples after 12 hours fasting. LDL cholesterol was estimated with Friedewalds formula (Friedewald et al 1972).

The Icelandic Sample

Icelandic blood samples were collected at The Icelandic Heart Association Research Centre after an overnight fast, and measured in their laboratory. Total serum cholesterol and serum triglyceride were measured by automated enzymatic colorimetry (Cobas Mira, Roche). HDL cholesterol was measured enzymatically after phosphotungsten/magnesium precipitation. Both internal and external laboratory controls were used. LDL cholesterol was estimated with Friedewalds formula (Friedewald et al 1972). Apo(a) was measured by radioimmunoassay (Pharmacia Diagnostics AB, Sweden) and 1 U/l of apo(a)

equals 0.1 mg/dl of Lp(a).

The EARS sample

All lipid analyses were performed in the Institute of Biochemistry, Royal Infirmary, Glasgow, using procedures recommended by the Lipid Research Clinic's Manual of Operations (1974). LDL cholesterol was calculated by the Friedewald formula (Friedewald et al 1972). The apoproteins AI and B were measured in Service de Recherche sur les Lipoprotéines et l'Athérosclérose (SERLIA), Institut Pasteur, Lille, by immunonephelometry on a Behring BNA nephlometer (Marburg, Germany), using Behring antisera and standards.

The NPHSII sample

A non-fasting blood sample was drawn by venepuncture using a 'vacutainer' system and minimal stasis. Cholesterol concentration in serum was measured by enzymic assay with reagents from Sigma (Poole, Dorset, UK).

2.7. Statistics

Statistical analysis was performed using the software package SPSS/PC+ for the analysis of lipid levels in FH patients Chapter 3., and for the analysis of the data for the Icelandic population Chapter 4. Section 4.2. The data from the EARS study were analyzed using the SAS statistical software package (SAS Institute Inc). Data was $\log_{10}$ transformed prior to carrying out parametric tests: regression, analysis of variance (ANOVA) and correlation analysis, if it was not already approximately normally distributed and if it appreciably reduced the kurtosis and skewness of the data.

Gene frequencies and Hardy-Weinberg equilibrium

In all the samples, the gene counting method was used for the estimate of allele frequencies. For the sample allele frequencies, 95% confidence intervals (CI) were calculated according to Colton (1974):

$$95\%\ CI = \pm 1.96\ \sqrt{p(1-p)/n},$$

where p = proportion of study sample with given allele and n = number of participants. Comparison of allele frequency between populations was performed by χ^2-analysis and statistical significance was considered to be at the 0.05 level.

Estimation of Hardy-Weinberg equilibrium was done by comparing the expected genotype distribution for a given allele frequency with the observed genotype frequency using χ^2-analysis. The expected genotype distribution in a sample is given by the formula:

$$p^2 + 2pq + q^2 = 1.0,$$

where p is the frequency of one allele and q is the frequency of the other allele and $p + q = 1.0$. p^2 = frequency of the commoner homozygous genotype, 2pq = frequency of the heterozygous genotype and q^2 = frequency of rarer homozygous genotype.

Calculation of 95% confidence limits for log_{10} transformed data

For traits that were not normally distributed, 95% confidence limits were calculated on the log_{10} transformed data as described by Sokal and Rohlf (1981), according to the formula:

$$95\%\ CI = \log_{10} \text{ of mean} \pm (t_{0.05\,(n-1)} \times \sqrt{(\log_{10} SD)^2/n}),$$

where CI = confidence interval, n = number of individuals, and SD = standard deviation.

Comparisons of differences between group means

For the FH (Chapter 3.) samples and the Icelandic samples (Chapter 4. Section 4.2.) the comparison of the group means of the traits examined was performed using the parametric tests: Student's t-test or analysis of variance (ANOVA), and when the data was not normally distributed, the Kruskall-Wallis one way ANOVA. Statistical significance was considered to be at the 0.05 level.

Statistical analysis of the EARS sample

The analysis of the data from the EARS sample (Chapter 3. Section 4.4.) was carried out by Dr. Viviane Nicaud at the EARS analysis team in Paris, using a strategy devised by myself.

Associations of lipid and apolipoprotein levels with genotype were tested by ANOVA with adjustments for the stratification criteria of the study (sex, region and case/control status and age) and for BMI as a covariates. Homogeneity of genotype effects according to status, gender and region were tested by introducing corresponding terms of interaction in the model. In all statistical analyses, triglyceride distributions were log-transformed to remove positive skewness. Statistical significance was taken to be $p < 0.05$.

Statistical analysis of the NPHSII sample

Statistical analysis of the NPHSII sample (Chapter 3. Section 4.5.) was carried out by Dr George Miller at St Barholomew's Hospital in London, using a strategy devised by myself. Association of cholesterol concentration with genotype was tested by ANOVA, with adjustments for age, BMI and smoking. Statistical significance was taken to be $p < 0.05$.

2.8. Subjects and materials

2.8.1. FH patient samples

2.8.1.1. Diagnostic criteria for FH

The criteria for diagnosis of familial hypercholesterolaemia used is the same as used for the Familial Hyperlipidaemia Register, which is a British register of patients with FH, and is published in Ball and Mann (1993) and shown in Table 2.8.1.

Table 2.8.1. Diagnostic criteria for FH

Definite FH			
Cholesterol	>7.5 mmol/l (Adult)		
or	>6.5 mmol/l (Child under 16)		
LDL cholesterol	>4.9 mmol/l (Adult)		
Plus			
Tendon xanthomas in patient or relative			
Possible FH			
Cholesterol	>7.5 mmol/l (Adult)		Family history myocardial infarction
	>6.5 mmol/l (Child under 16)		under 50 (2° relative)
		Plus	under 60 (1° relative)
or			or
			Family history raised
LDL cholesterol	>4.9 mmol/l (Adult)		cholesterol in 1° relative

Table from Ball and Mann (1993)

2.8.1.2. The FH 200 London sample

The patient sample consisted of 189 heterozygous and 11 homozygous patients with the clinical diagnosis of FH (as defined in Section 2.8.1.1.), selected randomly from a group of patients with that diagnosis and who had been attending one of three lipid clinics in the London area; Hammersmith Hospital (Dr Gilbert R Thompson), Charing Cross Hospital (Dr. Mary Seed), and St. Mary's Hospital (Dr. Mary Seed) (80 were definite of which 11 had homozygous FH, 120 were diagnosed as possible FH). The 11 patients that had homozygous FH, based on plasma cholesterol levels of > 15 mmol/l, the presence of cutaneous and tendon xanthomata, cardiovascular involvement before puberty, and hypercholesterolaemia in at least one parent. Clinical details of most of these homozygous FH patients have been described

previously (Allen et al 1980, Gudnason et al 1995b).

2.8.1.3. The UCH sample

The UCH sample consisted of 50 apparently unrelated heterozygous FH patients, (defined according to the criteria described in Section 2.8.1.1.) with the diagnosis of definite FH (n=35) and possible FH (n=15), attending the lipid clinic in the Department of Medicine, University College London Medical School (Dr. John Betteridge). In addition, further 22 patients (15 definite FH and 7 probable FH) were added to the sample for the analysis carried out in Chapter 3 Section 3.3.

2.8.1.4. The Southampton sample

The Southampton sample consisted of 50 FH patients (defined according to the criteria described in Section 2.8.1.1.), that were recruited from Southampton and South West Hampshire Health District (Dr. INM Day). 20 patients were diagnosed with definite and 30 with possible FH.

2.8.2. Population samples

2.8.2.1. The Icelandic sample

The subjects, 152 men and 166 women, age 15-78 years old, were a subset of randomly selected participants in The Icelandic National Diet Survey 1990. All subjects came from the south-west part of Iceland and the greatest proportion from the capital Reykjavik. All subjects completed a questionnaire concerning smoking habits. In this study smokers were defined as all those who currently smoked tobacco or had ceased smoking up to 14 days before blood sampling. Those on lipid lowering drugs, thyroxin,

or with diabetes were excluded. Blood samples were collected at The Icelandic Heart Association Research Centre after an overnight fast.

2.8.2.2. The EARS sample

Students from 14 Universities throughout Europe, aged between 18-26 whose fathers had a verified heart attack before the age of 55, were recruited for the study and represent the cases. Two age and sex matched controls for each case were recruited by computer selection from the same university population. The 14 recruitment centres were grouped into five regions for analysis, on the basis of IHD mortality rates, geographical and linguistic proximity: Finland (Helsinki and Oulu), Great-Britain (Bristol and Glasgow), Northern (Gothenburg, Aarhus, Hamburg), Middle (Innsbruck, Ghent, Zurich), and Southern Europe (Bordeaux, Napoli, Barcelona and Reus). Due to the small number of subjects genotyped in Barcelona and Napoli (due to failure of DNA extraction and/or difficulty with PCR), these centres were excluded from the analysis. Fasting venous blood was collected and details of lifestyle ie smoking, alcohol consumption and physical exercise, as well as personal and family history and physiological measurements were taken using standardised questionnaires and protocols. Detail of these protocols have been described elsewhere (The EARS group 1994).

2.8.2.3. The NPHSII sample

The men in this study were recruited from medical practices in Surrey, England, and St Andrews, Scotland, with their informed consent. All were Europeans of Caucasian origin, aged 50 to 61 years, and without clinical evidence of CHD.

2.9. Suppliers of materials and reagents

Oligonucleotides were purchased from Severn Biotech Ltd. (Kidderminster, UK), Oswell Scientific Ltd. (Edinburgh, UK), Advanced Biotechnology Centre (Charing Cross and Westminster Medical School, London, UK), and Genosys Ltd. (Cambridge, UK). All chemicals were obtained from BDH Ltd. (Poole, Dorset, UK) unless otherwise stated.

CHAPTER 3 - MUTATIONS IN EXON 4 OF THE LDL RECEPTOR GENE

3.1. Screening for known mutations

3.1.1. Introduction

Exon 4 codes for three binding repeats of the LDL receptor; repeats 3, 4 and 5. All these repeats have been shown to be essential for the binding of apoB containing lipoproteins to the receptor (Esser et al 1988, Russell et al 1989), and in addition, repeat 5 has also been shown to be essential for the binding of apoE containing particles (Esser et al 1988, Russell et al 1989). Functionally it may be expected that mutations affecting repeat 5 of the LDL receptor that destroy both the ability to bind apoB containing ligands as well as apoE containing ligands will be more deleterious than mutations affecting only the apoB binding ability. This might be reflected in a greater degree of hyperlipidaemia or more severe atherosclerosis. When the study, described in this thesis was started, 5 mutations were known in exon 4. These were D154N (Kotze et al 1989a), S156L (Hobbs et al 1989), D206E (Kotze et al 1989a, Leitersdorf et al 1989b, Kotze et al 1990), E207K (Leitersdorf et al 1990) and a 3 bp deletion G197 (Hobbs et al 1990), with the three last mutations being in repeat 5. Two of these mutations can be detected with restriction endonucleases, D154N with *Mbo*II and D206E with *Dde*I, while the other three needed detection by allele specific oligomelting.

3.1.2. Methods

The samples examined were the FH 200 London samples described in Chapter 2, Section 2.8.1.2. The PCR, digestion of the PCR fragments with restriction enzymes, allele specific oligomelting and sequencing were performed as described in Chapter 2, Sections 2.2.1., 2.3.2., 2.4.3., and

2.4.5. respectively

3.1.3. Results

A total of 211 LDL receptor defective alleles were screened for the known mutations in exon 4 of the LDL receptor gene. No patients were identified with the D154N or the E207K mutations. Figure 3.1.1. a) shows the amplification of the single 405 bp fragment I (the whole of exon 4) using primers p 4.1 and p 4.2 (see Figure 2.2.1. and Table 2.2.1. in Chapter 2, Section 2.2.1.). Figure 3.1.1. b) shows the *Mbo*II digestion of that fragment into two fragments of 225 bp and 180 bp, giving unambiguous results when electrophoresed on an agarose gel. No patient in this sample was found to carry this particular mutation.

Three patients were found to carry the D206E mutation. This mutation creates an additional restriction site for the restriction endonuclease *Dde*I. Figure 3.1.2. shows the PAGE of the *Dde*I digestion of the 234 bp long fragment II (for details of this fragment, see Figure 2.2.1. and Table 2.2.1. in Chapter 2, Section 2.2.1.) into two fragments of 70 bp and 64 bp in addition to the two constant fragments of 134 bp and 100 bp. Furthermore, the demonstration of the cosegregation of the restriction pattern for the D206E mutation with a particular haplotype of the LDL receptor gene in one of the families, is shown on Figure 3.1.2., and was shown to cosegregate with hypercholesterolaemia. This mutation was also confirmed by sequencing (Figure 3.1.3.) to be the same as that reported by Kotze et al (1989a), a transversion of a C to a G at base 695 in the LDL receptor gene altering codon 206 GAC to GAG.

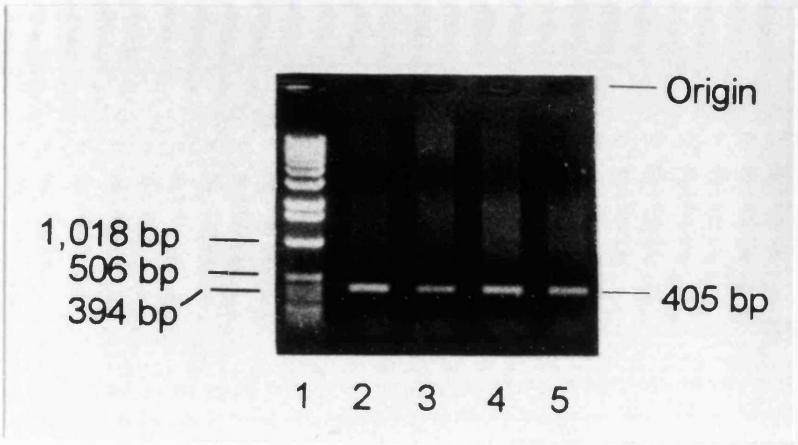

Figure 3.1.1. a) Amplified fragment of the whole of exon 4. Lane 1 contains 1 kb ladder and lanes 2 - 5 contain the 405 bp fragment, described in the text.

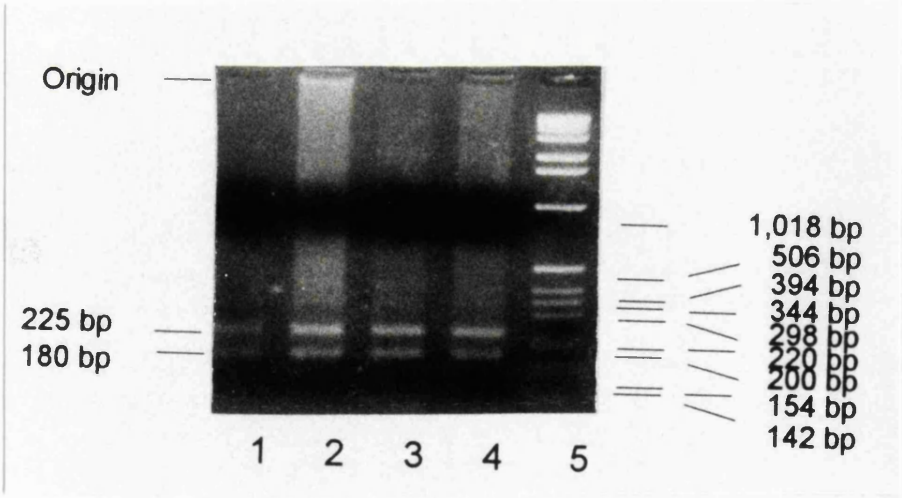

Figure 3.1.1. b) Amplified fragment of the whole exon 4 digested with *Mbo*II. Lane 5 contains 1 kb ladder and lanes 1 - 4 the 405 bp fragment completely digested into two fragments of 225 bp and 180 bp.

In 12 patients there was an unexpected pattern detected on the PAGE of a *Dde*I digested PCR fragment II. These patterns could be grouped into three distinctive patterns. Figure 3.1.4. shows a polyacrylamide gel with these patterns in addition to the pattern seen with the D206E (lane 4). The patterns were; firstly in lane 1, a new *Dde*I restriction pattern (seen in one patient), with two smaller fragments seen in addition to the fragments of 134 bp and 100 bp normally observed with this restriction enzyme, and different from the restriction fragments seen for the D206E mutation seen in lane 4. These were shown to be 77 bp and 57 bp long and proven by sequencing (Figure 3.1.5.) to be due to a novel single base substitution, a transversion of a C to an A. This substitution changes the TGC codon for cysteine in position 210 to a stop codon, TGA, designated C210X.

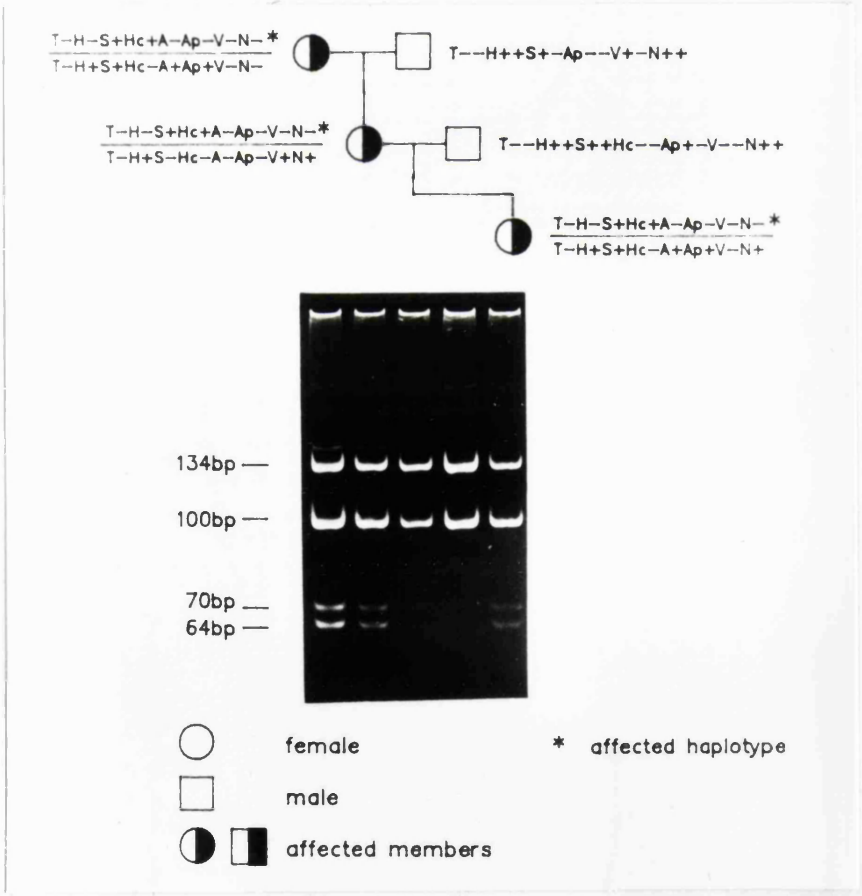

Figure 3.1.2. Cosegregation for three generations of the D206E mutation in a family with FH. The FH haplotype is marked with an asterisk and coincides with the *Dde*I restriction pattern, characteristic for the D206E mutation.

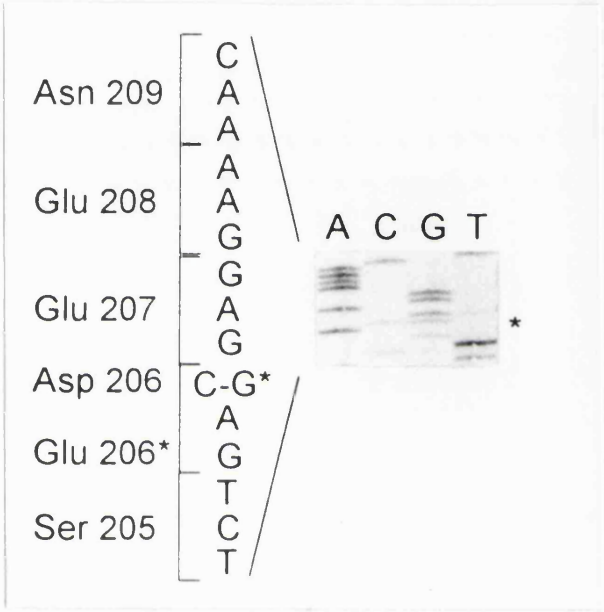

Figure 3.1.3. Sequencing of the (GAC) aspartate 206 to (GAG) glutamate mutation. The position of the base change is marked with an asterisk.

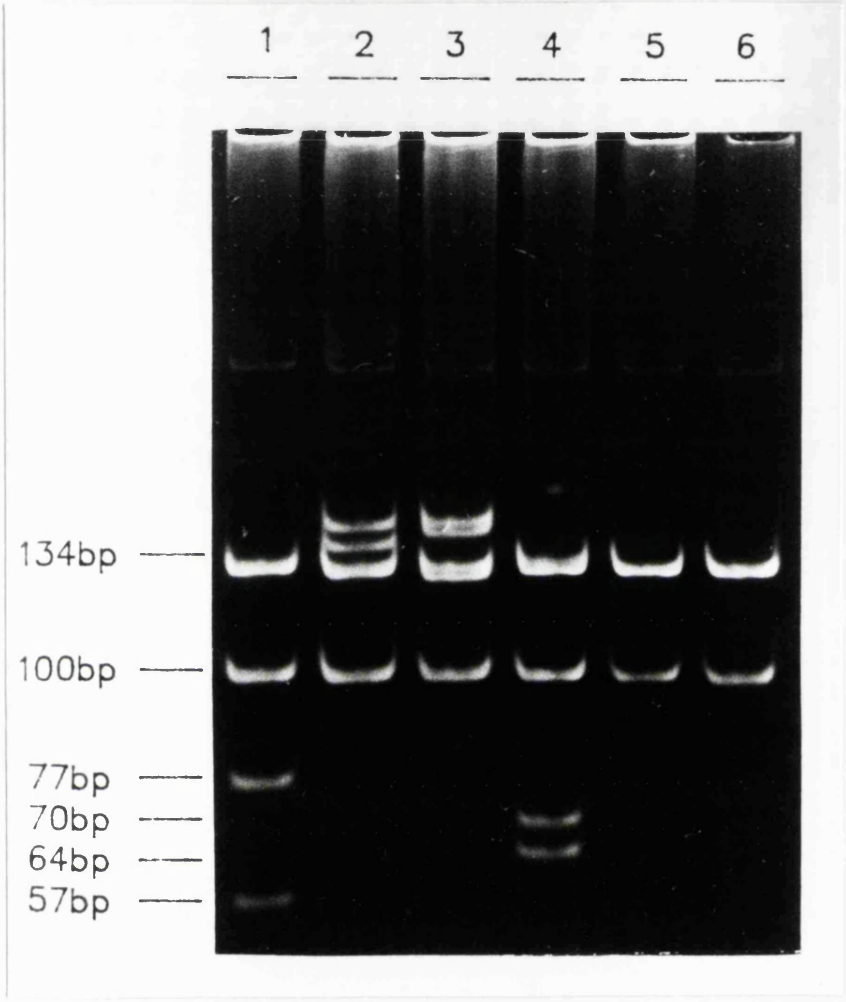

Figure 3.1.4. Ethidium bromide stained PAGE of DdeI digested PCR 234 bp fragment of the 3' part of exon 4, showing all the detected patterns (described in the text). Lanes 5 and 6 show the normal restriction pattern of the fragment into 134 bp and 100 bp. Lane 1 has additional fragments of 77 bp and 57 bp of the C210X, lane 2 has the heteroduplexes characteristic for the 3 bp deletion, ΔG197, lane 3 has the heteroduplexes characteristic for the 2 bp deletion, Fs206, and lane 4 is the D206E mutation with the additional restriction fragments of 70 bp and 64 bp.

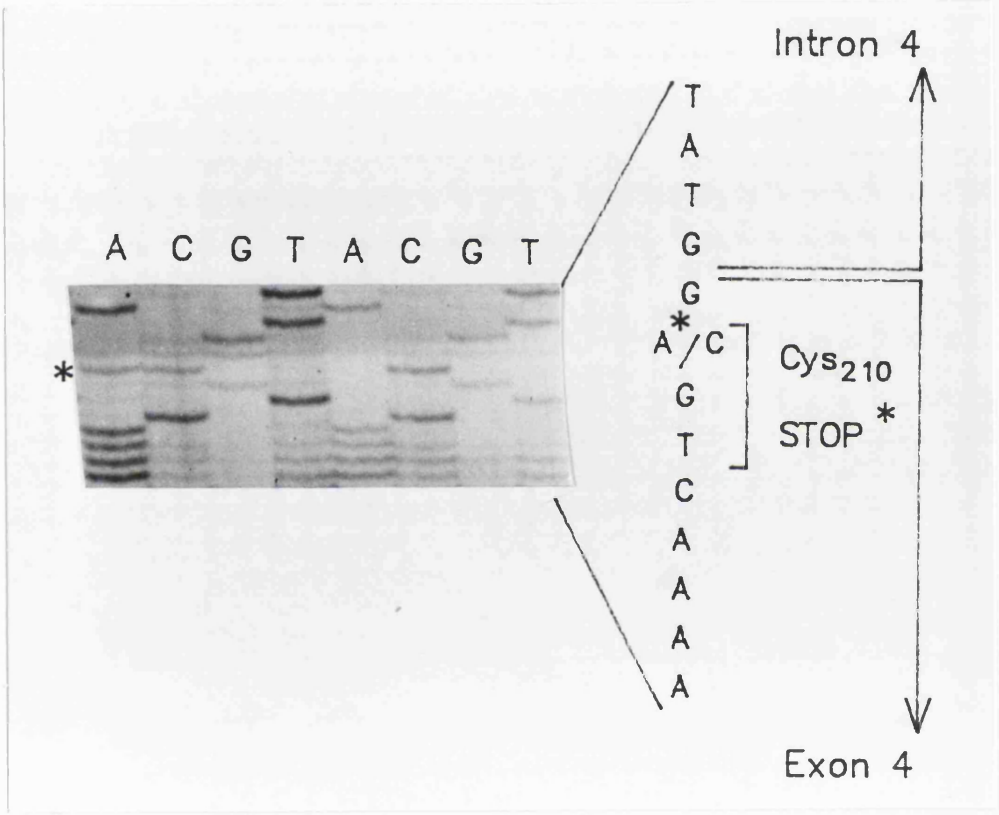

Figure 3.1.5. Sequence of the C210X mutation, on the left and of a normal sequence, on the right. The asterisk denotes the mutation.

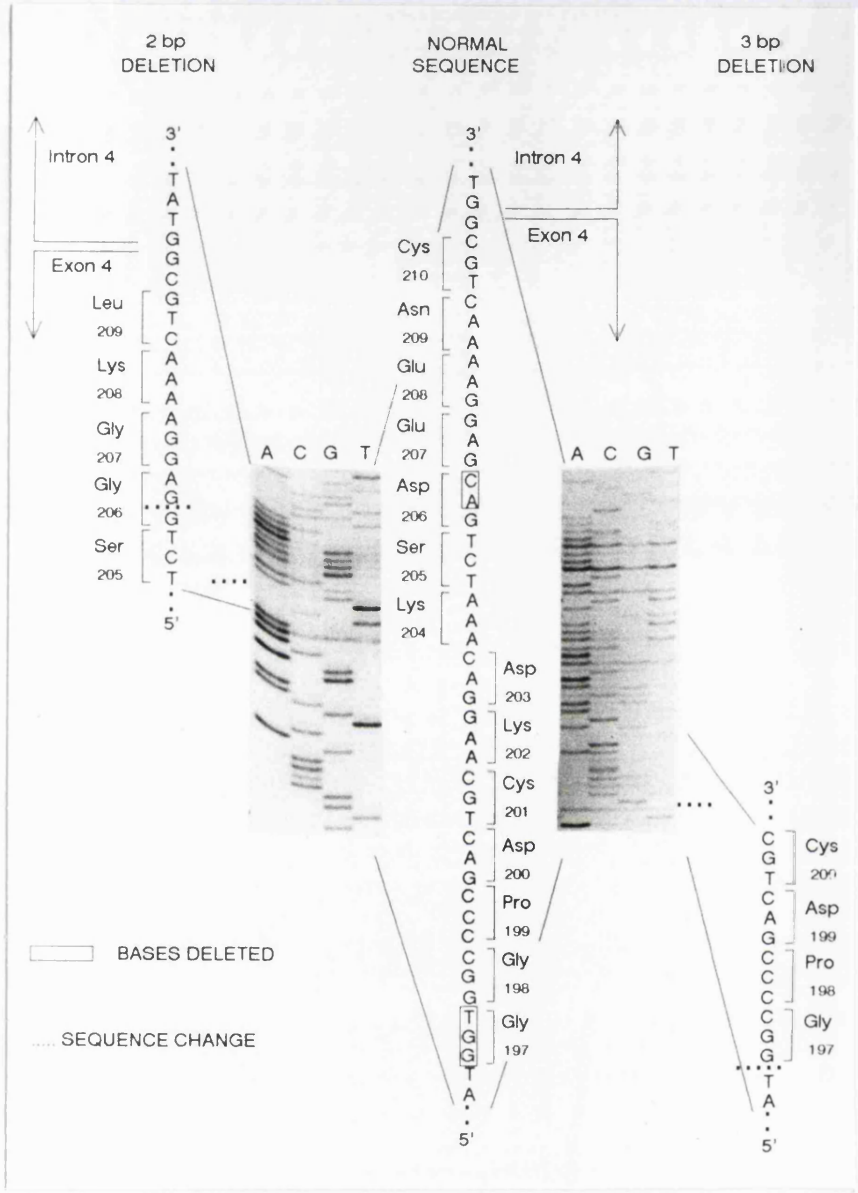

Figure 3.1.6. Sequences of the two small deletions; the ΔG197 on the right and Fs206 to the left. The normal sequence is written out between the two sequencings, with boxes around the deletions. The position of the sequence change is marked by dotted lines next to the pictures of the sequencing ladder and in the mutant sequences, written out to the left (for the 2 bp deletion), and to the right (for the 3 bp deletion). The normal amino acid sequence accompanies the normal DNA sequence and the mutant amino acid sequences accompany the mutant DNA sequences.

Secondly there were other patterns with larger extra bands Figure 3.1.4. lanes 2 and 3. Initially this was proposed to be a PCR artefact, but this "artefact" was seen to be present in two pairs of siblings. In each of these patterns there were two larger extra bands. One pattern was found in 5 apparently unrelated patients and the other pattern in 6 apparently unrelated patients. It was most likely to represent heteroduplexes, recently described for other genes, and seen accompanying small deletions (Nagamine et al 1989). This turned out to be the case as shown by sequencing (Figure 3.1.6.). One mutation, that was found in 6 patients was the previously described 3 bp deletion of GGT (Figure 3.1.6. right), deleting Glycine 197 and designated ΔG197 (Hobbs et al 1990). The sequence change can be clearly seen as the sequence reads A T G/G G/G T/C G/C G/C C/C G/G etc. This was also confirmed by using ASO analysis (Figure 3.1.7.). A doublet in the 134 bp fragment can be seen on the polyacrylamide gel in the sample with this mutation (Figure 3.1.4. lane 2).

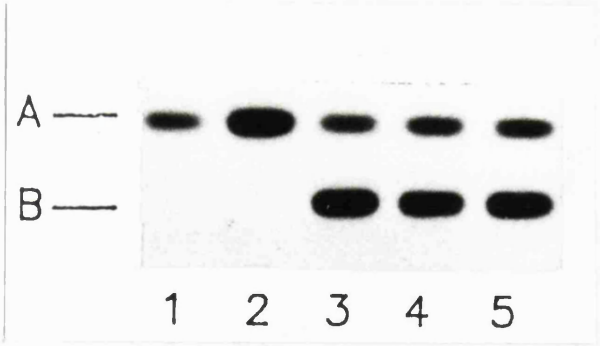

Figure 3.1.7. Analysis of the ΔG197 mutation by ASO technique. Lane A is hybridised with the probe for the normal allele and lane B with the probe for the mutant allele. Samples 3,4 and 5 have the ΔG197 mutation.

The other small deletion that was found was a novel deletion of an AC, the last two nucleotides (bases 694 and 695) in codon 206, designated Fs206. This was found in in 5 patients and is shown by sequencing in Figure 3.1.6. (left). The frameshifting of the sequence of the 2 bases is clearly seen; T C T G A/G C/A G/G A/G etc.

The other two known mutations in exon 4 were the S156L and the E207K, which are identifiable by hybridisation of allele specific oligonucleotide probes to PCR amplified fragment II. Excluding the presence of a mutation from a sample by this method requires a positive control for that particular mutation. Such a positive control was synthesised for both these mutations using the allele specific oligonucleotide probe as a PCR primer (described in Chapter 2, Section 2.4.3.3.), and the resulting product electrophoresed on an agarose gel along with PCR fragment II, and blotted onto the membrane and hybridised with the appropriate probe. No patient was identified with the E207K mutation (Figure 3.1.8.). One patient was identified as having the S156L mutation (Figure 3.1.9.). This was also confirmed by sequencing (Figure 3.1.10.) as a C to a T transition altering the codon TCG to TTG and changing serine to leucine.

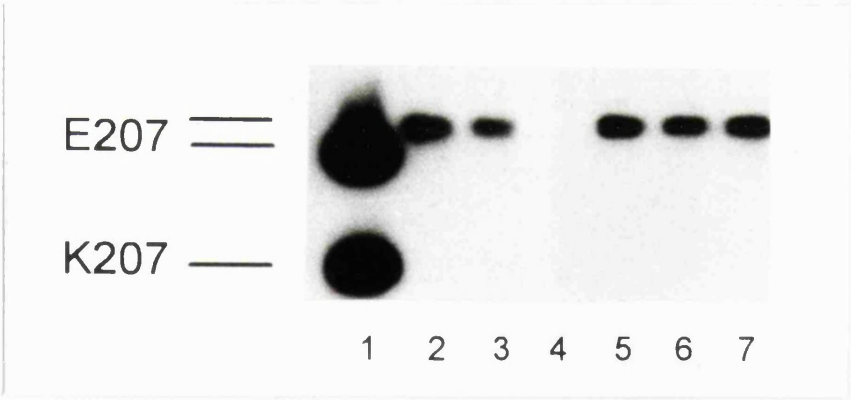

Figure 3.1.8. ASO analysis with the E207K probes. The synthesised control is in lane 1.

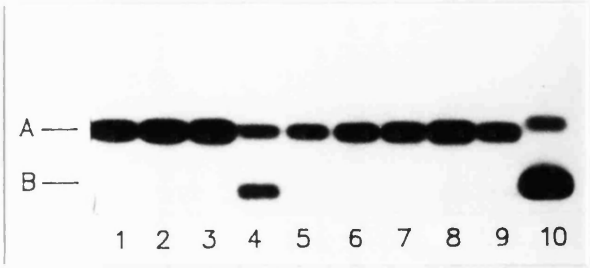

Figure 3.1.9. ASO analysis with the S156L probes. The synthesised control is in lane 10 and a positive sample in lane 4.

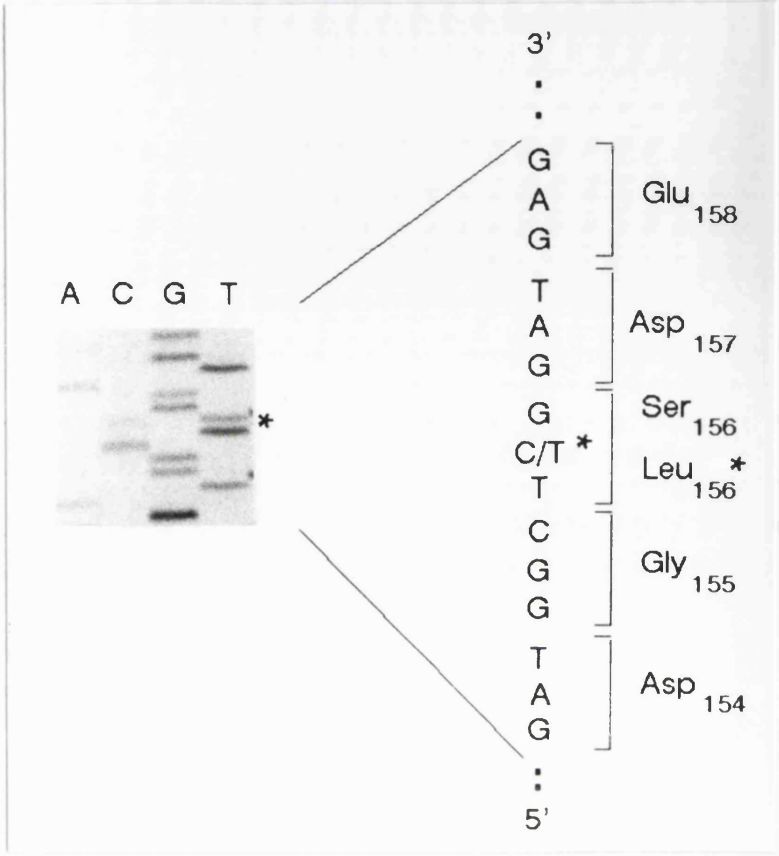

Figure 3.1.10. Sequencing of the S156L mutation. The position of the sequence change is marked with an asterisk.

Sixteen patients were found with one of the 5 different mutations identified in exon 4 in this sample of 200 FH patients. Table 3.1.1. shows the number of patients with each of these mutations, as well as their country of origin. The two deletions (ΔG197 and Fs206) were the most frequently found mutations in this part of the gene in this sample. The 3 bp deletion was only found in patients of Jewish origin. All the patients with the 2 bp deletion were of British origin. The D206E mutation was found in patients of British and South African origin, the C210X mutation in a patient of British origin, and the S156L mutation in a patient of Polish origin.

The RFLP genotypes of patients with detected mutations were determined at six polymorphic sites within the LDL receptor gene. Where a patient or a relative was homozygous for the RFLP the haplotype of the defective allele could be defined unambiguously. Where the patient was heterozygous for the RFLP the haplotype was deduced from information from available relatives, assuming no recombination had occurred. An example of this is shown in Figure 3.1.2. for the D206E mutation. The two other unrelated patients with this mutation have a haplotype compatible with that observed in the first family, though the haplotypes could not be determined unambiguously. For the ΔG197 all the six patients have the same haplotype for the defective allele, which differs from the haplotype that is deduced as common for all the 2 bp deletion patients (Table 3.1.1.).

The biochemical and clinical characteristics of the patients in whom mutations in exon 4 were detected are presented in Table 3.1.2. The untreated total plasma cholesterol and LDL cholesterol levels vary widely, even within groups of patients with the same mutation. It is though noteworthy that the plasma cholesterol concentration is higher than the diagnostic criteria for FH laid out in Chapter 2 section 2.8.1.1.

Table 3.1.1.

Frequency of mutations in Exon 4 of the LDL-R gene

MUTATION	No. IN THIS STUDY†	ETHNIC ORIGIN OF PATIENTS (No)	HAPLOTYPE OF THE DEFECTIVE LDL-R GENE‡						PREVIOUS DESCRIPTION
			T	S	H	A	V	N	
D154N	0								Kotze et al 1989a
S156L	1	Poland (1)	-	+	-	+	-	+	Hobbs et al 1989
D206E	3	Afrikaner (1)	-	+	+	-	-	-	Leitersdorf et al 1989b
		British (2)						¶	
E207K	0								Leitersdorf et al 1990
ΔG197	6	Jewish (6)	-	+	-	+	-	-	Hobbs et al 1990 Meiner et al 1991
C210X	1	British (1)	-	+	±	±	±	+	none
Fs206	5	British (5)	-	+	+	-	±	+	none

† Number found in the group of 211 LDL receptor defective alleles. ‡ Genotype as determined by haplotype. + or - indicate the presence and the absence of the cutting site respectively. Not able to determine unambiguously = +/-. T = *Taq*I, S = *Stu*I, H = *Hinc*II, A = *Ava*II, V = *Pvu*II, N = *Nco*I.

¶ Additional polymorphisms in the haplotype for this mutation are *Sph*I - and *Apa*LI-3'-.

Table 3.1.2

Clinical characteristics of the individuals with various mutations in Exon 4 of the LDL receptor gene.

Mutation	Sex	Age‡	TC¶ mmol/l	TG¶ mmol/l	HDL¶ mmol/l	LDL-C* mmol/l	TX**	CAD†
S156L	M	47	11.30	1.99	1.25	9.15	Y	N
D206E	F	29	7.90	0.90	1.24	6.25	Y	N
	M	50	11.20	0.90	0.83	9.96	Y	N
	F	61	13.30	1.70	1.70	10.83	Y	Y
C210X	F	43	8.90	1.13	1.45	6.93	Y	Y
ΔG197	F	39	11.00	0.80	0.96	9.67	Y	N
	M	52	10.70	1.50	1.29	8.62	Y	Y
	F	58	14.70	2.20	1.10	12.60	Y	Y
	M	36	10.40	0.73	1.24	8.80	Y	N
	M	49	12.40	1.45	1.14	10.60	Y	Y
	M	36	9.30	0.77	1.11	6.85	Y	N
Fs206	M	40	8.90	0.69	0.98	6.60	Y	N
	M	49	10.60	1.10	0.96	9.10	Y	Y
	M	43	12.80	1.15	1.48	10.79	Y	N
	M	46	9.60	1.20	1.10	7.95	Y	Y
	F	34	9.70	0.90	1.40	7.77	Y	N

‡ Age at cholesterol measurement. ¶ Untreated levels. * Calculated by Friedewald's formula: LDL-C = TC - HDL-C - TG/2.2 mmol/l. ** Y denotes the presence of tendon xanthomas. † +ve diagnosis of CAD as myocardial infarct or coronary artery bypass graft or angina pectoris diagnosed by coronary angiography or +ve exercise test.

3.1.4. Discussion

The spectrum of mutations in exon 4 found in this group of patients is likely to reflect the fact that the general population in London is mixed with regard to racial and ethnic origin and comes from a dispersed geographical background. It was thus not surprising that it exhibited the spectrum of mutations found elsewhere in the world. Of the five previously reported mutations in this exon, three were represented in the London sample. No patients were found with the D154N mutation reported in Afrikaners in South Africa (Kotze et al 1989a), or the E207K found in a French Canadian and a Mexican patient (Leitersdorf et al 1990). However, two of the previously described point mutations were found in the London sample, S156 and D206E.

The S156L change that has been described in a Puerto Rican family living in the United States (Hobbs et al 1989) was identified in one patient from London who is a second generation immigrant from Poland. The haplotype of the L156 allele was determined as *Taq*I$^-$, *Stu*I$^+$, *Hinc*II$^-$, *Ava*II$^+$, *Pvu*II$^-$ and *Nco*I$^-$. This allele differs in the *Nco*I and *Ava*II polymorphism from the haplotype reported in the initial description of this mutation (Hobbs et al 1989), and is thus likely to have arisen independently.

The D206E mutation, which occurs in 65% of the patients with FH in the Afrikaners population in South Africa (Kotze et al 1990), was found in three patients in this sample. One of the patients identified in the London sample is from South Africa but is not of Afrikaner origin, while the other two have lived in England for many generations with no evidence of recent migration. For all three patients the genotypes were consistent with a six polymorphism haplotype for the E206 allele determined unequivocally in one family as *Taq*I$^-$, *Sph*I$^-$, *Stu*I$^+$, *Hinc*II$^+$, *Ava*II$^-$, *Apa*LI-

3'-, *Pvu*II- and *Nco*I-. This is consistent with the haplotype for this mutation reported in the Afrikaner population (Leitersdorf et al 1989b). This mutation has also been reported in a FH patient in America of English ancestry where the mutation is on a haplotype differing from the South African patients only at the 3'*Apa*LI polymorphism which is downstream of the LDL receptor gene (Hobbs et al 1990). The majority of the Afrikaner population is of Dutch origin (Botha and Beighton 1983) but the D206E mutation has not been found in Dutch FH patients in Holland despite an extensive search but has recently been found in a FH patient there, who is of British ancestry (Defesche et al 1993). However, an estimated 5% of the Afrikaans-speaking population in 1867 were of English descent (Botha and Beighton 1983). It is therefore possible and widely accepted now, that this mutation occurred originally in an individual in England, although as it results from a mutation at a CpG dinucleotide, (though it is a transversion), it may have risen independently. Both the D206E and the S156L mutations occur in a CpG dinucleotide as does the third single base substitution identified in this sample, which is a transversion of a C to an A, which changes the TGC code for cysteine in position 210 to a stop codon, TGA.

The other mutations found in this exon were two small deletions, both of which were relatively common in the sample. The ΔG197 occurs in six of the FH patients in London who are all of Jewish origin and one of whom is a recent immigrant from South Africa. All alleles carrying the deletion have, or are consistent with, the same haplotype which is *Taq*I-, *Stu*I+, *Hinc*II-, *Ava*II+, *Pvu*II- and *Nco*I-. This mutation was originally described in an FH homozygous patient living in the United States (Hobbs et al 1990). The mutation causes a reduced rate of intracellular processing of the receptor protein, leading to a reduced number of receptors on the cell surface (Hobbs et al 1990). This deletion has recently been identified at a high frequency in patients of

Lithuanian Jewish origin (Meiner et al 1991), and the reported haplotype of the chromosome carrying the deletion is compatible with that observed in the patients from London.

The other small deletion (Fs206) is the next most frequent mutation in this sample, occurring in five of the patients, all of them consistent with the mutation being on the haplotype *Taq*I$^-$, *Stu*I$^+$, *Hinc*II$^+$, *Ava*II$^-$, *Pvu*II$^+$ and *Nco*I$^+$. All of these patients are of British descent. If the RNA is transcribed the deletion would lead to a frameshift and a stop codon and create a truncated protein of 217 amino acids. By comparison with other mutant forms of receptor protein it is likely that this protein would be rapidly degraded in the cell (Hobbs 1990), it is also likely that the RNA might be degraded rapidly as premature stop codons render the mRNA unstable (Urlaub et al 1989, discussed in McIntosh et al 1993).

A range of clinical expression and lipid levels was seen for the five mutations where there was more than one individual with the same defect. It was not possible from this data to draw any conclusions about the relationship between a specific mutation and the biochemical and clinical parameters, but this should be possible as more FH patients with defined mutations in the LDL receptor gene become available. At this stage, exon 4 of the LDL receptor gene had at least seven different reported mutations causing FH and because of the high CpG content, and the fact that exon 4 codes for a critical region in the binding domain of the LDL receptor, it was likely that others would be found in FH patients. In this FH sample, exon 4 mutations were detected in 16 alleles of 211 (~8%), of which 15 individuals had a mutation in repeat 5. It was thus essential to extend the sample and use a better method to identify previously unknown mutations, to find out whether there was any detectable bias towards mutations in the 3' part of exon 4.

3.2. Searching for unknown mutations by Single Strand Conformation Polymorphism (SSCP)

3.2.1. Introduction to SSCP

For identification of known mutations, such simple tests as restriction endonuclease digestion or detection by ASOs that are described above can easily be applied to large numbers of individuals. In some instances previously unidentified sequence changes may be found, as is described in Section 3.1.3. above, where new mutations were identified as a new restriction pattern of *Dde*I digests and as heteroduplexes. These are coincidental findings, but on a broader scale a systematic approach is needed to identify the underlying, currently unknown, sequence changes. Major gene rearrangements are still identified by Southern blotting but in the case of point mutations, other methods have to be used. The four techniques mainly used are; chemical cleavage of mismatch (CCM), denaturing gradient gel electrophoresis (DGGE); direct sequencing; or single strand conformation polymorphism (SSCP).

These published methods all allow comparison of the sequence of specific fragments of DNA amplified *in vitro* by PCR (Saiki et al 1985) from different individuals. The first uses chemical cleavage of mismatched bases (CCM) in the duplex formed between two heterologous DNA fragments after hybridisation (Montandon et al 1989). This is a robust technique which has been used successfully to look for mutations in the apoB gene (Dunning et al 1991). Because it is based on chemical methods, the technique is able to detect all mismatched bases irrespective of sequence (Michaud et al 1992), and individuals can be identified who are heterozygous for any sequence difference compared to the normal "probe" DNA, which is radiolabelled with [α-

^{32}P]dCTP. Fragments of DNA of about 500-600 bp give good results, but for longer fragments, the yield of amplified DNA is reduced, and cleaved mismatches within 50-60 bp of the ends of the DNA fragments may not be detected (as a size reduction). This can be overcome by using amplifying oligonucleotides that produce fragments that overlap by 100 bp. The method involves several time-consuming steps, some involving hazardous chemicals and is thus not feasible for large number of samples.

A second approach is the use of denaturing gradient gel electrophoresis (Myers et al 1985a), which again appears to be able to detect all possible mutations (Cariello and Skopek 1993). DGGE depends upon the difference in melting profile conferred by a single base substitution. DGGE employs a linear gradient of urea/formamide in a polyacrylamide gel where the DNA migrates into an increasing concentration of denaturant (Cariello and Skopek 1993). It is possible to predict the behaviour of a DNA fragment in a denaturing gradient gel, solely from the basepair sequence (Lerman & Silverstein 1987), and compute a calculated melting map. DGGE is only sensitive to mutations in the low-temperature melting domain of a molecule. The melting characteristics of a molecule can be altered by adding GC rich sequences to it, the so called GC clamp (Myers et al 1985b and 1985c). The method works best if a GC-rich sequence clamp is introduced at one end of the sequence (e.g.included on one PCR primer). As for chemical cleavage, the technique is somewhat inconvenient to set up and use. A recent report has used this method to screen the promoter region of the LDL receptor for mutations in 500 FH patients, but none were identified (Top et al 1992). In another study, Lombardi et al (1993) examined 32 patients with FH and used DGGE to detect a variation in 27 of the patients.

Direct sequencing of a PCR fragment as a primary method to search for unknown mutation is

difficult to use on large genes in large number of samples without a pointer as to where in the gene the mutation is to be found. The quality of the sequencing needs to be very high as a common problem is observing terminations in more than one track, which results in the detection of "heterozygotes" caused by artefacts. Of the two methods used, the Sanger chain termination method (Sanger et al 1977), that relies on the ability of dideoxynucleotides, which lack the 3'-hydroxyl group critical to phosphoribosyl chain extension, to terminate a strand synthesis from a specific oligonucleotide priming site by a DNA polymerase, is much more popular and easier than the Gilbert Maxam method, which uses chemical cleavage at specific bases (Maxam and Gilbert 1977). Automated DNA sequencers perform electrophoresis and detection on gels similar to manual sequencing gels, but the detection system is usually fluorescence, either using oligonucleotides with fluorescent labels attached, or incorporating fluorescent nucleotides into the product during PCR. With automated sequencing machines, this is a method preferred by some for a primary search for unknown mutations.

SSCP is an increasingly popular method to use for detection of unknown mutations, mainly because it is relatively simple to use and has the potential for mutation detection in a large number of samples. It has been an established technique for many years to separate the two strands of a duplex by non-denaturing electrophoresis (Maniatis et al 1982). However, the idea and demonstration that non-denaturing electrophoresis would resolve single nucleotide polymorphisms in either of the two strands of a duplex is much more recent (Orita et al 1989a). Under non-denaturing conditions a single strand of DNA will adopt a conformation (presumably dependent on internal base-pairing between short segments by foldback) which is uniquely dependent on its sequence composition. This conformation will usually be different if even a single base is changed, although the theoretical basis for the folding is not well understood.

SSCP is a method capable of identifying a large proportion of sequence variations in a single strand of DNA, typically between 150 and 250 nucleotides in length (Hayashi 1991, Sheffield et al 1993). Most conformations seem to alter the physical configuration or size enough to be detectable as a mobility difference upon electrophoresis though a retarding matrix such as acrylamide gel. The ability to detect differences in the migration pattern is reduced in fragments over 300-400 bp.

In many laboratories [α-^{32}P]dCTP is incorporated during the PCR, and diluted denatured PCR product is electrophoresed on a "long" (40 cm) acrylamide gel, but a number of systems using shorter gels and non-radioactive methods for detection have been developed (Mohaber et al 1991, Hongyo et al 1993), such as fluorescence (Makino et al 1992) and silver staining (Ainsworth et al 1991). Thus, SSCP is to date the easiest method to use for identification of unknown mutations in a large number of samples.

3.2.2. Methods: Adaption of SSCP for the 3' part of exon 4

SSCP was performed as described in Chapter 2, Section 2.4.4. A wide variety of different conditions for SSCP were evaluated, including track length, temperature and the presence or absence of glycerol in the gel. The specific conditions are described in the results below.

The samples used to carry out the SSCP evaluation for the 3' part of exon 4 were the ΔG197, D206E, Fs206 and C210X.

3.2.3. Results

3.2.3.1. The effect of track length

Figure 3.2.1., 3.2.2., and 3.2.3. show the increased resolution of the single strands with increased gel track length.

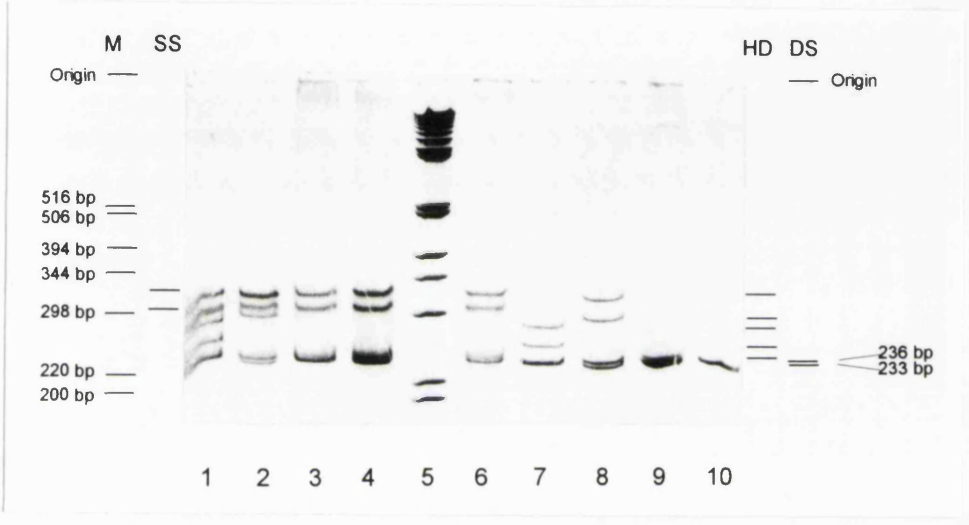

Figure 3.2.1. SSCP analysis on a small gel format; 6 cm long, 0.8 mm thick and 7% polyacrylamide, 10% glycerol, 1:49 bisacrylamide:polyacrylamide. The DNA is detected by silver staining. The double stranded DNA fragment is 234 bp long. SS and DS denote single strands and double strands, respectively and the relative positions are shown by the transverse lines. The relevant sizes of the 1 kb ladder (M) are displayed on the left of the Figure, and the sizes of the double stranded DNA is presented on the right. The samples in lanes 1 - 4 and 6 are all boiled. The first sample is the 2 bp deletion (Fs206), the second is the 3 bp deletion (ΔG197), 3 is the C210X, 4 is the D206E, lane 6 contains a control sample. In lane 5 the 1 kb ladder is used as a marker. Lanes 7-10 show the native 2 bp, 3 bp and the D206E and control, in that order.

The smallest gel (Figure 3.2.1.), the double stranded DNA samples have run 4.3 cm after 5 hours of electrophoresis at 10 mA and room temperature, the single stranded DNA has run 3.2 cm and 3.5 cm. Lanes 1-4 and 6 show samples denatured by boiling in formamide and SDS. Both the deletions show heteroduplexes (lanes 1,2 and 7,8) as does the D206E mutation (lanes 4 and 9). There is no indication of a difference in the migration of the single strands, except for the lower

strand of the 2 bp deletion (lane 1). The upper heteroduplex band for the 3 bp deletion falls on the slower migrating single strand for that sample and for that sample two bands can be seen of the double strand reflecting the 3 bp difference in size.

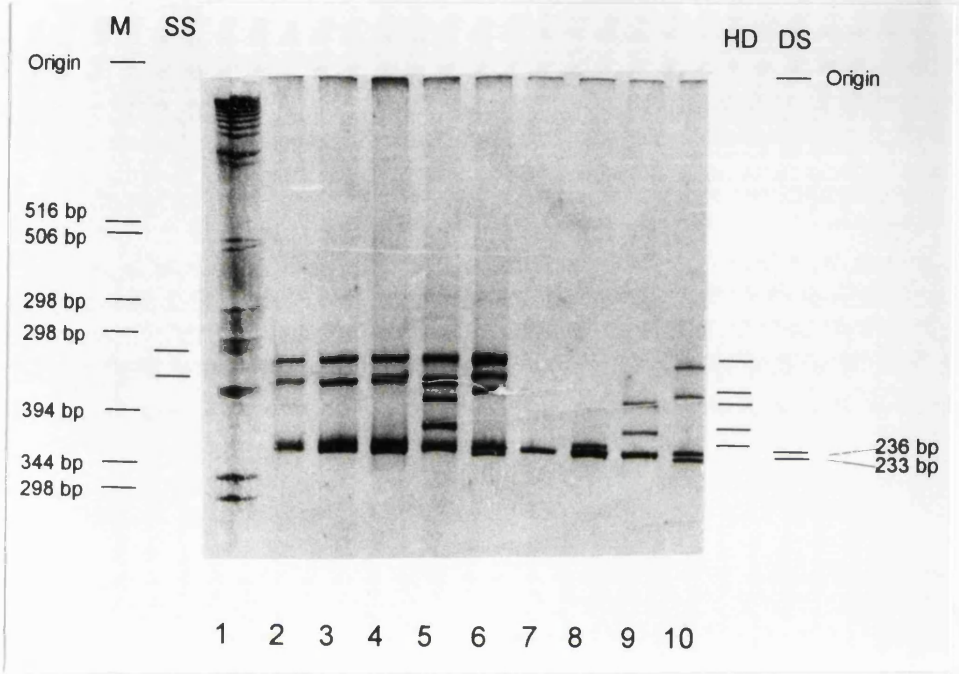

Figure 3.2.2. SSCP analysis on a medium gel format; 13 cm long, 1 mm thick and 7% polyacrylamide 10% glycerol 1:49 Bisacrylamide:polyacrylamide. The DNA is detected by silver staining. The double stranded DNA fragment is 234 bp long. SS and DS denote single strands and double strands, respectively and the relative positions are shown by the transverse lines. The first lane contains the 1 kb ladder, which sizes are presented to the left, shown by transverse lines (M). The next five lanes contain boiled samples, in lane 2 is the control sample, then in lane 3 the C210X, lane 4, D206E, lane 5 the 2 bp deletion (Fs206) and lane 6 the 3 bp deletion (ΔG197). The next four samples are unboiled; lane 7 is control, lane 8 is D206E, lane 9 is the 2 deletion and lane 10 is the 3 bp deletion.

On the medium sized gels (Figure 3.2.2.), the double stranded DNA samples have migrated 7.2 cm after 9 hours of electrophoresis at 30 mA at room temperature, the single stranded DNA has run 5.8 cm and 5.3 cm. There is no sign of different migration length of the mutant and the normal sized single strand, except for the lower strand of the 2 bp deletion sample (lane 5). The slower migrating heteroduplex for the 3 bp deletion migrate a little longer than the slower migrating single strand for that sample therefore it cannot be excluded that the mutant single

strand is not superimposed on that heteroduplex band. Interestingly here a heteroduplex can be seen for the C210X as for all the other mutant samples.

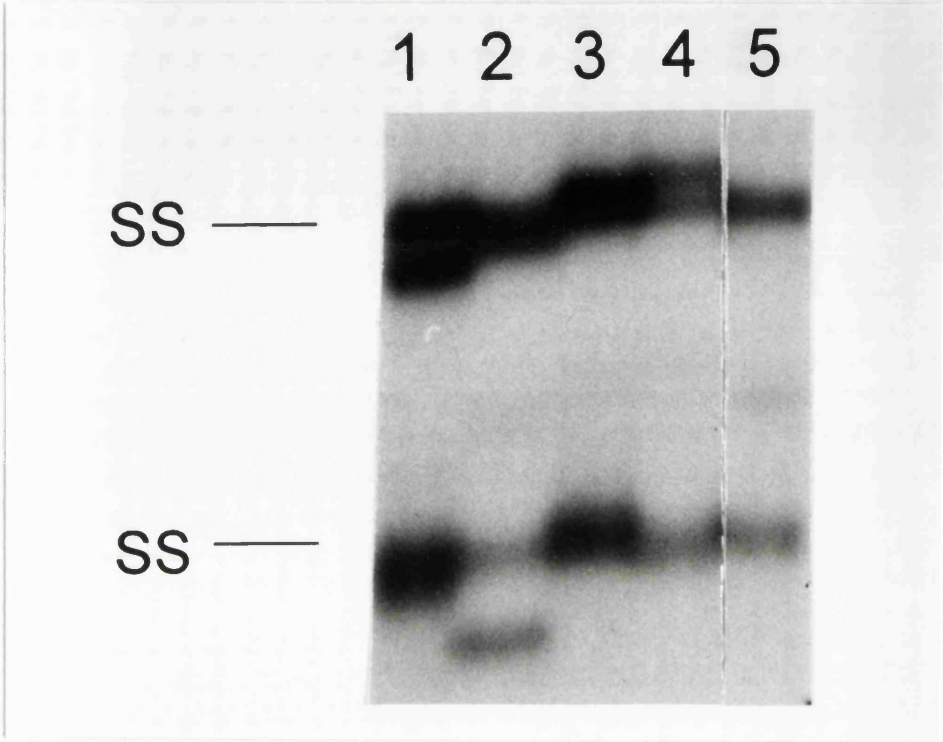

Figure 3.2.3. SSCP analysis on a large gel format; 40 cm long and 0.4 mm thick, 6% polyacrylamide 10% glycerol 1:49 bisacrylamide:polyacrylamide, electrophoresed at 7 W for 16 hours at room temperature. The DNA is detected by radioactivity. Lane 1 is the ΔG197, lane 2 is the Fs206, lane 3 is C210X, lane 4 is the D206E. SS denotes single stranded DNA.

On the largest gel, shown on figure 3.2.3. all the mutations show a different migration pattern between the mutant and the normal allele. Here the single strands have migrated for 22 cm for the slower migrating single strand and 25 cm for the faster migrating single strand. The double stranded DNA has run off the gel. The first sample is the 3 bp deletion showing a substantial difference in the migration of the upper strand as well as some resolution between the two faster migrating single strands. On the next sample, the 2 bp deletion, there is a major shift in the lower single strand migration pattern, whereas the upper single strand does not show any mobility shift. The third sample C210X shows the least mobility shift of all the mutations examined here, a

mobility shift of 1 mm on the 20 cm migration or a mobility shift of 0.5%. The fourth sample is the D206E showing a clear mobility shift on the upper single strand, but none at all on the lower one. The last sample is the normal control sample.

3.2.3.2. The effect of temperature and glycerol

Gels were run in the cold cabinet at 4°C without glycerol, and also at room temperature with glycerol. Electrophoresis of gels with glycerol in the cold cabinet retarded the run so severely that after 24 hours of electrophoresis the double strands had migrated about 15 cm into the gel. Electrophoresing gels fast at room temperature increases the temperature in the gel so that it can no longer be called non-denaturing gel electrophoresis. Figure 3.2.3. shows the migration pattern from a gel containing 10% glycerol, run at room temperature for 16 hours, described above (section 3.2.1.).

Figure 3.2.4 shows a gel electrophoresed in the cold cabinet. On this gel the samples have run a somewhat shorter distance into the gel than on the glycerol gel, 16 cm for the slower migrating single strand and 16.8 cm for the faster migrating strand. The single strands are thus migrating much closer to each other than when electrophoresed on a gel with glycerol at room temperature. In both the deletions, the alteration in single strand mobility is only seen for the faster migrating strand. In the C210X both single strand bands are somewhat thickened. The D206E mutation pattern does not differ at all from the pattern seen with the control sample. The resolution of the normal and mutant single strands is thus significantly better on the gel electrophoresed for a longer time with glycerol for these mutations, despite the fact that the bands on the slow running gel are broader than on the fast running gel.

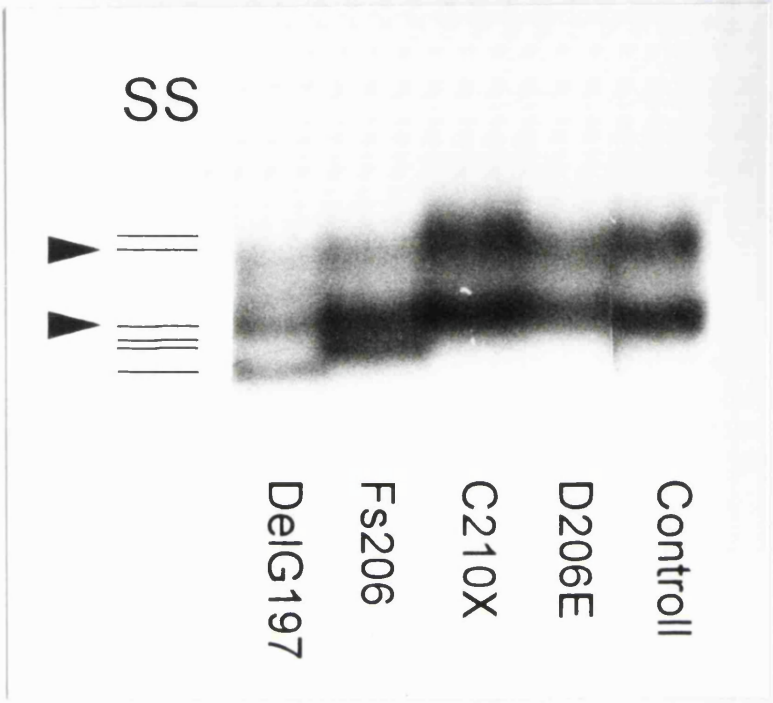

Figure 3.2.4. SSCP analysis on a large gel format without glycerol, 1:49 bisacrylamide:polyacrylamide, electrophoresed at 45 W and at 4 °C in the cold cabinet for 3 hours. The arrowheads indicate the position of the normal single strands (SS).

3.2.3.3. Application of SSCP to de novo mutation detection in the 3' part of exon 4 in a new set of FH patients

The aim of this experiment was to evaluate if the condition for the SSCP (6% polyacrylamide, 49:1 acrylamide:bisacrylamide,10% glycerol, electrophoresed at ambient room temperature for 16 hours) was applicable for mutation detection in a new sample of FH patients. A small sample of patients designated the UCH sample of FH patients (described in detail in Chapter 2 Section 2.8.1.3.) was examined.

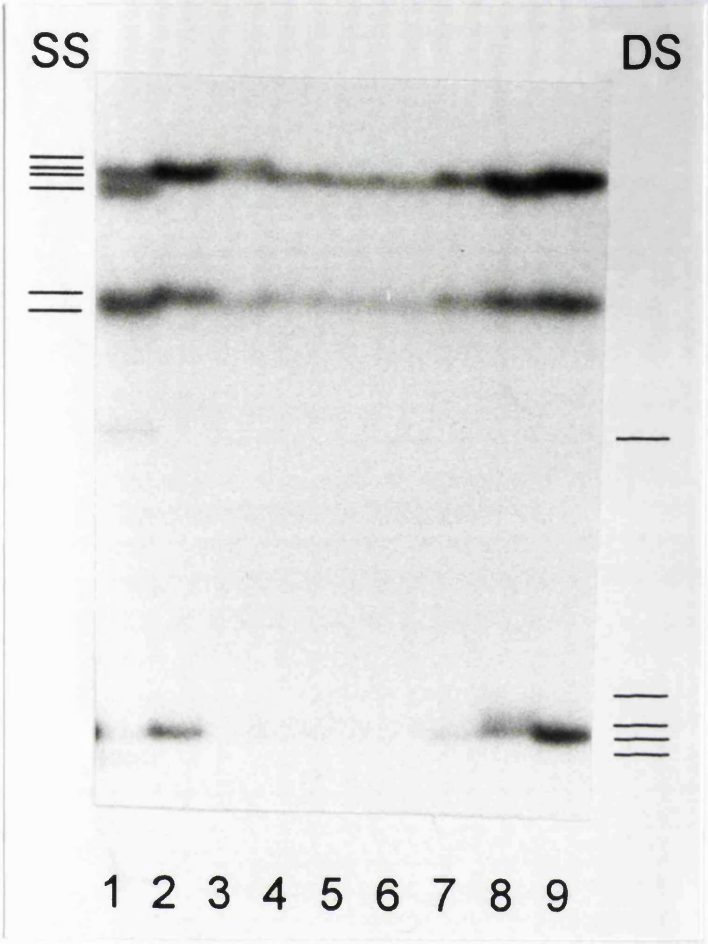

Figure 3.2.5. SSCP analysis on FH patients from the UCH sample. Lanes 1,3 and 8 contain samples with a SSCP variation.

Figure 3.2.5. shows the SSCP of the 3' fragment of the exon 4 in 9 FH patients. Three different pattern were observed. The typical pattern of the 3 bp deletion (ΔG197) is seen in lane 1, in lane 3, another more slowly migrating upper band, similar to that observed with the D206E mutation, is seen and in lane 8 a pattern of a more rapidly migrating upper band can be seen. For all these mutations heteroduplex bands of the double strands are also seen. One other patient with the classical pattern of the 3 bp deletion was also identified in the whole UCH sample. The intensity of the bands varied among the samples, presumably due to different PCR efficiency.

The 3' part of exon 4 from all these patients was amplified and sequenced. The sequencing confirmed the ΔG197 in two patients and the D206E in the third. The novel SSCP pattern (in lane 8 on Figure 3.2.5.) was shown to be due to a novel single base substitution of a G for an A, in codon 200 changing the amino acid aspartate (GAC) to glycine (GGC) (D200G) (Figure 3.2.6.).

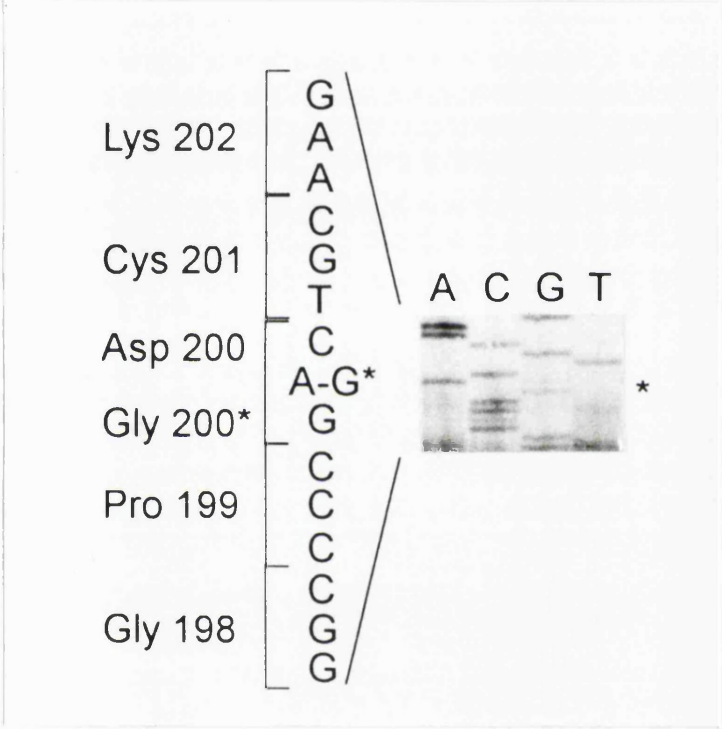

Figure 3.2.6. Sequencing of the D200G mutation. The position of the mutation is marked by an asterisk.

This novel mutation creates a site for the restriction enzyme *Msp*I (GGCC), with digestion of the PCR fragment II. This 234 bp fragment contains no cutting site for *Msp*I in the normal sequence, but in individuals with the D200G mutation *Msp*I cuts the fragment into 140 bp and 94 bp long fragments. The 3' part of exon 4 from the 200 FH patients described in section 3.2.1. was amplified and digested with *Msp*I and electrophoresed on an agarose gel. One additional patient carrying this mutation was identified in this sample (Figure 3.2.7. lane 7).

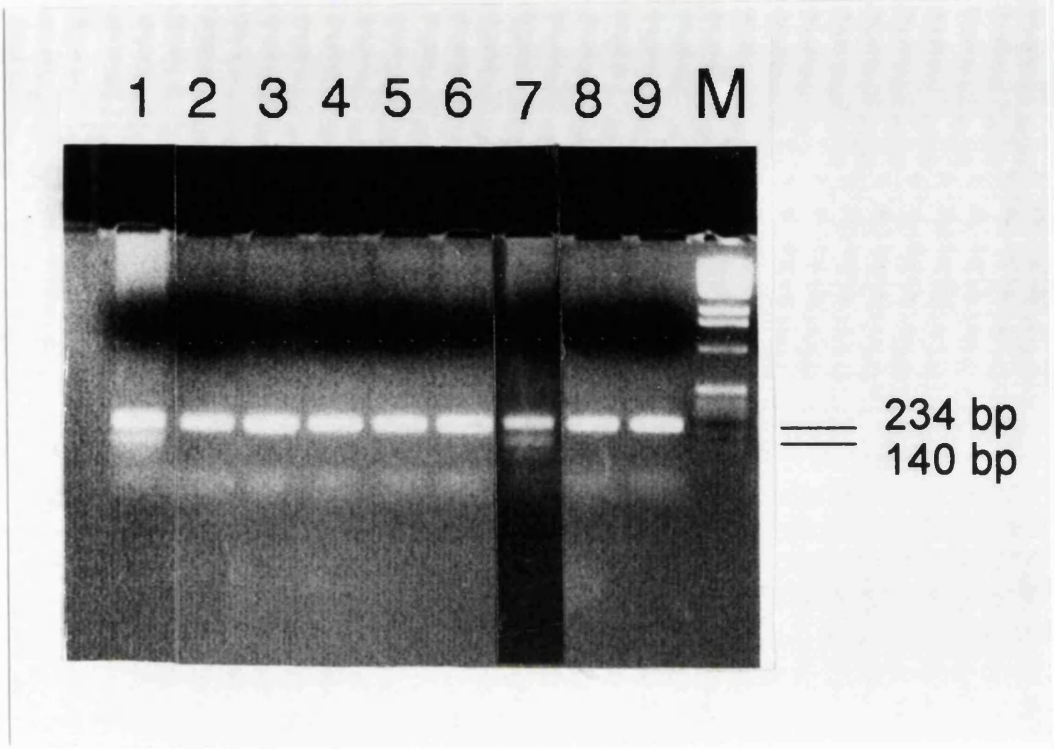

Figure 3.2.7. Demonstration of the D200G mutation by MspI digestion of the PCR. The 94 bp fragment is not seen on this figure. Two samples are positive for this mutation, the positive control in lane 1 and the sample from a patient from the FH 200 London sample, in lane 7.

3.2.4. Methods: SSCP analysis of the 5' and middle part of exon 4

Analysis of the 5' and middle part of exon 4 (coding for repeat 3 and 4) by SSCP (described in Chapter 2 Section 2.4.4.) was carried out on the sample of all the FH patients described earlier (the FH 200 London sample and the UCH sample described in chapter 2 sections 2.8.1.2. and 2.8.1.3.). A total of 239 individuals were examined, excluding the homozygous patients. The sample was examined by SSCP under three different condition; as a 340 bp fragment on a 6% gel with glycerol and on a 12% gel without glycerol, and in two separate, smaller fragments of 192 bp and 180 bp on a 6% gel with glycerol.

3.2.5. Results

3.2.5.1. The effect of gel porosity

A fragment of 340 bases was amplified from the 5' end of exon 4, using primers p 4.5 and p 4.6 (see Figure 2.2.1. and Table 2.2.1. in Chapter 2 Section 2.2.1.), coding for repeat 3 and 4 of the binding domain, and this fragment was subjected to electrophoresis of the single strands at the same condition as for the 3' fragment coding for repeat 5 (6% polyacrylamide, acrylamide:bisacrylamide 49:1, 10% glycerol at room temperature for 20 hours at 8 W). Under those conditions no SSCP change was seen in any sample from FH patients including DNA from a patient heterozygous for a mutation in this fragment (S156L), which was included as a positive control. The pattern of the normal single strands is shown on Figure 3.2.8. The double strand has migrated 33.5 cm. There are three single strands clearly visible in all the samples. They probably represent different conformations of the single strands as they were not seen in the unboiled samples. The slowest migrating single strand had moved 26.3 cm and the fastest had moved 28.7 cm.

The PCR amplified fragment was then electrophoresed on 12% polyacrylamide gel without glycerol in a cold cabinet at 4°C and 45 W for 20 hours, as increased concentration of polyacrylamide has been shown to increase the resolution for larger PCR fragments in SSCP analysis (Savov et al 1992). Under these conditions three different SSCP patterns were observed in three patients, which were analyzed by direct sequencing of the amplified exon 4 to identify the sequence change involved. The SSCP patterns are shown on Figure 3.2.9.

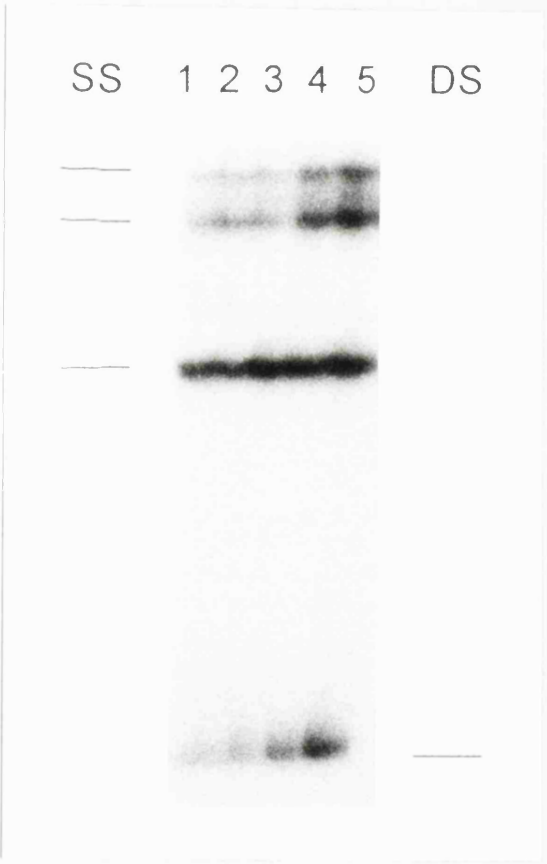

Figure 3.2.8. SSCP analysis of a 340 bp fragment of the 5' and middle part of exon 4. The conditions were; 6% polyacrylamide, acrylamide:bisacrylamide 49:1, 10% glycerol at room temperature for 20 hours at 8 W. SS denotes single strands and DS denotes double strands.

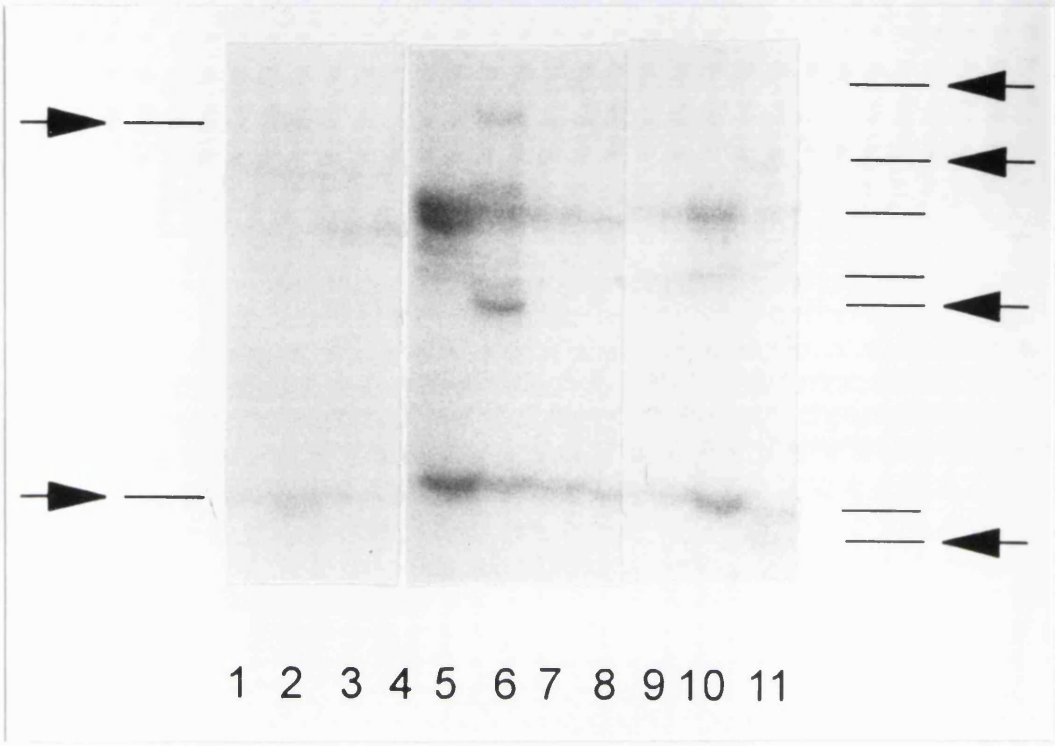

Figure 3.2.9. SSCP analysis of the 340 bp fragment of the 5' and middle part of exon 4 on 12% polyacrylamide gel without glycerol in a cold cabinet at 4°C and 45 W for 20 hours. Only single strands are visible on this gel. The arrows denotes the variant single strands and the transverse lines denotes the normal single strands. Variations from the normal pattern are seen in lanes 2,6, and 11.

The normal single strands have migrated 6.8 cm and 10 cm with an additional band at about 7.5 cm seen in a number of the samples. The first pattern, shown in lane 2 has a slower migrating upper strand and slightly faster migrating lower strand than the normal strands. This was shown by sequencing to be the S156L mutation, previously identified in another FH patient and described in Section 3.1.3. The second SSCP pattern in lane 6 on Figure 3.2.9. is somewhat more complex, with a number of bands in addition to those normally seen. Above the slower migrating single strands are two bands not seen in other samples. Whether both of these are

caused by the mutant strand is not possible to say. Below the slower migrating strand are three bands, of which the two upper ones are frequently seen in other samples, either together as in lane 5 (which was shown by sequencing to contain only the normal sequence), or as either the one or the other as in lanes 3 ,4, and 9, 10 and 11. The third band was not seen in any other sample and is probably representing a variant faster migrating single strand DNA.

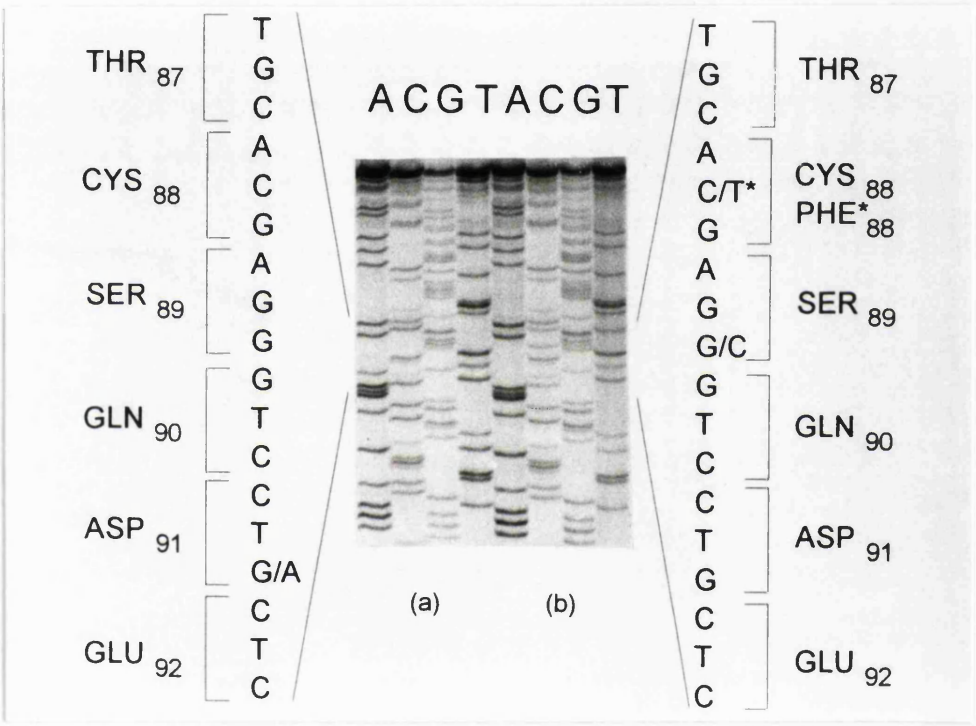

Figure 3.2.10. Sequencing of the SSCP variations observed after electrophoresis on a 12% polyacrylamide gel.

When DNA from this individual (sample lane 6), was amplified and analyzed by direct sequencing both an A to a G transition in nucleotide 339, which alters a cysteine to phenylalanine in codon 88 (C88F), and a C to a G transversion in nucleotide 343, which is a silent substitution in codon S89 were seen (Figure 3.2.10. b)). Whether, in this patient, both these mutations occurred on

the same allele is not readily possible to determine, as there were no other family members available for studies, but the SSCP pattern observed was that predicted for an individual, heterozygous for one normal and one mutant allele. The third SSCP pattern seen in lane 11 on Figure 3.2.9., showed a slower migrating upper band and a faster migrating lower band. This was shown by direct sequencing to be a C to T transition in nucleotide 349 (Figure 3.2.10. a)), which is in the wobble position of codon D91 and is thus unlikely to be deleterious. All these sequence changes were novel.

3.2.5.2 The effect of the fragment length

Trying to find more mutations in the DNA coding for repeat 3 and 4, the fragment of 340 basepairs was divided into 2 fragments of 192 bp and 180 bp, using the oligonucleotide primers p4.5 and p4.7 for repeat 3 (the 5' part of exon 4) and oligonucleotide primers p4.8 and p4.6 to include repeat 4 (the middle part of exon 4) respectively (for details of the primers and the fragments see Table 2.2.1. and Figure 2.2.1. in Chapter 2 Section 2.2.1.). These fragments were subjected to SSCP analysis on 6% polyacrylamide gel, 49:1 acrylamide:bisacrylamide and 10 % glycerol and electrophoresis for 16 - 18 hours at ambient room temperature at 8 W. Figure 3.2.11. a) shows the two SSCP patterns (lane 1 and lane 5) observed for the mutations in the 5' part of exon 4 previously identified on the 12% gel (C88F and S89S in lane 1 and D91D in lane 5). As can be seen on the figure, there are number of bands in addition to the double strand (ds) which has migrated 29 cm on this gel. There are two sets of single strands presumably with at least two conformations each. These two that have migrated furthest are at 27.1 cm and 25.2 cm on this gel. On the upper strand, a mobility shift (z) can be seen for the sample in lane 5 (the C to T transition in codon D91, as shown by sequencing on Figure 3.2.10. a). There is evidence

for another mobility shift in connection with the faster migrating band (x) of the set of single strands that have migrated a much shorter distance 21.3 and 21.6 cm (seen faintly on the photographs). The C88F and the C to G silent transversion in codon S89 are seen as a mobility shift (y) on sample 1. It is difficult to tell whether the shifted strand belongs to the faster migrating pairs or the slower migrating pairs, but its intensity implicates it rather with the fainter upper bands, where the faster migrating one can hardly be seen. In neither of these samples was there evidence of heteroduplex double strands when the film was exposed for a shorter length of time (Figure 3.2.11. b)). No other pattern was identified in this fragment.

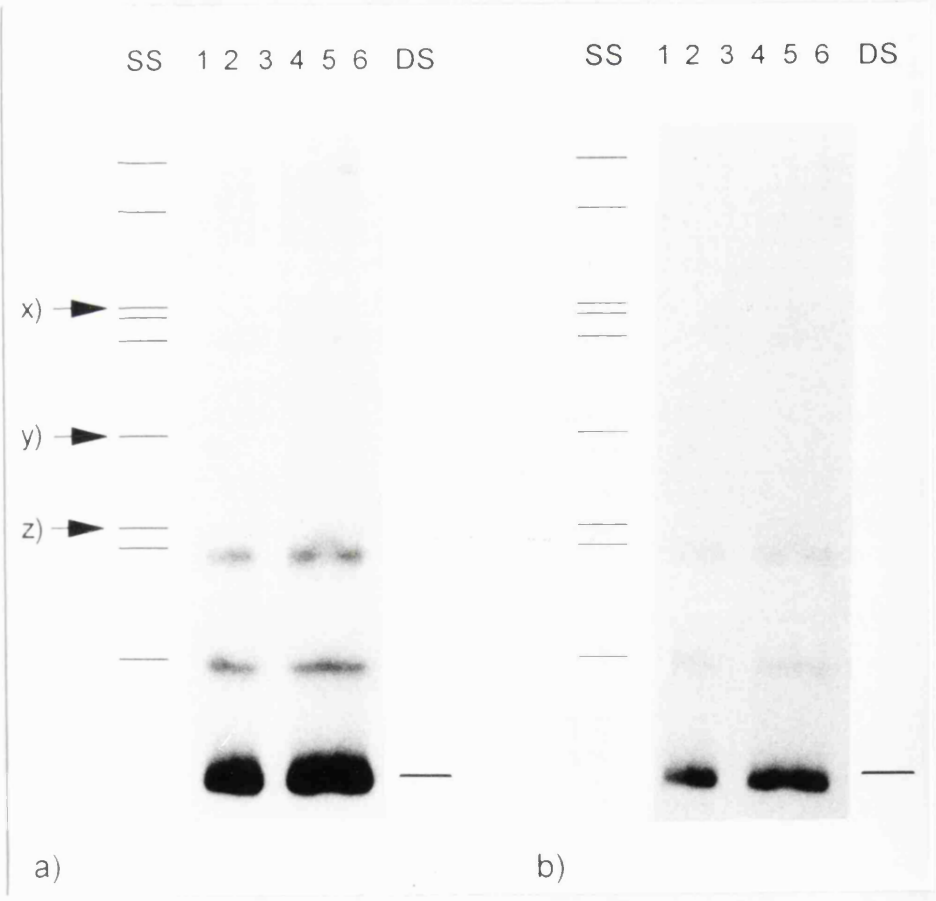

Figure 3.2.11. SSCP of the 192 bp fragment of the 5' part of exon 4. a) and b) represent the same gel exposed for different length of time. The single strand (SS) variation bands are indicated by the arrows. x), z), represent the variations seen in lane 5, and y) represents the variation seen in lane 1. The double stranded DNA is labelled DS and its position indicated by the transverse bar.

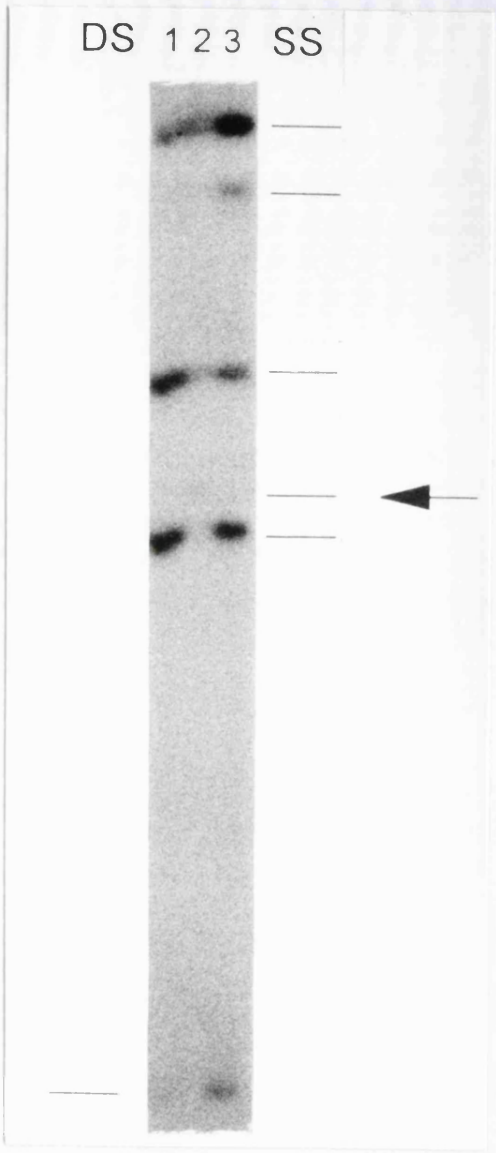

Figure 3.2.12. SSCP analysis of the 180 bp fragment of the middle part of exon 4. DS denotes the double stranded DNA and the SS denotes the single strands. The arrow shows the position of the SS variation in the sample in lane 2.

In the fragment coding for repeat 4 or the middle part of exon 4, only one variant pattern was observed. Figure 3.2.12. shows an SSCP analysis on three individuals of which the sample in lane 2 shows a variation. Here, as with the 5' fragment, the single strands show as four main bands in addition to the double strand (ds) that has migrated on this gel for 34.3 cm. The fastest

migrating single strand shows a mobility shift of 0.8 cm on 27.2 cm of migration, or 3%. The other single strands, which have migrated 25.3, 23.0 and 22.2 cm did not show any alteration of mobility. This sample did not show a heteroduplex.

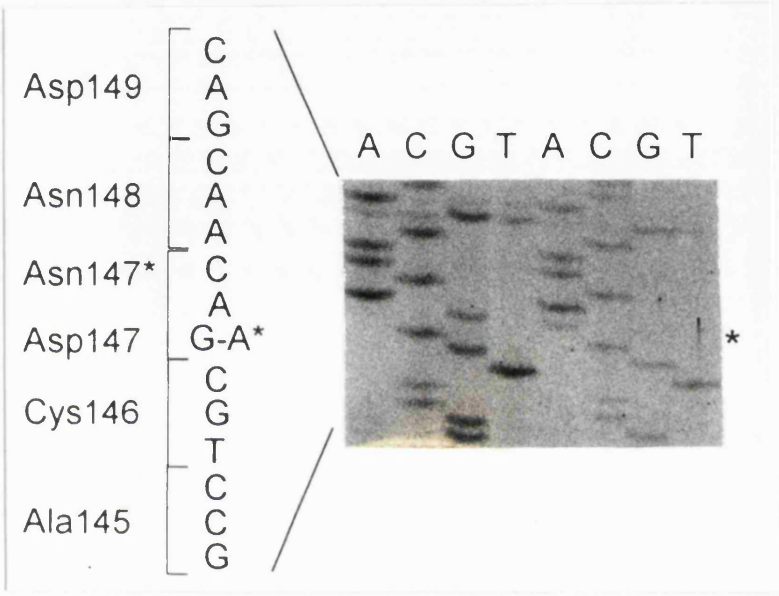

Figure 3.2.13. Sequencing of the D147N mutation is shown on the sample to the right. The position of the mutation is marked by an asterisk. The sample on the left is of a normal sequence.

When this sample was amplified and sequenced it revealed a mutation in codon 147, an aspartic acid GAC to AAC, an aspargine (D147N), shown on Figure 3.2.13. This mutation has not been reported before. The S156L mutations in this sample were not identified by these conditions, and an example of this is shown on figure 3.2.14. There is also no clear evidence of a heteroduplex for the sample containing the S156L mutation.

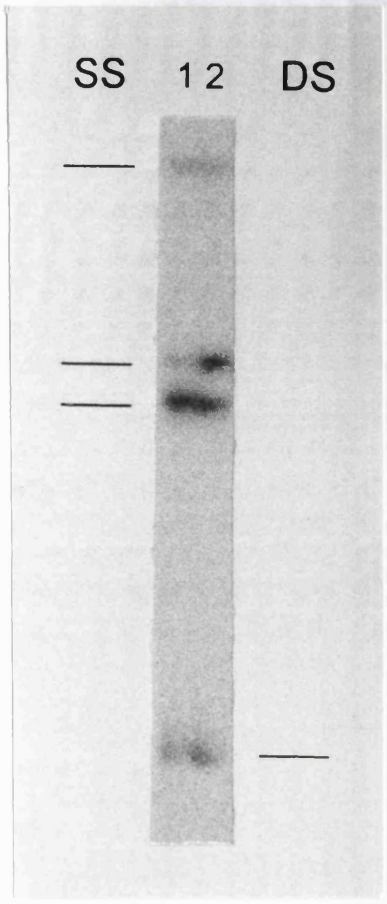

Figure 3.2.14. SSCP analysis of the S156L in the 180 bp fragment. The S156L is in lane 1. Single strands (SS) and double stranded DNA (DS) are indicated by horizontal lines.

3.2.6. Heteroduplexes

Heteroduplexes of double stranded DNA, form between two single strands when there is a mismatch of one or more bases. These are frequently seen as bands of retarded migration upon electrophoresis of PCR amplified fragments in a matrix such as polyacrylamide or modified matrixes like Hydrolink or Hydrolink-MDE (Keen et al 1991, Soto and Sukumar 1992). The sensitivity in polyacrylamide is dependent on the differences between the single strands and are most obvious when there are small deletions or small insertions. In the studies described here

heteroduplexes were observed for both the small deletions (ΔG197 and Fs206) as shown the small format gel in Figure 3.2.1 and on the medium size format gel on Figure 3.2.2. In addition, heteroduplexes can be seen for the D206E mutation on both the small and on the medium sized gels. On these gels there is no detectable heteroduplex for the C210X. On the large gel format all the single base substitutions are seen having heteroduplexes.

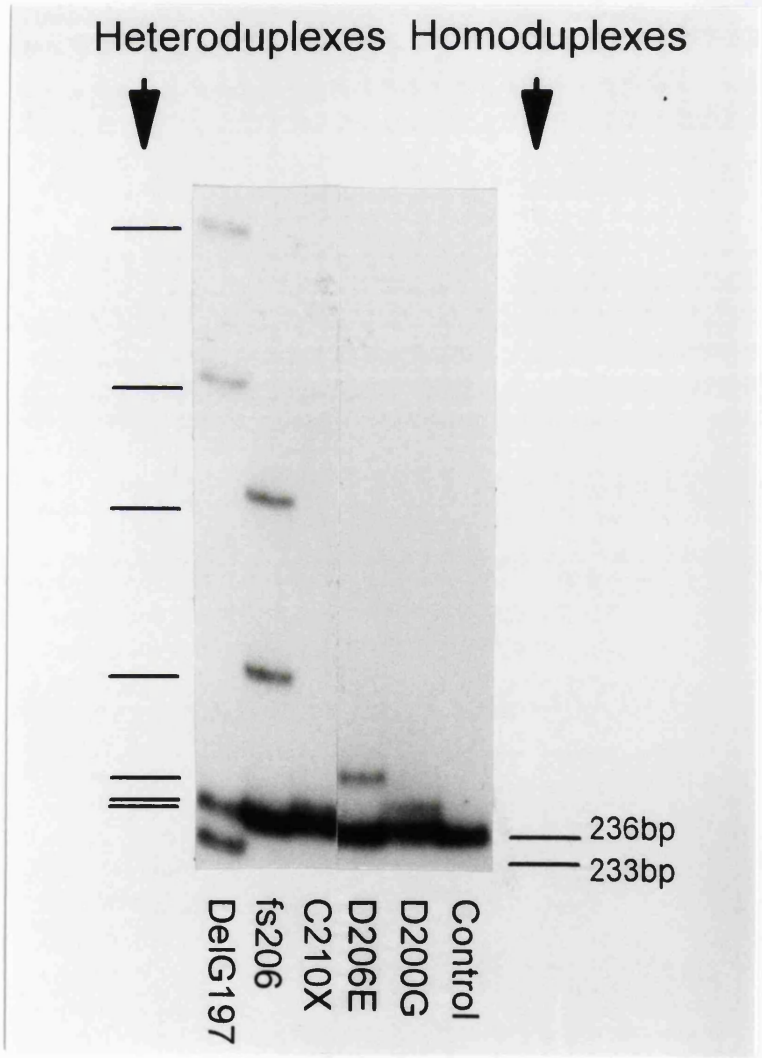

Figure 3.2.15. Heteroduplexes in the 3' part of exon 4.

Figure 3.2.15. shows the heteroduplexes for the five mutations identified in the 3' part of exon 4, the slowest migrating heteroduplex for the single base substitutions is seen for the D206E, and the heteroduplex for the C210X is closest to the double strand DNA.

No heteroduplexes were seen for the 340 bp fragment neither under the conditions the 6% gel was electrophoresed (Figure 3.2.8.) nor when the polyacrylamide concentration was 12% (Section 3.2.4.1. no data shown). There were no heteroduplexes seen for the smaller fragments representing the 5' and middle part of exon 4 (Figures 3.2.11., 3.2.12., and 3.2.14.).

3.2.7. Detection methods

There were two different techniques used to detect single stranded DNA after electrophoresis in polyacrylamide in this thesis. These were detection by labelling with radioactivity and silver staining. Both these techniques are described in detail in Chapter 2, Sections 2.2.3.1., and 2.4.4.2. The results from their usage are described below.

Silver staining of the small and medium sized gels revealed the single strands very clearly and the manipulation of the gels was easy, whereas silver staining the fragile large gels was cumbersome and difficult. Figure 3.2.16. shows a silver-stained 30 cm x 40 cm large gel. Three samples show a variation from the normal pattern of the single strands, those are samples 5, 8 and 10 all representing the D206E mutation. The single strands were more difficult to see on this large gel when silver stained compared to when detected by radiolabelling (Figure 3.2.3.) due to high background staining of the gel.

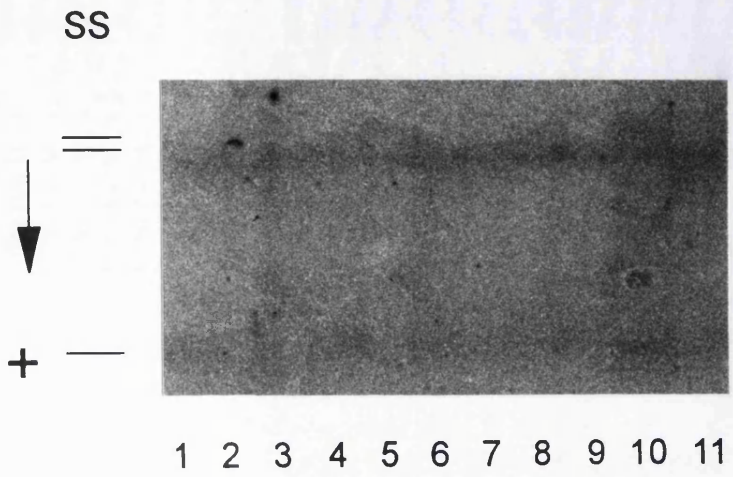

Figure 3.2.16. Silver staining of a large format SSCP gel. The samples that show some variation are in lanes 5, 8, and 10. The variations can be seen on the upper strand. The high background staining makes the interpretation of the results very difficult. The arrow denotes the direction of the single strands (SS), towards the anode +.

3.2.8. Discussion

SSCP is an established method to detect single base variations in PCR amplified fragments (Orita et al 1989a, Orita et al 1989b, Hayashi and Yandell 1993). A number of reports have described its use to identify known and unknown mutations in various genes such as p53 (Condie et al 1993) and the LDL receptor (Leren et al 1993a). The sensitivity of the method to detect single base variation is estimated to be around 80 - 95 % (Hayashi and Yandell 1993, Sheffield et al 1993), dependent on the conditions used.

Reported gel lengths range between 5 cm and 50 cm. In the present study 7 cm, 13 cm and 40 cm long gels were used. At present, most results are read by eye and therefore visible resolution of the single strands is necessary. Although clear cut mobility shifts (*e.g.* 10%) would be

demonstratable on short gels, a long electrophoresis may be necessary to resolve 0.5% mobility differences such as that for the C210X mutation (Figure 3.2.3.). Longer track length has been shown in other studies to be essential for detection efficiency (Fan et al 1993). In addition, the composition of the gel and the conditions of the electrophoresis are important, as is shown by the D206E mutation which was not seen when gels were electrophoresed at a high voltage without glycerol in a cold cabinet.

The use of SSCP to detect the known mutations in the 3' part of exon 4 of the LDL receptor was not sensitive using 7 cm and 13 cm long gels. The gels were run at 10 mA and 30 mA respectively for 5 and 9 hours, which may have affected the single strand mobility. Despite more difficulty in handling, the 40 cm gels showed excellent sensitivity. For the 3' part of exon 4 the best results were obtained when the samples were electrophoresed slowly over-night on gels containing 10% glycerol, which is in agreement with other reports (Hayashi and Yandell 1993).

SSCP depends on non-denaturing conditions in the gel for the detection of mobility shifts of the single strands introduced by single base substitutions that affect their conformation. To achieve those non-denaturing conditions, gels have to be electrophoresed at a voltage that does not heat the gel to such a degree as to denature the single strands. The exact temperature when single strands stop migrating according to conformation and start migrating according to size is not known. The precise regulation of temperature in large gels is possible with recycling buffer systems or fans and metal plates (Hayashi 1991) but cooling plates are complex and expensive. Most studies are thus done either by electrophoresing at room temperature or in a cold cabinet or cold room (Orita et al 1989a, Leren et al 1993a). This is not very accurate but according to most studies, sufficient. In the studies described here no major problems were encountered with

use of ambient room temperature 18 - 22° C or the cold cabinet at 4° C.

The use of glycerol gave better results for the 3' part of exon 4 fragment when the four known mutations were compared, and those conditions were chosen for the search for mutations in that part of the gene. There are number of reports that have described improved detection in sensitivity by using two gel conditions, with and without glycerol (O'Rahilly et al 1991, Orita et al 1989a). When searching for mutations in large genes and in a large number of individuals it is a major inconvenience to have to use many different conditions for the detection. If the object of the studies is to identify 100% of the mutations under such conditions, direct sequencing might be considered as an option.

The effect of increased acrylamide concentration has been described for improving the sensitivity of detection of mutations in longer fragments of amplified DNA (Savov et al 1992). The results obtained for the analysis of the 340 bp fragment, representing the 5' and the middle part of exon 4 of the LDL receptor gene, is in agreement with these reports. Although only one condition was tried before trying to increase the acrylamide concentration (6% polyacrylamide, 49:1 acrylamide:bisacrylamide with 10% glycerol), the results of finding four SSCP variations instead of none, indicate clearly that a higher concentration of acrylamide in the gels improves the sensitivity to detect SSCPs in larger fragments. However one mutation, identified by SSCP in the smaller fragment (D147N), was not seen under these conditions. So increasing the concentration of acrylamide does not necessarily allow the identification of all mutations.

The handling of the 12% acrylamide, large format gels is very difficult, mainly as it is hard to get the gels off the glass plates and onto 3MM papers for drying, and a number of gels were lost

during this procedure. Interestingly, the Pharmacia PhastSystem, which uses small format gels for the SSCP analysis relies on using high concentrate gels (12.5% acrylamide generally) (Vidal-Puig and Mollar 1994). Whether the use of high concentration gels in the Pharmacia PhastSystem is the reason for its ability to detect variations is not known as this was not systematically examined here, but again with the Pharmacia PhastSystem it is standard to use up to six different conditions for the SSCP analysis to improve the sensitivity, as well as the use of smaller fragments (Vidal-Puig and Mollar 1994).

The size of the fragments has been shown to be important with respect to the sensitivity of the detection of single base differences in PCR DNA fragments (Hayashi and Yandell 1993). Generally, a diminished sensitivity is observed with longer fragments (Orita et al 1989a). In the case of the middle part of exon 4 fragment the S156L mutation was not detected in a smaller fragment, but was detected in the longer fragment when electrophoresed on a 12% polyacrylamide gel. As one condition only was used for analysis of this smaller fragment it is not unlikely that under some other conditions that the S156L mutation would be detected. These studies were not undertaken as analysis of the efficiency of SSCP was not the main object of the project.

In addition to variation in single strand mobility, heteroduplexes of the double strands were observed in number of cases. For the 3' fragment of exon 4, heteroduplexes were seen for both the deletions as well as for all the single strand substitutions, when the samples were electrophoresed on a long gel. Detection of heteroduplexes on non-modified polyacrylamide gels is well established for small deletions and insertions (Nagamine et al 1989), but for single base substitutions it is less sensitive, unless electrophoresed on modified matrixes like Hydrolink or

Hydrolink-MDE (Keen et al 1991, Soto and Sukumar 1992). In the studies described here, the single base substitutions in the 3' fragment of exon 4 under investigation all showed heteroduplexes when electrophoresed on a long gel, and in the case of the D206E on the smaller format gels as well. For the 340 bp fragment, heteroduplexes were not seen when samples were electrophoresed on a 6% polyacrylamide gel with 10% glycerol at ambient temperature. Interestingly, no heteroduplexes were observed for any of the mutations in the 5' and middle part fragments either when run on 12% polyacrylamide gels as a large fragment or on the smaller fragments. Probably it is related to the fragment itself rather than the gel composition, though it would be of interest to electrophorese the samples in systems with Hydrolink or Hydrolink-MDE matrix, which have been developed to improve heteroduplex detection (Keen et al 1991, Soto and Sukumar 1992). It is generally accepted that the use of heteroduplex analysis in conjunction with SSCP increases the effectiveness of detecting mutations in some PCR fragments (Soto and Sukumar 1992, Cotton 1993). This was observed with the D206E mutation, in which case, when the SSCP was done at 4°C in the cold cabinet no SSCP was seen for that mutation (Figure 3.2.4), however, at these same conditions, a clear and distinctive heteroduplex could be seen for that mutation (Figure 3.2.15.). On the other hand the SSCP analysis of the large (340 bp) fragment, showed no heteroduplexes on either the 6% or the 12% gels for the mutations previously identified in this fragment, as was indeed the case when the same mutations were screened for in the smaller fragments of the 5' and middle part of exon 4.

A number of reports have described the detection of the single strands by silver staining after separation by electrophoresis (Dockton-Dwerniczat et al 1991, Ainsworth et al 1991). Silver staining is very convenient for staining the small (7 cm) and the medium (13 cm) gels. However the 40 cm long gels were much more difficult to handle because they are very fragile, and the

high background staining made the interpretation of the SSCP more difficult (see Figure 3.2.16.).

The study of the 3' part of exon 4 in the UCH sample, confirmed the frequency of 8% of mutations in this part of the LDL receptor gene in British FH patients. Three of the mutations were previously seen in FH patients in the UK. One mutation was novel and was found in one other individual in the sample of 200 FH patients previously screened for known mutations in this part of the gene. It was clear that this method of mutation detection was successful and could be used to detect known and unknown mutations in the 3' part of exon 4.

When compared with the results from the rest of exon 4 it raises the question of whether SSCP analysis is sensitive enough for those fragments. Certain conditions did not identify all the known mutations, though the combination of three conditions with different fragment size and gel porosity managed to identify all previously known mutations.

When all known mutations (based on Hobbs et al 1992) are plotted out on a schematic drawing of the repeats of the ligand binding domain of the LDL receptor (Figure 3.2.17.), it is obvious that there is a clustering of mutations in the 3' part of repeat 5. Mutations in other repeats also tend to cluster at the 3' part of the repeats, though in much fewer numbers. This may well indicate a functional basis for the distribution of the mutations in the ligand binding domain, as the highly conserved amino acids, DGSDE, shown to be involved in the binding of ligands, are in this part of the domains (Goldstein et al 1985). The concentration of mutations in the 3' part of repeat 5 raises the question of whether there is a bias towards a more clinically severe phenotype in individuals carrying mutations in that part. To attempt to answer whether the number of mutations in this part of the LDL receptor gene could be explained by such a bias,

studies on a larger sample of individuals with definite and probable FH was undertaken. Based on the results obtained to date, the protocol that was chosen for the study of the 3' part of exon 4 was; ^{32}P incorporation into the PCR fragment, denaturing of the sample and long track length on a 6% polyacrylamide gel with 10% glycerol.

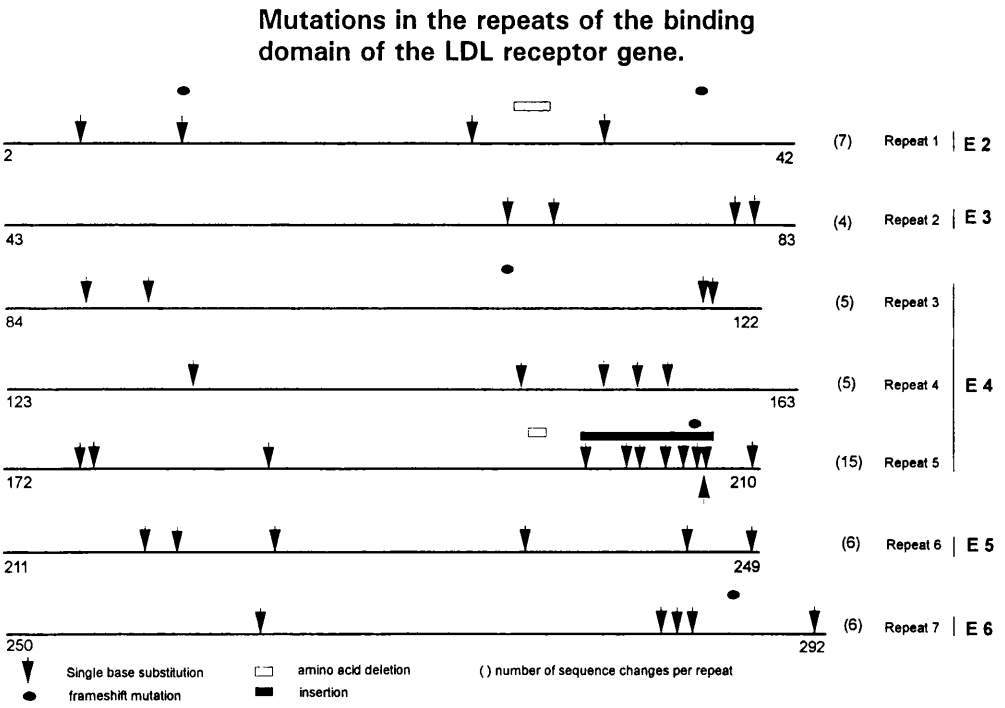

Figure 3.2.17. A schematic drawing of the ligand binding repeats of the LDL receptor showing the distribution of all known mutations in that part of the receptor. One line equals one repeat. The exons coding for the repeats are shown on the right side of the figure.

3.3. The effect of mutations in the 3' part of exon 4 of the LDL receptor gene on the clinical phenotype of familial hypercholesterolaemia

The aim of this experiment was to ask whether the high frequency of mutations in the 3' part of the LDL receptor gene represents a greater clinical severity of the disease (and hence possible clinical selection bias) in patients carrying such mutations.

The sample consisted of a total of 311 (apparently unrelated) patients with heterozygous FH. They were from four Lipid Clinics in London (Hammersmith Hospital, Charing Cross Hospital, St. Mary's Hospital (n = 189, the patients described in Chapter 2 Section 2.8.3.1. less the 11 homozygotes) and the Department of Medicine, University College London Medical School (n = 72, the 50 described in section 3.2.2. plus an additional 22 patients). The patients from the first three clinics have been described in earlier studies (Sun et al 1992; Gudnason et al 1993a, Gudnason et al 1993c). In addition, 50 patients were recruited from Southampton and South West Hampshire Health District.

3.3.1.Results

3.3.1.1. New mutations

Five different patterns of SSCP were detected in 31 patients in this sample. All the patterns were compatible with the five previously identified mutations (see sections 3.2.1. and 3.2.2.); theΔG197, D200G, D206E, Fs206 and the C210X mutations. Samples from all the individuals identified with SSCP patterns and where the sequence change had not been confirmed previously

(n = 11), were amplified and sequenced. In addition to the ΔG197, D206E, and the Fs206 mutations, two previously unknown sequence variations were discovered. These were; Glu 207 to stop (designated E207X) and a single base substitution of a C to a T 24 bases into intron 4, designated C+24T. Figure 3.3.1. shows the sequencing of those two variations.

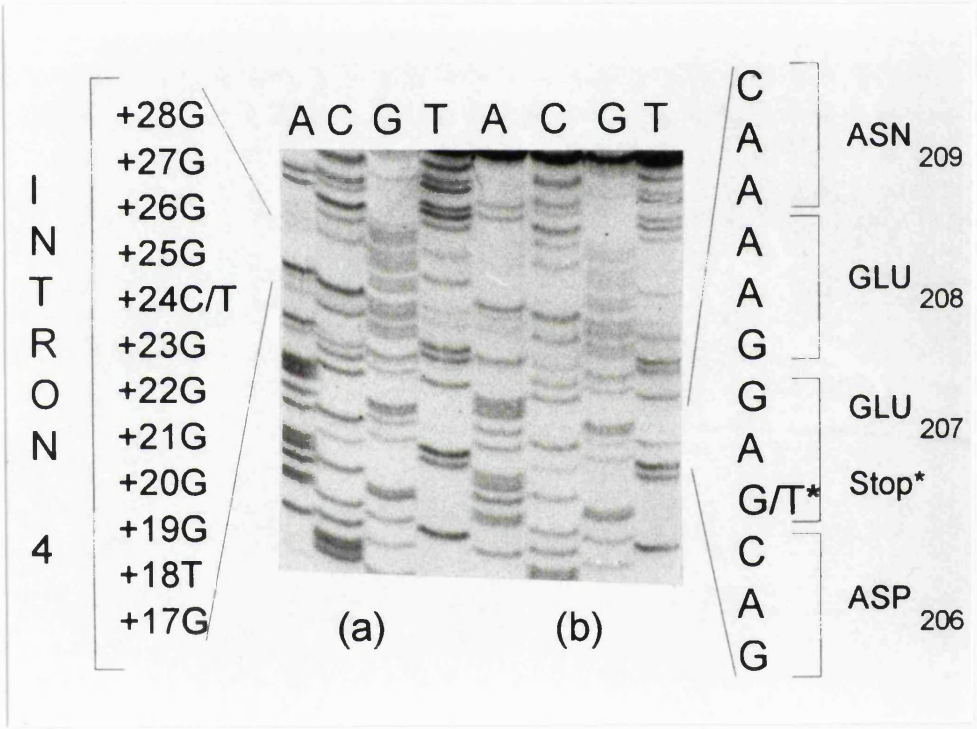

Figure 3.3.1. Sequences of additionally identified variations in this sample. a) shows the C+24T substitution in intron 4. b) shows the E207X mutation.

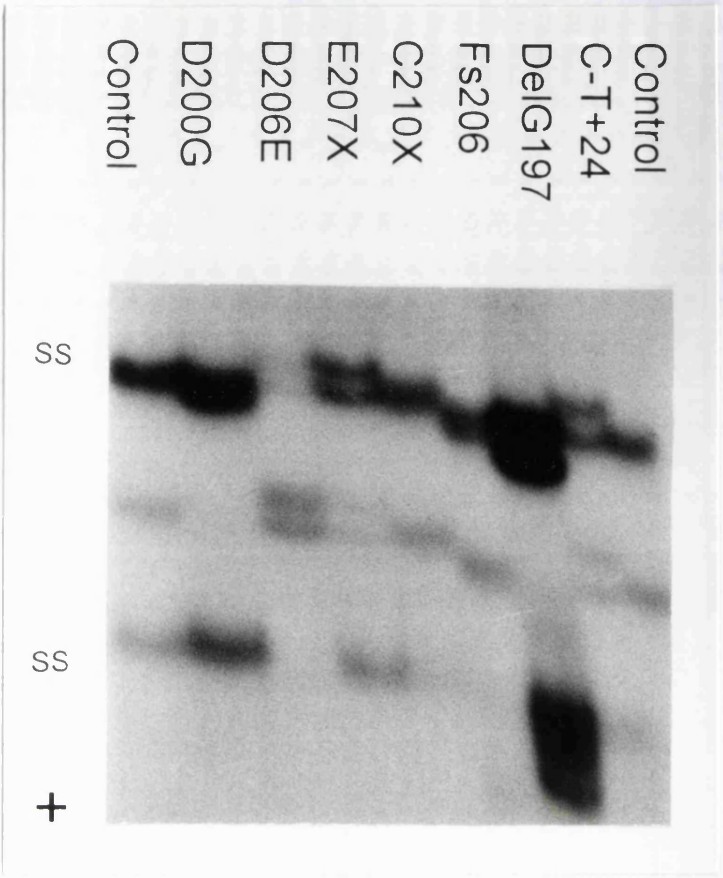

Figure 3.3.2. SSCP analysis of all the seven identified variations together. SS denotes single strands. The samples were electrophoresed towards the anode +.

The seven SSCP variation patterns obtained are shown together on Figure 3.3.2. All the variations are easily detected by changes in the upper strand migration pattern. The three variations, D206E, E207X and C+24T show very similar changes in mobility and cannot easily be distinguished from each other by the SSCP pattern alone. On this particular picture the D206E is somewhat fainter, which most likely is due to a diminished efficiency of the PCR, and in a number of the samples the lower single strand is faint or even absent. There are other fainter "shadow" bands seen between the normal single strands, and this is most likely due to PCR artefacts, described by Condie et al (1993).

To evaluate whether the SSCP method had detected all the mutant alleles present in this part of the gene, PCR amplified DNA from 30 of the patients with definite FH but no SSCP in exon 4 were chosen and the DNA reamplified and directly sequenced. No additional mutations were detected.

3.3.1.2. Plasma lipid levels

The clinical parameters and the lipid levels of the patient groups with different mutations affecting repeat five are shown in Table 3.3.1. Although there are some differences among the groups there were too few patients for these differences to be analyzed statistically. In further analysis the individuals with the C+24T variation were not included in any specific functional class of mutations, as it is not known what the effect, if any this mutation may have on the function of the protein. Three of the mutations (occurring in 12 patients) are predicted to cause "null alleles", FS206, E207X and C210X, and lipid data from these patients were compared with that from the 17 patients with mutations predicted to cause a defective protein in repeat 5; del G197, D200G and D206E (designated defective protein repeat 5), and with data from a group consisting of four patients with the missense mutation E80K (Webb et al 1992), three with the P664L mutation (King-Underwood et al 1991), two with the S156L mutation (see section 3.2.3.) and two patients with gross deletions causing a defective protein (Sun et al 1992) (designated defective protein excluding repeat 5).

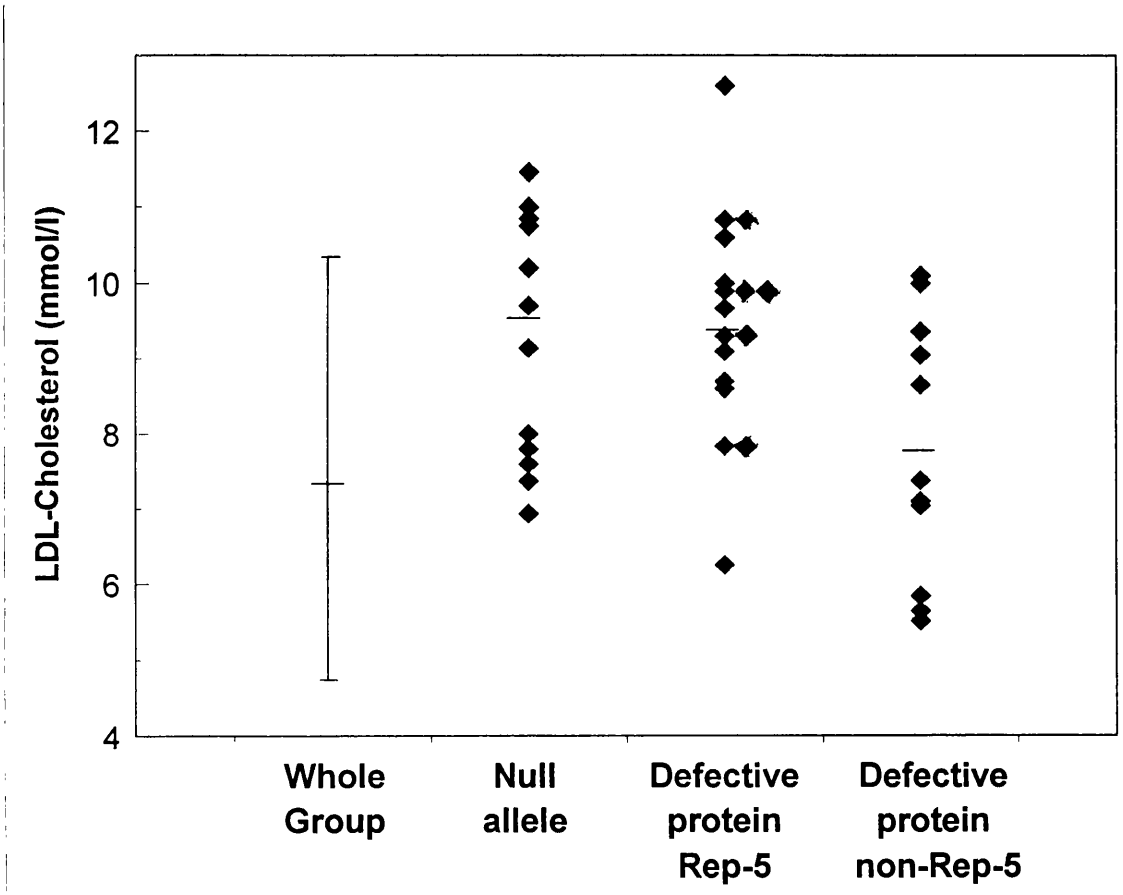

Figure 3.3.3. A scatterplot of LDL cholesterol levels in the different groups of FH patients. The filled dimonds present individuals. The mean LDL cholesterol concentration in each group are shown by transverse bars. The vertical line in the whole group represents SD.

Figure 3.3.3. shows a scatterplot of the LDL cholesterol levels in the different patient groups. It is clear that although the distribution of the plasma LDL cholesterol concentration vary, the individuals with either a mutation in repeat 5 that leads to the production of a defective protein, or with a mutation leading to no protein at all, tend to have much higher LDL cholesterol than individuals with a defective protein due to a mutation outside repeat 5, which show a distribution of plasma cholesterol concentration much more similar to the whole group. This is further seen in Table 3.3.2., which shows the plasma lipid concentrations and characteristics of the patients.

The patients with "any" detected mutation had total cholesterol and LDL cholesterol that were 15% and 22% higher respectively ($p<0.0001$) and the TG 18% lower ($p<0.05$), compared with the rest of the FH patients with no detected mutation. This difference was also highly significant ($p<0.0001$ and $p<0.05$ respectively) when the analysis was done on sex and age adjusted values (data not shown).

To examine the possibility that the common apoE variation might explain the differences in plasma lipids, apoE genotype was determined. The frequency of the apoE alleles did not differ between those with a mutation in repeat 5 compared to the rest of the FH patients (E2 0.054, E3 0.786, E4 0.016 and E2 0.047, E3 0.774, E4 0.0179 respectively), nor between those in the subgroups of null alleles and defective proteins.

Table 3.3.1. Mean lipid levels of FH patients with mutations in repeat five of the LDL receptor gene.

Mutation	No of Proband	TC (mmol/l)[a]	TG (mmol/l)	HDL (mmol/l)	LDL (mmol/l)[b]	Lp(a) (mg/dl)[c]	Age at diagnosis (years)
ΔG197	9	11.20 (1.72)	1.56(0.73-3.9)	1.10 (0.09)	9.39 (1.56)	33.6(5-93) (n=7)	42.3 (13.0)
D200G	2	10.90(0.99)	1.70 (1.2-2.2)	1.20 (0.0)	8.99(1.40)	39.0 (39) (n=1)	48.0 (9.9)
Fs206	6	10.72 (1.67)	1.02 (0.69-1.2)	1.21 (0.23)	9.04 (1.47)	21.2 (10-27) (n=4)	46.5 (11.35)
D206E	6	11.38 (1.95)	1.72 (0.9-3.20)	1.15 (0.38)	9.46 (1.74)	18.0 (16-20) (n=2)	37.6 (8.2)
E207X	5	11.90 (1.36)	1.03 (0.4-1.75)	1.37 (0.42)	10.06 (1.41)	20.7 (6-46) (n=3)	32.4 (9.1)
C210X	1	8.90 (NA)	1.13 (NA)	1.45 (NA)	6.94 (NA)	13.0 (NA)	43 (NA)

a) Values are given as mean (± SD) except for TG and Lp(a) where it is mean (range). b) Calculated by Friedewald's formula: LDL = TC-HDL-TG/2.2 mmol/l. c) Number of individuals measured are in brackets. c) N/A - not applicable.

Table 3.3.2. Comparison of unadjusted lipid levels between various groups of probands with FH.

	Whole group	Any mutation	Null mutations	Defective protein repeat five	Defective protein excluding repeat five
Number	118[a)]	44	12	17	11
Male/Female	75/43	25/19	5/7	11/6	7/4
Age (years)[b)]	44.9 (14.5)	41.1 (12.8)	40.1 (11.2)	41.4 (11.1)	42.0 (16.7)
TC mmol/l	9.37 (1.77)	10.74(1.90)*	11.31 (2.11)†	11.23 (1.65)‡	9.64 (1.77)¶
LDL mmol/l[c)]	7.31 (1.86)	8.93 (1.82)*	9.54 (2.05)†	9.37 (1.52)‡	7.78 (1.73)¶
TG mmol/l	1.65 (0.65-3.36)	1.38 (0.67-2.55)*	1.03(0.4-2.55)†	1.63(0.73-3.90)§	1.47 (0.67-2.09)
HDL mmol/l	1.30 (0.42)	1.19 (0.28)	1.29 (0.31)	1.13 (0.22)	1.20 (0.36)
Lp(a) mg/dl[d)]	39.1(3-105) (n=70)	30.5 (5-93) (n=25)	22.2 (4-60) (n=8)	31.0 (5-93) (n=10)	64.7 (20-102) (n=5)

a) Only patients with untreated plasma lipid values available were included in the analysis. B) All values are mean (±SD) except TG and Lp(a) showing mean (95% confidence interval) c) Calculated by Friedewald's formula: LDL = TC-HDL-TG/2.2 mmol/l. d) Number of individuals assayed for Lp(a) are given in brackets. *$p < 0.0001$ in comparison with the whole group †$p < 0.05$ in comparison with the whole group ‡$p < 0.01$ in comparison with the whole group §$p<0.05$ between defective protein in repeat five and null. ¶$p<0.05$ between defective protein in repeat five and defective protein excluding repeat five.

3.3.1.3. Increase of plasma LDL cholesterol concentration with age

Figure 3.3.4. shows the distribution of plasma LDL cholesterol in all heterozygous FH patients in whom the mutation has been identified including those previously described (King-Underwood et al 1991, Sun et al 1992, Webb et al 1992, Gudnason et al 1993a, Gudnason et al 1993c). Patients with mutations in the LDL receptor gene were combined according to the predicted class of mutation, of either a defective protein or a null allele. It can be seen that those individuals with a defective protein (Figure 3.3.4.a) display a statistically significant correlation of plasma LDL cholesterol with age, whereas there is no statistically detectable correlation for these variables in the group with null alleles (Figure 3.3.4.b). The slope of the regression line for the defective allele group is 0.8 mmol/l per decade.

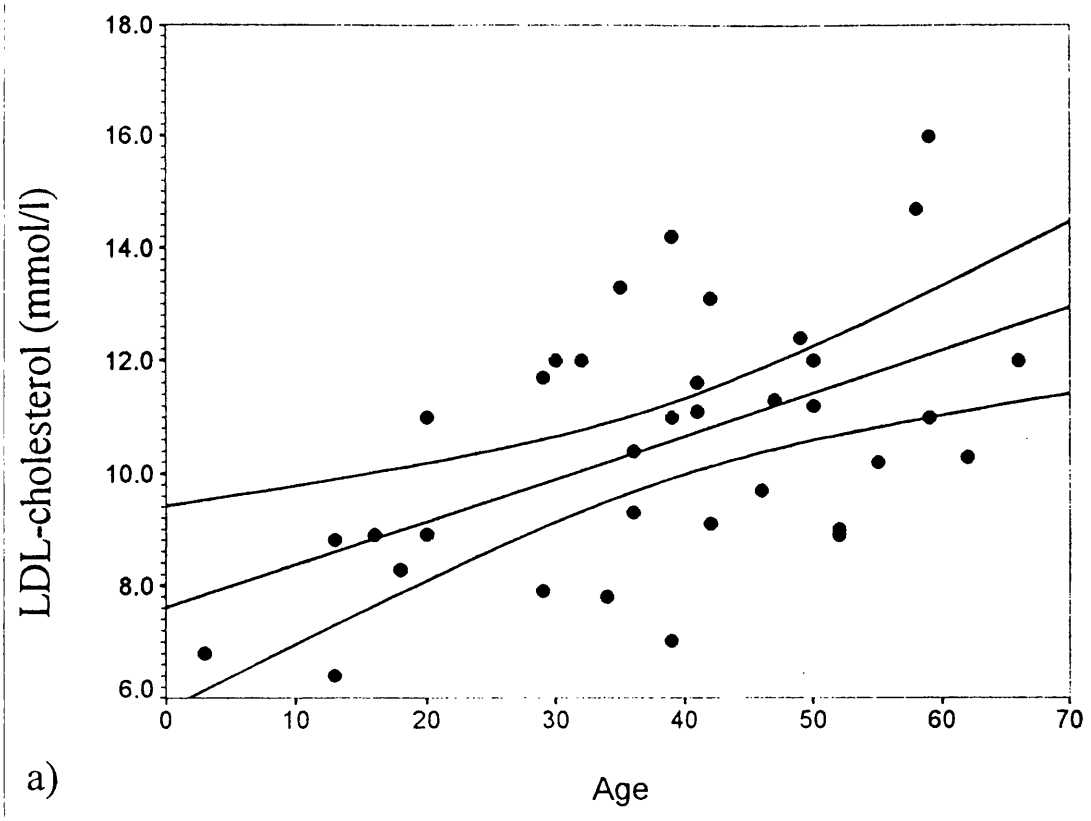

Figure 3.3.4. a) scatterplot of the plasma LDL cholesterol concentration values in individuals with mutations causing a defective protein. $(r = .53, p = .001)$.

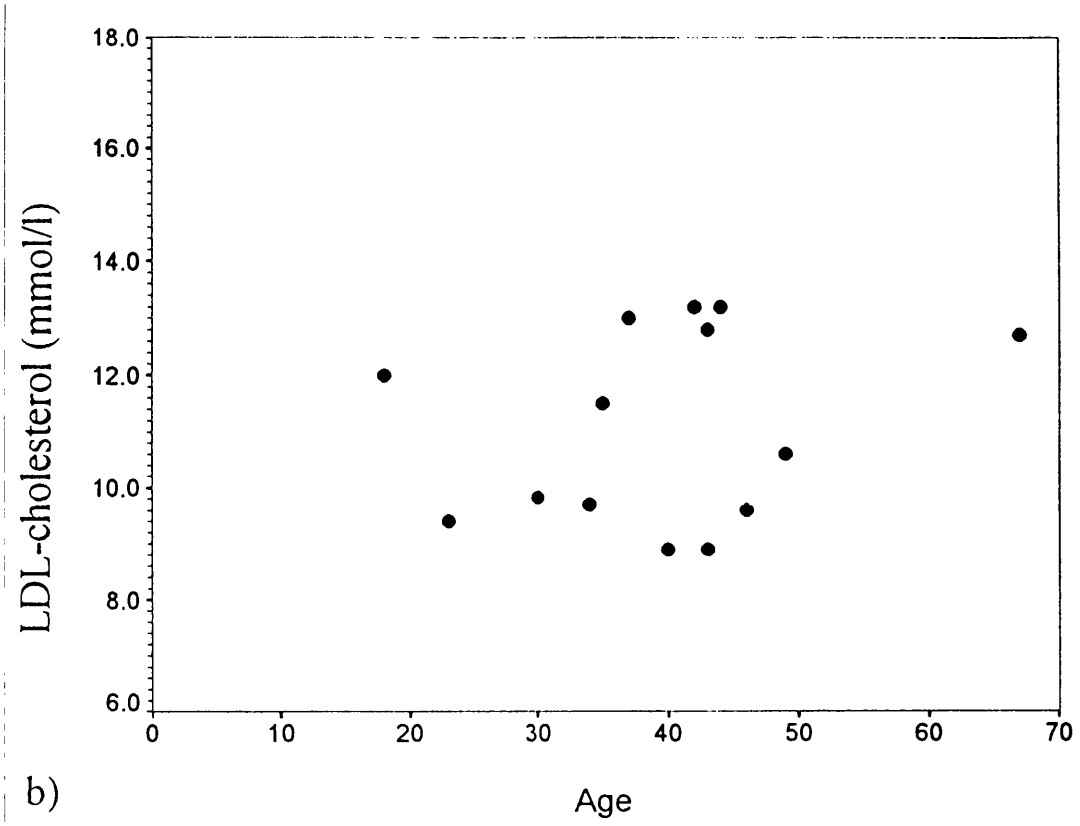

Figure 3.3.4. b) scatterplot of the plasma LDL cholesterol concentration values in individuals with a null mutation. (r = .24, p = .4).

3.3.2. Discussion

SSCP is shown here to be very useful in detecting mutations in the 3' part of exon 4 of the LDL receptor gene, coding for repeat 5 of the ligand binding domain. Out of the 311 individuals studied here 31 (or 10%) had an SSCP. Of those 29 (9.3%) had a mutation that clearly is of functional importance, and thus, as a single test to identify the mutation in FH patients in the UK, SSCP on the 3' part of exon 4 in the LDL receptor gene may be able to identify up to 10% of the mutations responsible for FH in this country. As to being able to determine the exact

mutation by examining the SSCP pattern, then for the two small deletions (ΔG197 and Fs206) the combination of the double strand and the single strand DNA both give a pathognomonic pattern. This is not the case for the other mutations in this fragment of the receptor gene, and for D206E, E207X and the C+24T, it is impossible to predict the mutations, as the SSCP patterns are indistinguishable. An alternative strategy to SSCP would be to use ASOs to identify the mutations specifically. The conclusion that SSCP on this 234 bp fragment will detect all or the vast majority of the mutations in it, is further supported by the fact that in the 30 patients (approximately 10% of all the individuals in this group) with tendon xanthomata but no sequence variation detected in exon 4, that were subjected to sequencing, no additional sequence variation was found.

For analysis of the impact of specific mutations in the LDL receptor gene on the phenotypic expression of FH in a heterogenous population like in the UK, it is a reasonable approach to group the mutations according to functional classes as there are not enough numbers of individuals with specific mutations to allow for mutation-specific analysis. Of the 29 FH patients with mutations in repeat 5 that were used in the analysis, 12 had a mutation causing a premature stop codon. Of these, the E207X mutation has been shown to produce a null phenotype (Hobbs et al 1992), and it is thus a reasonable assumption that the other two stop mutations will behave in the same way. For the defective proteins in repeat 5 there is evidence for some production of a detectable protein (Hobbs et al 1992). This is also the case with the other defective protein alleles used in the analysis (Webb et al 1991, King-Underwood et al 1991, Hobbs et al 1992). In the case of the C+24T mutation in intron 4 there is no evidence as to what effect the mutation has on the receptor activity. It was found in two, apparently unrelated probands, and one proband had an affected sibling who had the C+24T allele. This

sequence change was not found in any of the more than 300 non FH chromosomes in this sample. It is possible that this mutation has an effect on splicing, but the sequence GGGGGTGGGG as an alternative to GGGGGCGGGG bears no similarity to either 3' T/CT/CT/CT/CT/CT/CT/CT/CNCAGG or the 5'AGGTA/GAGT sequences involved most frequently in splicing (Shapiro and Senapathy 1987). Thus there is no suggestion from the sequence change as to any specific mechanism of the effect, though only experiments on cells from these patients will be able to address this question.

The results from the comparisons between various groups of mutations according to functional classes shows that patients with a mutation causing a null allele had the highest levels of total and LDL cholesterol, with very similar levels to those seen in subjects with a defective protein due to a mutation affecting repeat 5. In contrast, those with a defective protein mutation outside repeat 5 had levels of total and LDL cholesterol which were similar to those seen in the group with an unknown mutation. As repeat 5 is the sole repeat of the ligand binding domain responsible for binding both the ligands of the LDL receptor, it is not inconceivable that those with a mutation in this region will in effect behave as though no protein was produced at all, and thus have no residual receptor activity, where as mutations elsewhere affecting only the binding of apoB containing lipoproteins, will have residual receptor activity with respect to the binding of remnant particles like VLDL and IDL that contain apoE. In addition, there are number of mutations that lead to the production of defective proteins, that are either so rapidly degraded or completely blocked on their way to the cell surface (Hobbs et al 1992), that they may be called null alleles in a similar way as those caused by mutations in repeat 5. However, no attempt was made, in the studies described here, to further divide the patients into more groups.

In homozygous FH there is clear evidence that those with lower levels of receptor activity have higher LDL cholesterol (Sprecher et al 1985) and a more rapid progress of atherosclerosis (Goldstein and Brown 1989). However, although there is some evidence of a relationship between serum cholesterol levels and age of onset of CAD in heterozygous FH individuals, a clear correlation has not been demonstrated between the level of cholesterol and CAD (Jensen et al 1967, Beaumont et al 1976, Gagné et al 1979, Hill et al 1991). In a study from Canada of 364 FH heterozygous individuals from 283 families representing at least 38 countries (Hill et al 1991), it was shown that men with CAD had significantly higher LDL cholesterol (7.13 mmol/l vs 6.51 mmol/l). It has also been shown that in patients with FH that there is a significant positive correlation between intima-media thickening in the femoral artery and total serum cholesterol levels (Wendelhag et al 1993). In a group of French-Canadian FH patients, individuals with the W66G mutation in exon 3 of the LDL receptor gene had lower plasma cholesterol levels (7.2 mmol/l) than those individuals that were heterozygous for the French-Canadian 10 kb deletion (8.0 mmol/l) (Moorjani et al 1993). Even though there was no difference in the frequency of CAD between the individuals and the families, those that carried the 10 kb deletion had CAD at an earlier age, supporting the view that the plasma level of cholesterol has an influence on the onset of CAD. In addition in a recent study of the Afrikaner population, FH patients with CAD had 8% higher cholesterol than those without CAD (Kotze et al 1993).

In the general population there is a clear relationship between the magnitude of hypercholesterolaemia and the prevalence and incidence of CAD (Grundy 1986, Stamler et al 1986) as well as the onset of symptoms, with an increase of 1.3 mmol/l in plasma cholesterol

associated with an onset of CAD 10 years earlier. Thus the 1.78 mmol/l higher total cholesterol in those individuals with mutations in repeat five raises the possibility that they would be more severely affected with CAD than the other FH patients. With the clinical information currently available a complete analysis of this could not be carried out at this time. When patients who have mutations with different effect on the protein were compared, those with the defective protein had higher TG and somewhat lower HDL levels compared with those patients with a null allele mutation. This raises the possibility of the influence of this part of repeat five in binding with apoE, where a negatively-charged motif in this part of each repeat of the binding domain has been suggested to be responsible for the binding to positively-charged amino acids of the apoE protein (Goldstein et al 1985). It has been shown that the LDL receptor binds more than one apoE containing particle (Innerarity et al 1984, Tabas et al 1991), and it has been suggested that the LDL receptor protein may function as an oligomer (Grant et al 1990). Thus in the cell of a patient who is heterozygous for a defective receptor the defective protein could interfere with the normal protein. These possibilities await further investigation.

There was no difference in the distribution of the apoE alleles in the patients with a mutation in repeat five compared with the rest of the patients, and because of the limited number of patients identified with mutations in exon 4, it was not possible to carry out an analysis of the effect of apoE genotype on plasma lipids in the two groups of null allele and defective protein. The observation that the individuals with a defective allele have cholesterol levels that increased with age while cholesterol levels are high at all ages in those with a null allele is in agreement with results from others (Kotze et al 1993). The increase in plasma cholesterol levels is about 0.9 mmol/l per decade in the group with the defective protein compared with about 0.5 mmol/l

per decade in the general British population (Tunstall Pedoe et al 1989).

In the light of the observations in this report, as well as by others (Koivisto et al 1992, Moorjani et al 1993, Leitersdorf et al 1993, Kotze et al 1993), it is now clear that different mutations associate with differences in lipid levels and it is likely that this will be associated with clinically different effects.

CHAPTER 4 - THE ALANINE 370 TO THREONINE POLYMORPHISM IN THE LDL RECEPTOR

4.1. Introduction

To date, only one amino acid substitution in the LDL-receptor has been described that does not cause FH. This is the alanine to threonine change in codon 370 (A370T), caused by substitution of an A by a G in exon 8 (Kotze et al 1989b). This nucleotide change causes a loss of a restriction site for the *Stu*I endonuclease (Kotze et al 1986), and the frequency of the loss of this restriction site ranges from 0.04-0.08 in most populations studied (Taylor et al 1988, Kotze et al 1989b, Leitersdorf et al 1989a). This change involves the amino acid residue next to the last cysteine in repeat B of the EGF precursor homology domain of the LDL receptor (Südhof et al 1985). This amino acid is not highly conserved between species (Figure 4.1.1.), which suggests that the change is unlikely to have a major effect on the function of the LDL receptor protein, although this residue is either an alanine (human and rabbit) or a valine (mouse, hamster and rat) in the species investigated (Südhof et al 1985, Yamamoto et al 1986, Hoffer et al 1993, Bishop 1992). No studies have addressed the question as to whether this amino acid change has an effect on plasma cholesterol concentration in the general population.

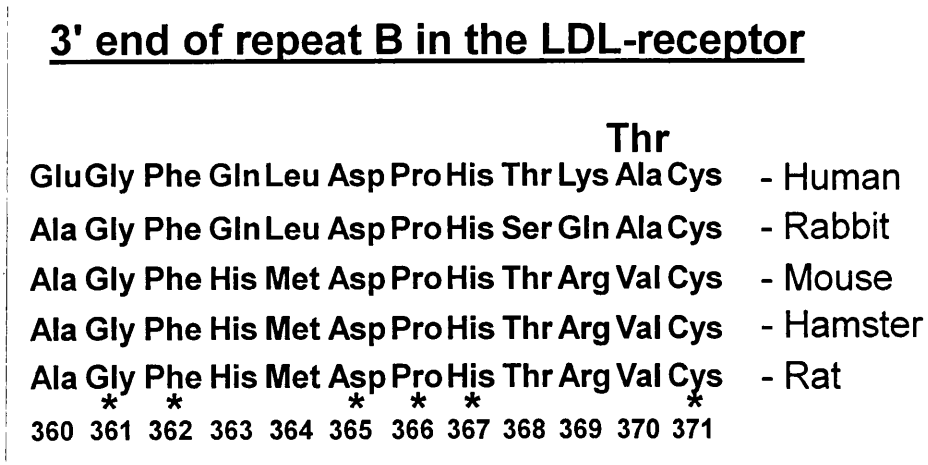

Figure 4.1.1. Comparison of amino acid sequences of repeat B in the EGF precursor homology domain of the LDL receptor in various species. Asterisks denote conserved amino acids between species. The number of the amino acids in the human protein are shown below.

4.2. The effect of the A370T polymorphism on plasma lipids in the Icelandic population.

4.2.1. Methods

The A370T polymorphism was determined in the Icelandic sample (described in detail in Chapter 2 Section 2.8.2.1.) by digestion with *Stu*I of amplified DNA as described in Chapter 2 Section 2.4.2.2. Statistical analysis was carried out as described in Chapter 2 Section 2.7. The genotyping was carried out at the MRC Lipoprotein Team laboratories at the Hammersmith Hospital in London.

4.2.2. Results

Lipid and lipoprotein concentration of 318 individuals from the general Icelandic population are shown in table 4.2.1. The women have significantly lower triglycerides and higher HDL cholesterol than men in addition to having lower plasma LDL cholesterol concentration.

All of the individuals were genotyped for the A370T polymorphism by restriction enzyme analysis of an amplified fragment of the LDL receptor using the restriction endonuclease *Stu*I. Figure 4.2.1.a) shows a schematic drawing of the fragment sizes of the cut 144 bp and 49 bp and uncut 193 bp long PCR of the whole of exon 8 of the LDL receptor gene.

Table 4.2.1. Unadjusted lipid concentrations and characteristics of the sample of 318 individuals from the general population of Iceland. Mean (± SEM)

	Men	Women	All
n	152	166	318
Age (years)	41.1 (1.2)	42.6 (1.2)	41.7 (1.2)
BMI (kg/m^2)	25.1 (0.3)	24.1 (0.3)	24.6 (0.3)
TC (mmol/l)	5.58 (0.09)	5.51 (0.09)	5.54 (0.09)
TG (mmol/l)	1.15 (0.05)	0.98 (0.04)*	1.06 (0.05)
HDL-C (mmol/l)	1.16 (0.02)	1.44 (0.03)**	1.31 (0.03)
LDL-C (mmol/l)	3.89 (0.08)	3.63 (0.09)*	3.76 (0.09)
ApoB (mg/dl)	120.10(2.08)	111.82(2.22)**	115.78 (2.15)
Lp(a) (mg/dl)	24.6(0-92.4)†	26.3 (0-100.6)†	25.5 (0-93.7)†

* $p < 0.05$ ** $p < 0.01$ † 95% Confidence Interval

Figure 4.2.1.b) shows a silver stained polyacrylamide gel, showing the pattern of the restriction fragments after digestion with *Stu*I. Lane 1 shows a digest pattern from an individual heterozygous for the *Stu*I cutting site, lane 2 shows the 1 kb ladder markers, and lanes 3 and 4 are from individuals homozygous for the cutting site.

Because of the rarity of the T allele, samples were combined into groups of 5 after checking the PCR, incubated with *Stu*I and electrophoresed on a polyacrylamide gel. Those groups that showed evidence of a uncut fragment were digested individually by *Stu*I. Figure 4.2.2. shows such a gel, the samples in lane 2, 3, 5 and 7 are positive for an uncut fragment, and upon digestion with the restriction endonuclease proved to contain a single positive sample for the T370 allele except for the sample pool in lane 2 in which, upon digestion with *Stu*I of individual samples, two positive samples for the T370 allele were identified out of the five samples in the pool. This can clearly be seen as an uncut band of stronger intensity.

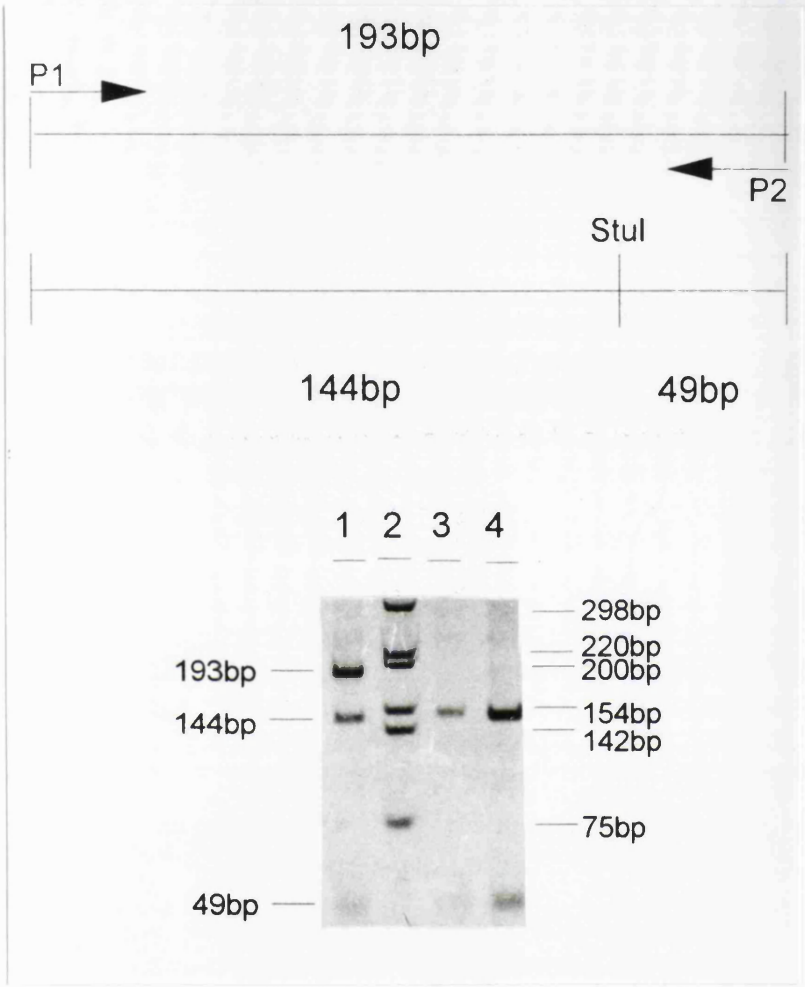

Figure 4.2.1. Schematic drawing of the PCR fragment amplified for exon 8 of the LDL receptor gene and the expected fragments obtained upon digestion with *Stu*I in a). In b) the digestion of the PCR fragment is shown, giving the correct fragment sizes. The marker used was 1 kb ladder. The gel was silver stained.

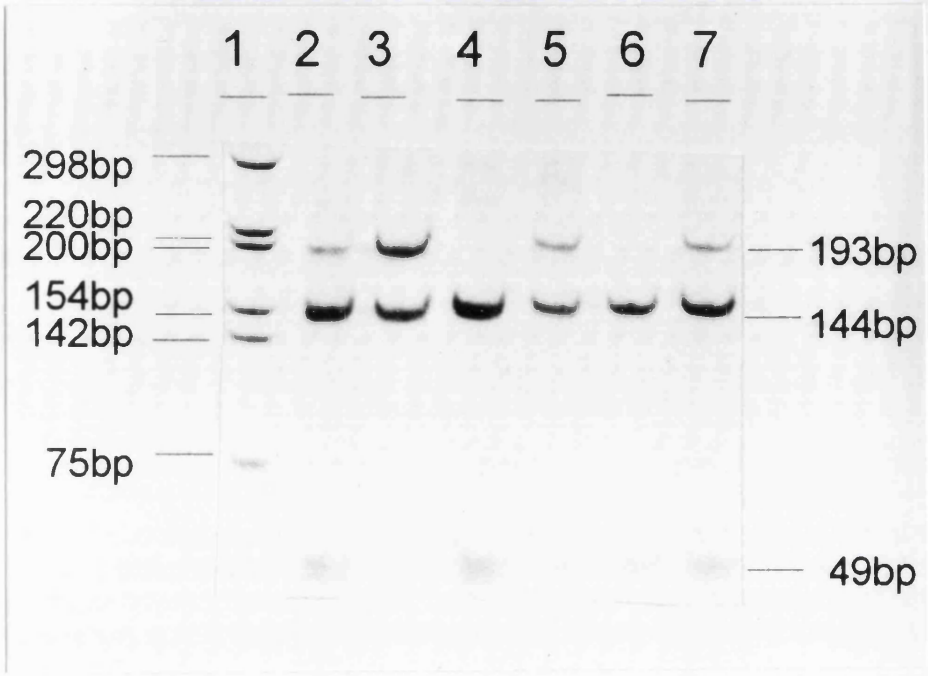

Figure 4.2.2. Digestion of the pooled samples. The samples in lane 2, 3, 5 and 7 are positive for an uncut fragment. The marker used was 1 kb ladder. The gel was silver stained.

The frequency of the T370 allele (*Stu*I-) in the sample was 0.06 (95%CI=0.022-0.098) for men and 0.04 (95%CI=0.010-0.070) for women and was not significantly different between the two groups (Table 4.2.1.). There were no subjects homozygous for the T370 allele in these groups.

To examine the distribution of plasma LDL cholesterol concentration in the population, the values were plotted on a scattergram. A scattergram of the LDL cholesterol values according to age for both men and women is shown in Figure 4.2.3. Generally, LDL cholesterol concentrations in subjects carrying the rare T allele were above the regression line in the sample of men but below the line in the sample of females. Scattergrams of total cholesterol and apoB concentrations showed similar effects (data not shown).

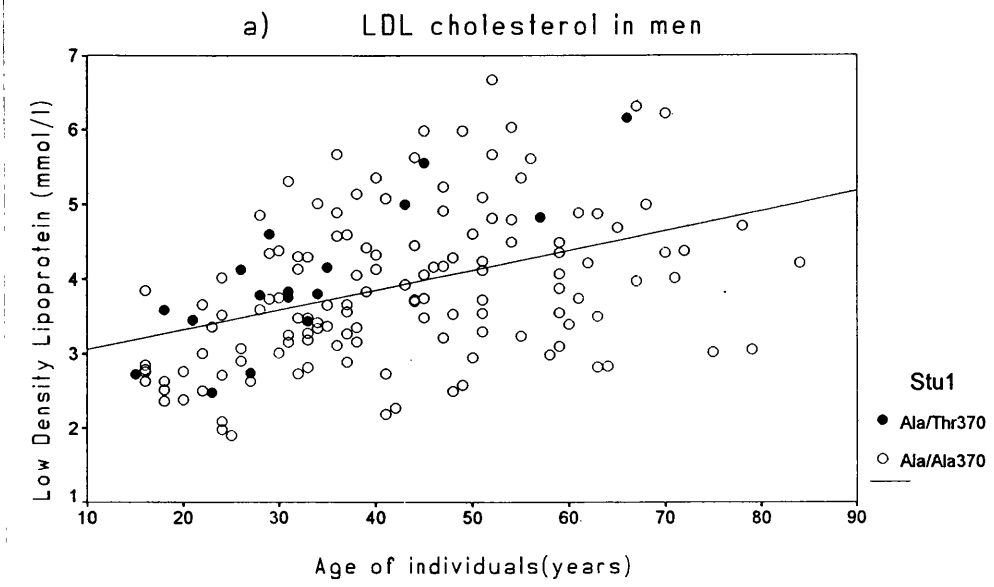

Figure 4.2.3. a) scattergram of LDL cholesterol with age and the A370T genotype in men. The regression line is drawn.

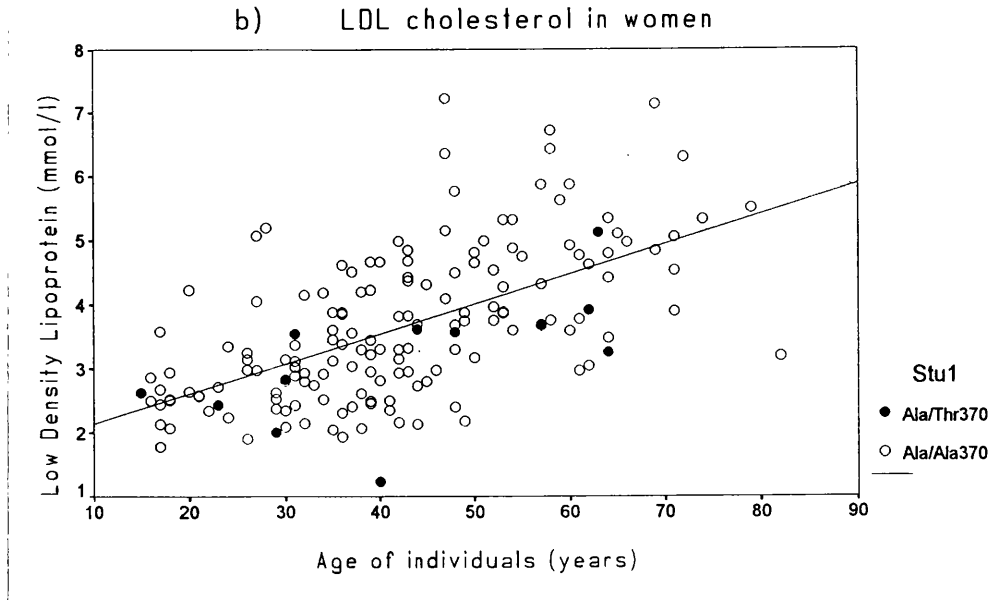

Figure 4.2.3. a) scattergram of LDL cholesterol with age and the A370T genotype in women. The regression line is drawn.

Table 4.2.2. Adjusted lipid and lipoproteins characteristics of men and women according to their genotype.

Genotype		Ala/Ala	Ala/Thr
Men	(n)	134§	18
	Age (years)	42.4 (15.6)**	32.8 (12.9)
	TC (mmol/l)	5.52 (0.96)*	6.02 (0.83)
	LDL-C (mmol/l)	3.83 (0.91)*	4.34 (0.85)
	ApoB (mg/dl)	118.50 (21.92)	132.02 (19.41)
	TG (mmol/l)	1.18 (0.54-2.34)	1.20(0.26-3.16)
	HDL (mmol/l)	1.17 (0.29)	1.13 (0.26)
	Lp(a) (mg/dl)	23.6 (0-93.8)	32.2 (5.6-91.8)¶
Women	(n)	154	12
	Age (years)	42.1 (14.8)	42.1 (16.8)†
	TC (mmol/l)	5.54 (0.97)	5.13 (0.52)†
	LDL-C (mmol/l)	3.69 (0.94)	3.20 (0.74)†
	ApoB (mg/dl)	112.64 (22.63)	101.26 (19.81)†
	TG (mmol/l)	0.95(0.46-1.71)‡	0.90 (0.5-1.58)
	HDL (mmol/l)	1.43 (0.32)‡	1.54 (0.41)‡
	Lp(a) (mg/dl)	25.9 (0-99)	30.7 (0-111.8)

All values are mean (±SD) except for Lp(a) which is mean (95%CI), § X^2 for frequency of genotypes NS, * $p < 0.05$ between genotypes, ** $p < 0.01$ between genotypes, † $p < 0.05$ between men and women, ‡ $p < 0.01$ between men and women, ¶ $p < 0.05$ between genotypes by Kruskal Wallis 1-way ANOVA

Plasma lipid levels were studied in greater detail after values had been adjusted for age and body mass as shown in Table 4.2.2. Men with the T370 allele had 8.9% higher mean total cholesterol, 13.6% higher LDL cholesterol, and 11.4% higher apoB concentrations compared with those with only A alleles ($p<0.05$). By contrast, women carrying the T370 allele have lower mean total and LDL cholesterol (6%), and 8% lower apoB levels than those with only A370 alleles, though these differences did not achieve statistical significance. In the group as a whole, there was a statistically significant difference between

men and women in both unadjusted (Table 4.2.1.) and adjusted LDL cholesterol values (table 4.2.2.). These gender differences were maintained when men and women carrying the allele for the T370 were compared, but was not observed when men and women with the common genotype A370 only were compared (Table 4.2.2.). Lp(a) levels were consistently higher in individuals carrying the allele for T370 (Table 4.2.2.), though this was only statistically significant for men.

All the individuals were also genotyped for the apoE polymorphism as published (Gudnason et al 1993b). When the analysis was carried out on men with the apoE 3/3 genotype only, those with the genotype A370T (n=13) still had significantly higher levels of TC, LDL-C and apoB compared with those with only the allele for A370 (n=74)(6.02 mmol/l, 4.36 mmol/l and 132.3 mg/dl compared with 5.46 mmol/l, 3.73 mmol/l and 117.5 mg/l respectively).

4.2.3. Discussion

Because of the expected low frequency of the T370 allele (Taylor et al 1988, Kotze et al 1989b, Leitersdorf et al 1989a), it was decided to do the initial analysis by restriction endonuclease digestion on the samples in groups of five. This meant that for a predicted frequency of 10% carriers, every other pool would contain a positive sample and only the individuals in that pool would thus need to be reanalysed separately for the polymorphism. This pooling of previously checked PCR's saved the redigestion of about half of the samples and only for 2/3 of the entire sample were digestion and electrophoresis of the samples carried out.

The study was carried out in a sample of 318 healthy men and women from the general Icelandic population, which is genetically relatively homogenous, and which has been analyzed previously to confirm that variations at the apoAI, and apoE gene loci affect lipid and lipoprotein levels (Sigurdsson et al 1992b, Gudnason et al 1993b). In this sample, the allele for T370 was associated with raised levels of total plasma cholesterol, as well as LDL-cholesterol and apoB, although the effect was only seen in men. By contrast women who carried the T370 allele had lower total plasma cholesterol, as well as LDL-cholesterol and apoB, though this did not reach statistical significance. This holds, even when the analysis is carried out only on those individuals with an apoE 3/3 genotype, indicating that the effect is independent of the apoE genotype. A difference between the effect of genotype on lipid levels in men and women has been observed with the effect of the common apoE gene polymorphisms (Reilly et al 1991, Gudnason et al 1993b) and the effect of an apoAI gene polymorphism (Sigurdsson et al 1992b). Although the precise molecular mechanism for these gender-specific effects is unknown, it is likely to be mediated by hormonal differences, and it is not inconceivable that the different effect of the A370T polymorphism on plasma cholesterol concentration in men and women is through a similar mechanism.

Genetic variation in the LDL receptor that is associated with plasma cholesterol concentration but which in itself does not cause FH, has been described (Pedersen & Berg 1988, Schuster et al 1990b, Humphries et al 1991). This variation is identified as the *Pvu*II polymorphism in the LDL receptor gene. This polymorphism is associated with about 0.5 mmol/l lower LDL cholesterol in those carrying the less common allele (Pedersen & Berg 1988, Schuster et al 1990b, Humphries et al 1991), which is a similar to the difference observed here in men with the A370T polymorphism in the Icelandic population. None of

these studies of the *Pvu*II effect analyzed the data for men and women separately, so at this stage it is not known if there is a difference between sexes.

There was also a difference in Lp(a) levels with respect to the A370T genotypes. Those with a T370 allele have markedly higher levels of Lp(a), though it only reaches statistical significance in men (23 mg/dl versus 32 mg/dl, $p < 0.05$). This has been observed before in a sample of Finnish children (Lindahl 1991) but has not been examined elsewhere. However, a number of studies on heterozygous FH patients have demonstrated an increased plasma Lp(a) concentration (Utermann et al 1989, Seed et al 1990, Wiklund et al 1990, Mbewu et al 1991), although this has not been confirmed by all studies (Soutar et al 1991). Furthermore, studies on transgenic mice overexpressing the LDL receptor show that this results in a markedly increased clearance of injected Lp(a), compared to control mice (Hofmann et al 1990). On the other hand, upregulation of the LDL receptor by HMG CoA reductase does not affect the Lp(a) plasma concentration (Kostner et al 1989), casting doubt on a major role for the LDL receptor in Lp(a) clearance, and plasma Lp(a) concentration seems to be dependant mainly on the production rate rather than on the rate of removal (Rader et al 1993). Binding of Lp(a) to the LDL receptor has been demonstrated but with lower affinity (Floren et al 1981, Havekes et al 1981, Krempler et al 1983, Armstrong et al 1990, Steyrer and Kostner 1990), so it is possible that the LDL receptor could participate in the removal of Lp(a) from the circulation and thus a genetic variation in the receptor gene may participate in the determination of the plasma levels of Lp(a). Further studies are required to answer this.

4.3. *In vitro* analysis of the A370 and the T370 proteins of the LDL receptor

It is possible that the change of the alanine for threonine in codon 370 is in itself responsible for the association observed in the Icelandic sample by affecting LDL receptor function. Alternatively, the A370T change may be in linkage disequilibrium with another functional, as yet unidentified sequence change in the LDL receptor gene. To address the possibility, *in vitro* analysis of the two alleles was undertaken. This was carried out by site-directed mutagenesis and transfection into CHO cells, with the examination of internalisation and degradation of LDL. All of this work was carried out at the MRC Lipoprotein Team laboratories at the Hammersmith Hospital in London. The work was carried out with Dr. Xi-Ming Sun, Dr Dilip Patel, Dr Anne K. Soutar and Dr Brian L. Knight who are co-authors of the publication describing this study (Gudnason et al 1995a, a copy of this paper is bound at the back of the thesis).

4.3.1. Methods

Site directed mutagenesis was performed as described in Chapter 2 and Section 2.5. The analysis of receptor function in CHO cells transfected with the A370 and the T370 alleles of the LDL receptor were carried out as described in Chapter 2, Section 2.5

4.3.2. Results

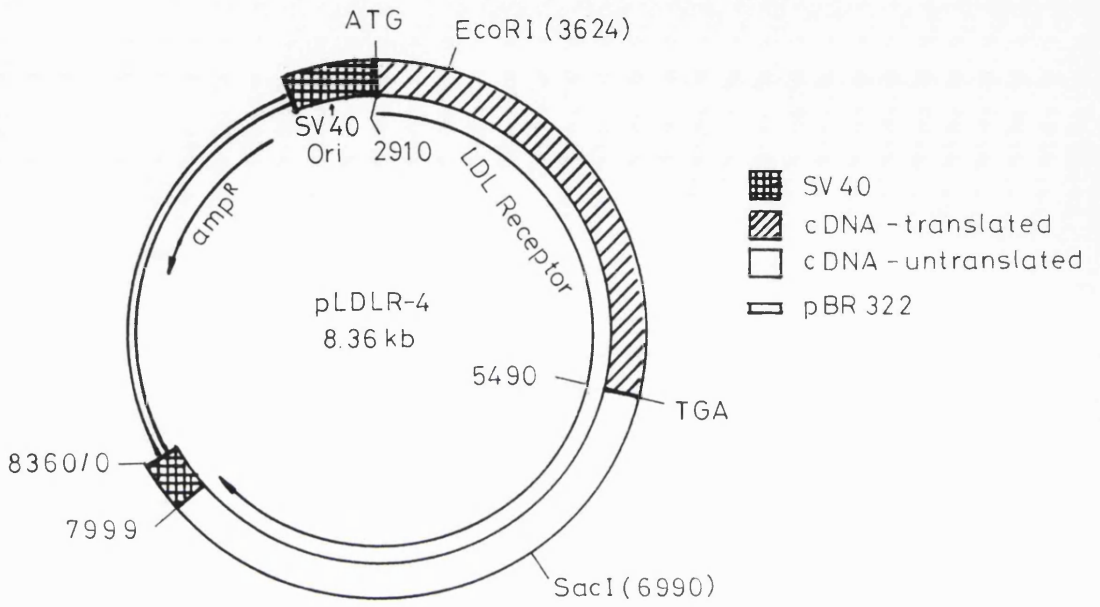

Figure 4.3.1. Structure of the pLDLR4, which contains the full length cDNA for the human LDL receptor. The coding region (hatched area) encompasses 2580 nucleotides (from 2910 to 5490) between codons ATG and TGA. The EcoRI and the SacI sites of the 3.2 kb fragment used in the pALTER for the synthesis of the T370 allele are shown (at bases 3624 and 6990, respectively).

Figure 4.3.1. shows a schematic drawing of the LDLR4 plasmid which contains the full length cDNA for the human LDL receptor, in a mammalian expression vector driven by the SV40 promoter (kindly provided by Dr. David Russell). LDLR4 has the A370 allele and the T370 allele had to be introduced into this plasmid. The construct with T370 allele in was made in a two step process. The pALTER-1 phagemide containing a 3.2-kb *Eco*R1-*Sac*I fragment comprising the 3' part of the LDL receptor cDNA (kindly provided by Dr Anne K. Soutar, see Figure 4.3.1.) was used for introducing the T370 allele into the human LDL receptor cDNA by site directed mutagenesis. Two oligonucleotides was annealed simultaneously to the single stranded DNA (obtained from the pALTER-1 phagemide with

the *Eco*RI-*Sac*I LDL receptor. insert transfected into JM 109 cells with the R408 helperphage). In addition to the oligonucleotide containing the T370 site, an oligonucleotide containing a sequence restoring an ampicillin resistance to the vector was used to allow the identification of the vectors that were successfully mutated, on the notion that if one oligonucleotide was incorporated into the vector, the other one would as well. The presence of the T370 allele was then confirmed by sequencing of a miniprep plasmid DNA (data not shown). After inserts from positive clones had been ligated into the LDLR4 plasmid their presence was confirmed by sequencing of a large scale preparation of the mutant plasmid The plasmids containing either the A370 allele or the T370 allele were then expressed in ldl-A7 cells (kindly supplied by Dr. M. Krieger) transfected with the plasmids. Figure 4.3.2. shows an immunoblot of the human LDL receptor expressed in these cells.

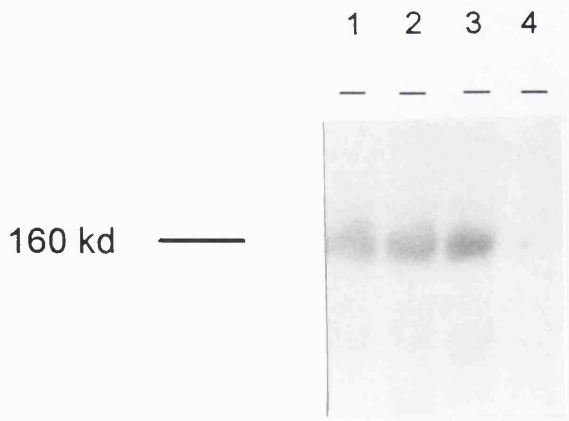

Figure 4.3.2. An immunoblot of the human LDL receptor in the CHO ldl-A7 cells. Lanes 1and 2 contain the T370 receptor protein, lane 3 contains the A370 receptor protein and lane 4 is extract from untransfected ldl-A7 cells, negative for the human LDL receptor.

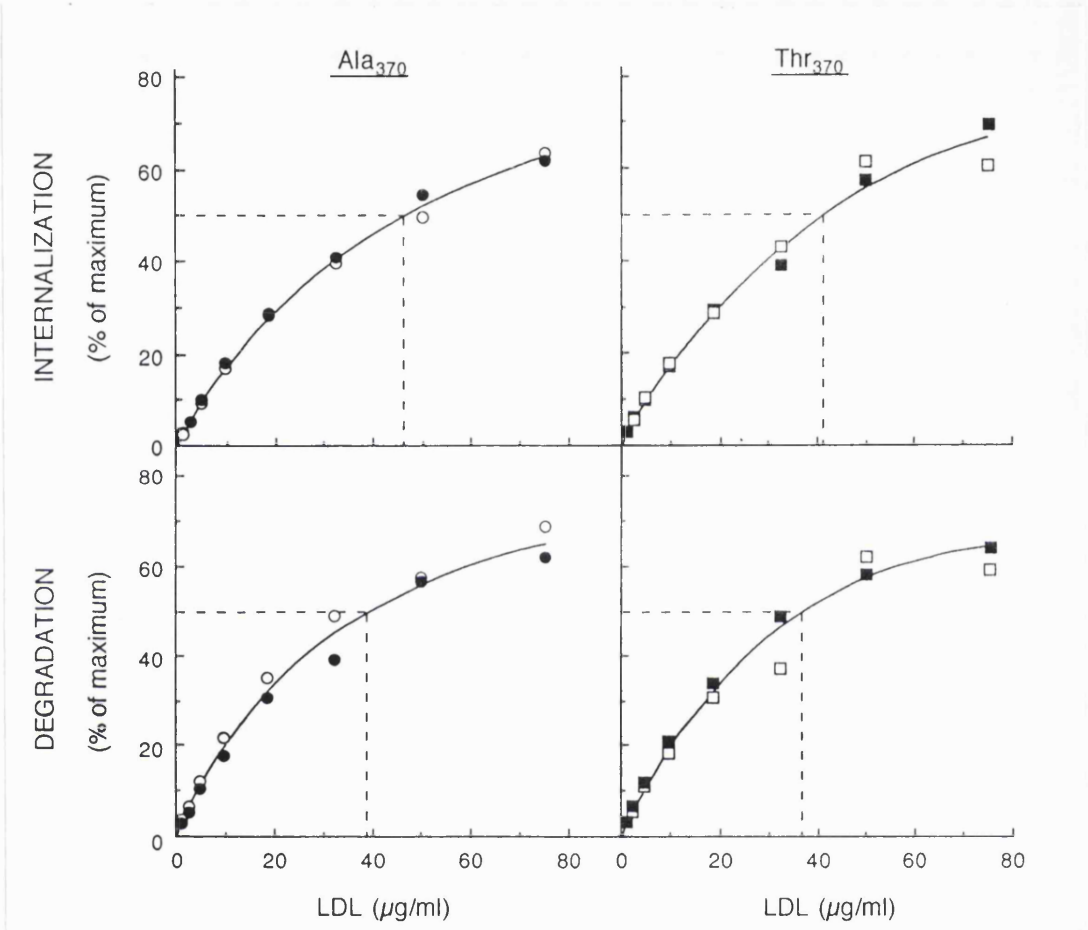

Figure 4.3.3. The internalisation and degradation of LDL by the transfected ldl-A7 CHO cells. Points are the averages of duplicate incubations for two separate cell lines carrying the A370 allele (○,●) and two carrying the T370 allele (□,■).

Figure 4.3.3. shows the internalization and degradation of LDL by CHO cells transfected with human LDL receptor cDNA. Cells were incubated with indicated concentrations of labelled LDL for 4 hours at 37°C and the amount of LDL internalized or degraded was determined. Values were correlated for non-saturable internalization or degradation observed in the presence of an excess of unlabelled LDL and were expressed as percentage of the maximum, calculated with the assumption that the points followed a rectangular hyperbola. Points are the averages of duplicate incubations for two separate cell lines carrying the A370 allele (○, ●) and two carrying the T370 allele (□, ■). Individual values for internalization and degradations (ng/mg protein) and for concentration giving one-half maximum (μg/ml) and shown in Table 4.3.1.

Table 4.3.1. Individual values for internalisation and degradations of LDL and for the concentration giving one-half maximum.

Cells	Internationalisation		Degradation	
	Maximum ng/mg protein	Concentration giving 1/2 maximum μg/ml	Maximum ng/mg protein	Concentration giving 1/2 maximum μg/ml
○A370-1	767	47	1640	35
●A370-2	129	45	265	44
□T370-1	96	41	208	42
■T370-2	101	42	226	37

4.3.3. Discussion

To see if the observed difference in plasma lipid and lipoprotein levels associated with the A370T change was caused by differences in receptor activity an expression vector was

constructed carrying the T370 and the A370 sequence, which was then transfected into CHO cells which were tested for the presence of a human LDL receptor as well as for uptake and degradation of LDL. No difference in receptor activity was seen between the transfected CHO cell lines in the receptor activity. The cell experiments thus do not support the contention that the differences observed in men are related to a large difference in LDL receptor activity. However, this does not entirely exclude the possibility that there is a functional difference *in vivo*, as some LDL receptor mutations that are known to cause definite FH, such as the W66G mutation, result in only a minimal loss of activity when studied *in vitro* (Hobbs et al 1992). In the Dallas collection of fibroblasts from homozygous FH patients three different cell lines homozygous for the W66G mutation were examined with respect to the LDL receptor activity, which was reported to be from 25% to 100% (Hobbs et al 1992). Studies such as those performed here may thus not detect small differences in receptor activity, and it is possible that more accurate assays of different aspects of LDL receptor function *in vitro* will be needed to analyze the effect of some mutations in the LDL receptor gene.

The A370T change described here affects repeat B of the EGF homology region. Mutations in repeat A, B and particularly in the YI of the RGF homology region have been found to cause class 5 mutations at the functional level (Hobbs et al 1992). This is a defect in the recycling of the receptor, and deletion of the EGF homology region has, by *in vitro* studies, been shown to result in a defective recycling of the LDL receptor, in addition to causing its rapid degradation (Davis et al 1987a). To answer this question for the A370T mutation different experiments would have to be performed.

It is also a possibility that this polymorphism is not functional at all, but is in linkage disequilibrium with another functional variation somewhere else in the gene. It was thus of importance to examine this amino acid change in another sample to see whether it was consistent. The A370T effect was examined in a sample of young individuals from various places in Europe.

4.4. The effect of the A370T polymorphism in the European Atherosclerosis Research Study (EARS) sample

4.4.1. Methods

Genotyping of the EARS sample (described in detail in Chapter 2 Section 2.8.2.2.), was performed by allele specific oligomelting (as described in Chapter 2 Section 2.4.3.). The statistical analysis was carried out by Dr. Viviane Nicaud at the EARS analysis team in Paris as described in Chapter 2 Section 2.7., using a strategy devised by myself.

4.4.2. Results

Genotype was determined by allele specific oligonucleotide melting as shown in Figure 4.4.1. The genotype for individuals identified as heterozygous for the T370 allele were confirmed by digestion with *Stu*I as described in Section 2.4.2.2. of Chapter 2.

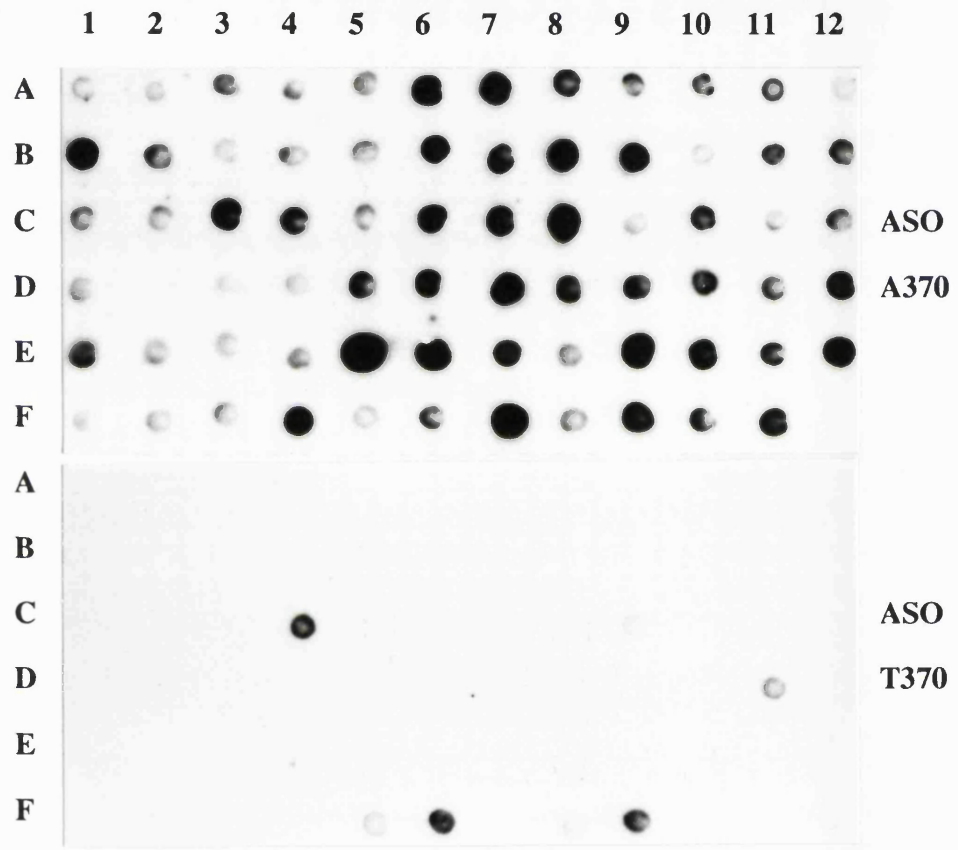

Figure 4.4.1. Genotyping with ASO detection of the A370T polymorphism in exon 8 of the LDL receptor gene. The format is that of a 96 well PCR plate. Heterozygotes are in lanes C4, C9. D11, F5, F6, and F8, and a homozygote positive control for the T370 is shown in lane F12. D2 is a no-DNA negative control. The variation in the intensity of the signal reflects the variation in the PCR yield.

In Table 4.4.1. the number of genotypes obtained and the relative allele frequencies in cases and controls in the five different regions is presented. There was no departure from Hardy-Weinberg equilibrium in any region in either cases or controls. In the controls, the frequency of the T370 allele varied from 0.061 in Great Britain to 0.027 in the South, and overall was 0.048(95%CI=0.040-0.056) with similar values in men and women (0.045 and 0.052 respectively), and no statistical evidence for heterogeneity over the five regions. In the cases, the frequency of the T370 allele was higher than in the controls in four of the five regions, with an average frequency of 0.060 (95% CI= 0.044-0.073), and overall this difference was statistically significant (p = 0.02). In the middle region, the frequency of the T370 allele in the controls is similar to the frequency in the controls in other regions (0.043), whereas the frequency of the T370 allele in the cases is much lower (0.03). There was no difference in allele frequency between cases with a father who had had a fatal or a non-fatal event. There were two subjects homozygous for the T370 allele, one in the cases from Gothenburg and one control from Oulu. Overall, the relative risk associated with the T370 allele of having a father with an MI under the age of 55 in this sample was 1.49 (CI 1.03-2.15, p = 0.03).

The relationship between genotype and plasma lipid levels was next examined separately in males and females and by status (cases and controls), and the data are presented in Table 4.4.2., after values had been adjusted for the effects of region and BMI. In the controls there were no differences in any measured lipid traits associated with A370T genotype. However in the cases, the women carrying the T370 allele had lower mean plasma total (8.8%) and LDL cholesterol (15.43%), and 13.0% lower plasma apoB concentration and 20% lower LDL/HDL ratio than those with the A370 allele, and these differences were all

statistically significant ($p < 0.01$). By contrast, in the male cases those with the T370 allele had 5.6% *higher* mean total cholesterol, 6.8% higher LDL cholesterol, and 6.4% higher apoB concentrations compared with those with the A370 allele, although these differences did not achieve statistical significance.

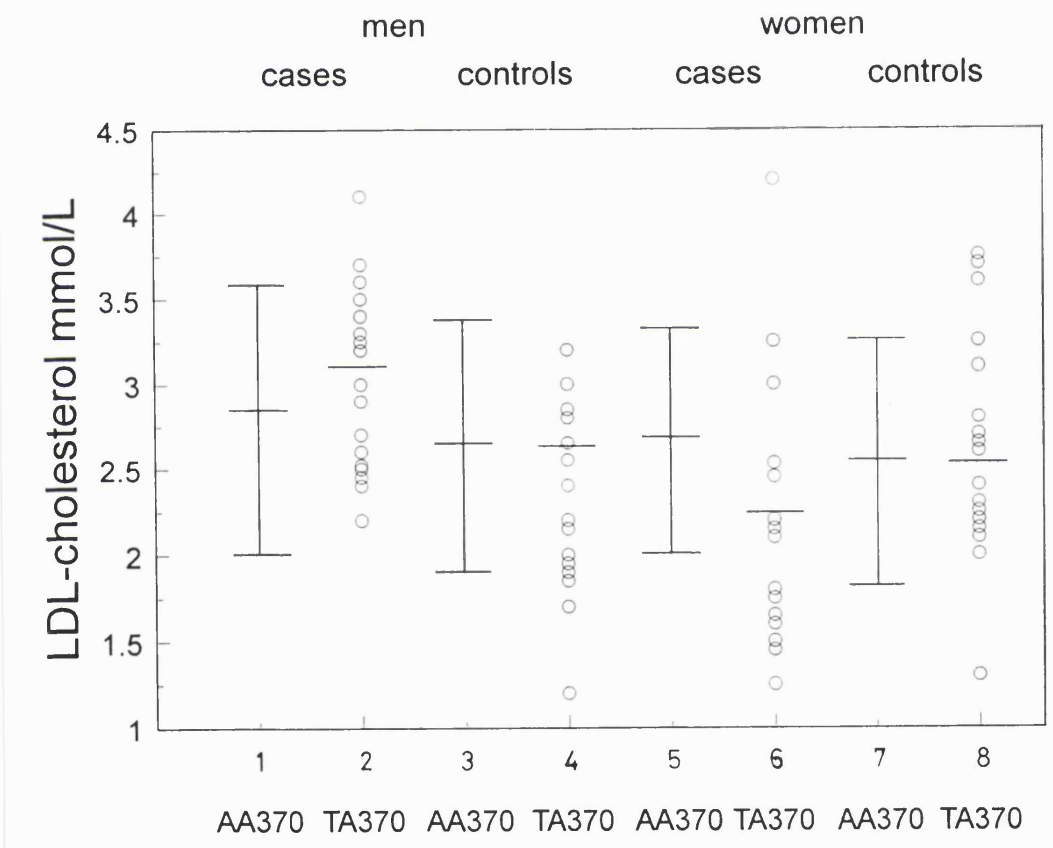

Figure 4.4.2. Scattergram of the distribution of plasma LDL cholesterol in the A370T individuals (circles) (lanes 2, 4, 6, and 8). The mean levels ± the standard deviation are shown for the groups of individuals homozygous for the A370 allele (lanes 1, 3, 5, and 7). The data male offspring of cases and controls are shown in lanes 1 and 2, 3 and 4, respectively and for the female offspring of cases and controls in lanes 5 and 6, 7 and 8, respectively.

Table 4.4.1. Number of individuals genotyped (cases and controls) and relative frequency of the T370 allele in five different regions.

	Genotype Cases (%)		Genotype Controls (%)		T370 Allele frequency	
Region	A/A	A/T + T/T	A/A	A/T + T/T	Cases	Controls
Finland	69	8 (10.4%)	153	12 (7.3%)	0.052	0.039
Gt Britain	45	7 (13.5%)	72	10 (12.2%)	0.067	0.061
North	117	20 (14.6%)	242	24 (9.0%)	0.077	0.045
Middle	140	9 (6.0%)	213	20 (8.6%)	0.030	0.043
South	73	15 (17.1%)	160	9 (5.3%)	0.085	0.027
All	444	59 (11.7%)	820	* 75 (8.2%)	0.060	*0.042

Test on differences between centres (adjusted for Status) = NS
* Test on difference between cases and controls (adjusted for Centres) p = 0.02

Table 4.4.2. Total-cholesterol, LDL-cholesterol, LDL/HDL ratio and apoB levels (mean, SEM) according to LDL-A370T genotype, separately by sex and case/control status.

Lipid trait	Geno-type	Females N=689		Males N=730	
		Cases (216:27)†	Controls (404:42)	Cases (228:32)	Controls (437:33)
Total-Chol (mmol/l)	A/A	4.66 (0.06)	4.50 (0.04)	4.50 (0.06)	4.34 (0.04)
	A/T	4.25 (0.15)‡	4.46 (0.12)	4.75 (0.15)	4.30 (0.14)
LDL-Chol (mmol/l)¶	A/A	2.69 (0.05)	2.53 (0.04)	2.80 (0.05)	2.65 (0.04)
	A/T	2.28 (0.14)*	2.50 (0.11)	2.99 (0.14)	2.63 (0.13)
LDL/HDL Ratio¶	A/A	1.81 (0.05)	1.67 (0.03)	2.36 (0.06)	2.13 (0.04)
	A/T	1.46 (0.13)**	1.68 (0.09)	2.43 (0.16)	2.25 (0.13)
ApoB# (mg/l)	A/A	891 (16)	847 (12)	943 (15)	881 (11)
	A/T	771 (41)§	818 (32)	1003 (39)	904 (38)

Means are adjusted for age, region and BMI, †numbers of A/A and A/T individuals. ‡p = 0.009, *p = 0.006, **p = 0.013, §p = 0.005, ¶interaction between case-control status and A370T genotype in females, p = 0.05
interaction between sex and A370T genotype p = 0.01

Figure 4.4.2. shows the distribution of the plasma LDL cholesterol in the A370T individuals on a scattergram in both the men and the women, cases and controls as well as the mean plasma LDL cholesterol ± the standard deviation for those homozygous for the A370 allele. The association of the A370 allele with lower levels of plasma lipid levels in women cases and not men was consistent over the five regions of Europe studied (test of homogeneity, p = 0.22), and the apoB data for women are presented in Figure 4.4.3. In the females the interaction between A370T genotype and case control status was on the borderline of statistical significance for the effect on LDL-cholesterol (p = 0.054) and for the LDL/HDL ratio (p = 0.053), and for apoB there was a significant interaction between genotype and sex (p = 0.01). In male cases, the plasma concentration of total cholesterol, LDL cholesterol and apoB was not as consistently higher, and Figure 4.4.4. shows the data for the apoB in men. There was no association with A370T genotype and any other lipid trait.

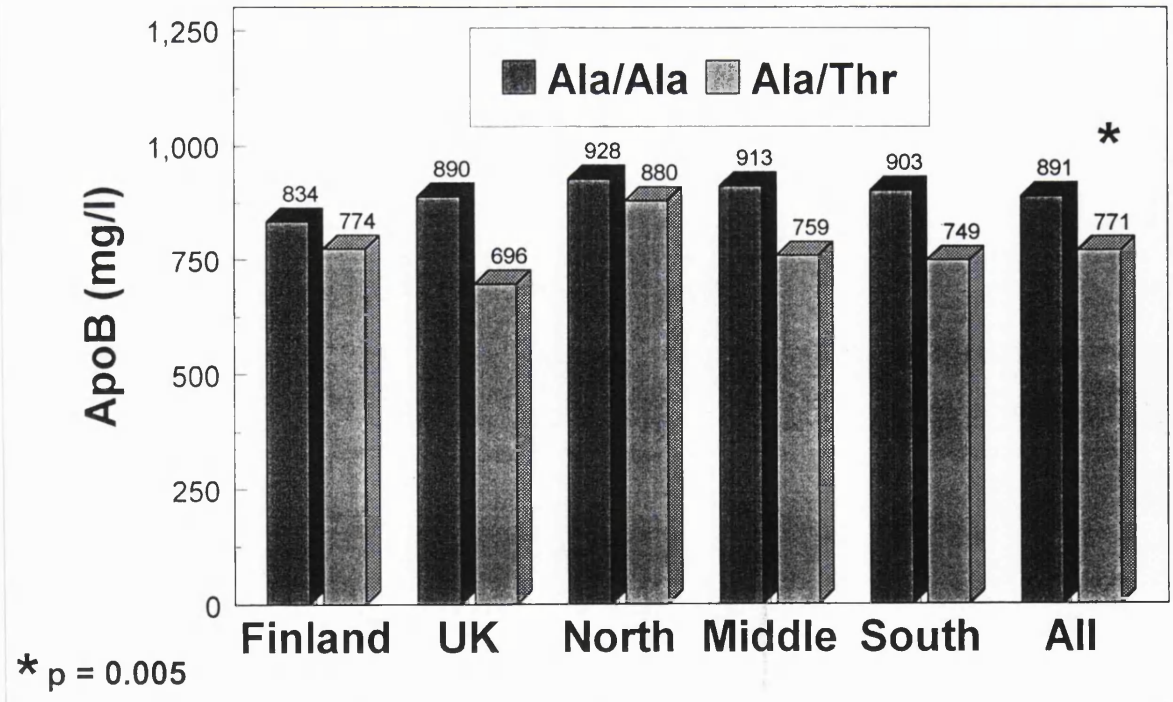

Figure 4.4.3. Mean levels of apoB in female offspring of cases from five regions of Europe, with different A370T LDL receptor genotype.

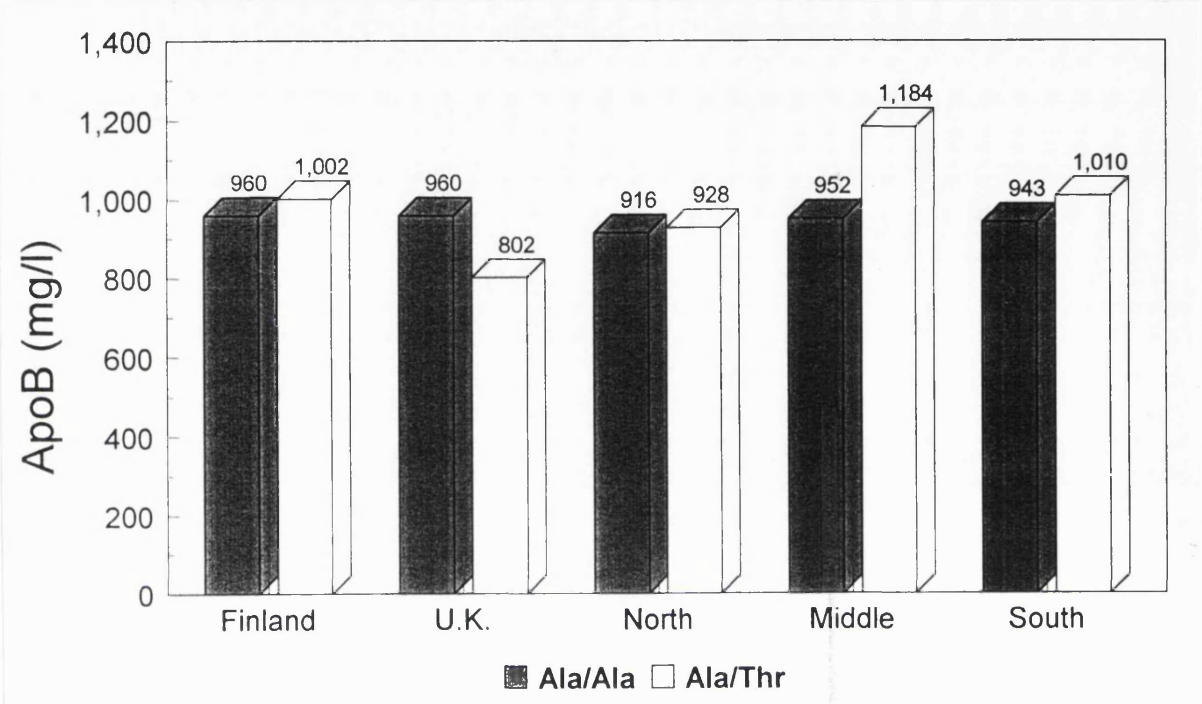

Figure 4.4.4. Mean levels of apoB in male offspring of cases from five regions of Europe, with different A370T LDL receptor genotype.

To explore possible explanations for this interaction with genotype, the relationship between plasma lipid traits was examined in females with different genotypes who reported the use of oral contraceptive. As expected, women who reported use of oral contraceptives had higher levels of lipid traits and in this group the lowering effect associated with the T370 genotype was not seen (Table 4.4.3.). Of the other measured factors, there was no evidence for interaction that were consistent between males and females, between genotype and BMI, blood pressure, alcohol consumption, smoking, physical activity, measures of plasma cholesterylesters or glucose in the determination of plasma lipid levels.

Table 4.4.3. Lipids according to LDL-A370T genotype and contraception (women) - adjusted for age, region, case/control status and BMI.

Lipid trait	Genotype	No contraceptive (n = 349 : 43)	On contraceptive (n = 269 : 26)	Interaction
Total-chol	A/A	4.50 (0.04)	4.67 (0.05)	
(mmol/l)	A/T	4.23 (0.12)*	4.67 (0.15)	NS
LDL-chol	A/A	2.61 (0.04)	2.59 (0.05)	
(mmol/l)	A/T	2.32 (0.11)*	2.64 (0.14)	NS (p=0.06)
LDL/HDL	A/A	1.76 (0.04)	1.69 (0.04)	
	A/T	1.56 (0.09)*	1.72 (0.12)	NS
ApoB	A/A	829 (12)	915 (13)	
(mg/l)	A/T	738 (31)*	920 (40)	NS (p=0.07)

(For 2 women, contraception was unspecified) * $p < 0.05$

4.4.3. Discussion

The EARS study compares a sample of young healthy men and women selected from different regions in Europe, with the aim of the study being to compare genetic and environmental factors in the offspring of fathers with and without early MI (cases and controls). The prevalence of the T370 allele was similar in all regions of Europe studied, showing no significant evidence for a North-South gradient, however, except for the middle region and Gt Britain, there was a consistently higher frequency in the cases, with the estimated relative risk of 1.5 associated with the T370 genotype. Because of the "offspring" design of the EARS study, it has a low power to detect such frequency differences (Tiret et al 1993); thus all "cases" have a father with premature CAD, but for each father who carries a high risk allele there is only a 50% chance of that allele being passed to his offspring. This suggests that the actual effect associated with the T370 allele on risk of MI

is likely to be larger, and this could be tested in a prospective study.

The mechanism of the effect on MI risk associated with the T370 allele is unclear. If the mutation is causing a "mild" form of FH it would be expected to be associated with higher levels of plasma lipid traits and thus act through promoting atherosclerosis. In the sample of middle-aged (mean age 32 years) healthy individuals from Iceland studied previously, the men with the T370 allele had significantly higher levels of plasma lipid traits, while the women had lower levels of these traits (see Section 4.1.). The observations made in the much younger healthy EARS group are qualitatively similar, but the higher lipid levels in the carrier males was smaller, which raises the possibility that age may have an effect on the expression of the LDL-receptor gene in those with this genotype. It has been suggested that the increase in plasma lipid levels seen in both men and women with increasing age may be a result of down-regulation of the LDL receptor expression in the liver (Miller 1984), and some evidence in both rats and humans in support of this has been reported (Nanjee and Miller 1988, Nanjee and Miller 1989). If men with the T370 allele are predisposed to develop higher levels of plasma lipids (and thus greater risk of atherosclerosis) as they become older, this would explain the higher frequency of the T370 allele seen here in the offspring whose fathers had CAD before 55 years.

To examine further the possible effect of the A370T change on plasma lipid levels and thus on CHD risk, a sample of men participating in the Northwick Park Heart Study II were genotyped.

4.5. The effect of the A370T polymorphism in the Northwick Park Heart Study (NPHS) II sample

4.5.1. Methods

Genotyping of the NPHSII sample (described in detail in Chapter 2 Section 2.8.2.3.) was done by PCR amplification and allele specific oligonucleotide melting as in section 4.4.

Statistical analysis was carried out by Dr George Miller at St Bartholomew's Hospital in London as described in Chapter 2 Section 2.7., using a strategy devised by myself.

4.5.2. Results

Genotype was determined in 707 individuals. There was no departure from Hardy-Weinberg equilibrium in this sample. The genotypes obtained were 638 AA, 68 AT and 1 TT, with allele frequencies of the T370 allele of 0.053 (95% CI=0.036-0.070). There was no difference in allele frequency between the two practices.

Figure 4.5.1. shows the changes in mean total plasma cholesterol concentration at baseline and over the four years period in the groups of men with different genotype. The mean total plasma cholesterol concentration rises 0.86 mmol/l for those with the A370A genotype (5.79-6.66) over the four years period or about 0.2 mmol/l/year which is similar as for those with the A370T genotype 0.82 mmol/l (5.84 -6.65). Those individuals with the A370T genotype have the highest average total plasma cholesterol concentration at all

points, though it is not statistically significant (p = 0.35). However, when only data was analyzed from the individuals for whom complete 4 years results were available (n = 503), those with a T370 allele had consistently lower mean total plasma cholesterol concentration at the baseline and years 1-3, although they have risen to the same values as those with the A370 allele by the fourth visit. Figure 4.5.2. shows the results for those individuals with complete data. Mean total plasma cholesterol concentration in those with the A370T genotype show a rise of 1.03 mmol/l over the period compared to 0.86 mmol/l in those with the A370 genotype only. The rise in mean total plasma cholesterol concentration with age in both analysis is highly significant ($p < 0.0001$). When the relationship between age and total plasma cholesterol concentration is analyzed by regression at baseline, $r = -0.015$ ($p = 0.70$) for those homozygous for the A370 allele, and $r = -0.04$ ($p = 0.74$) for carriers of the T370 allele.

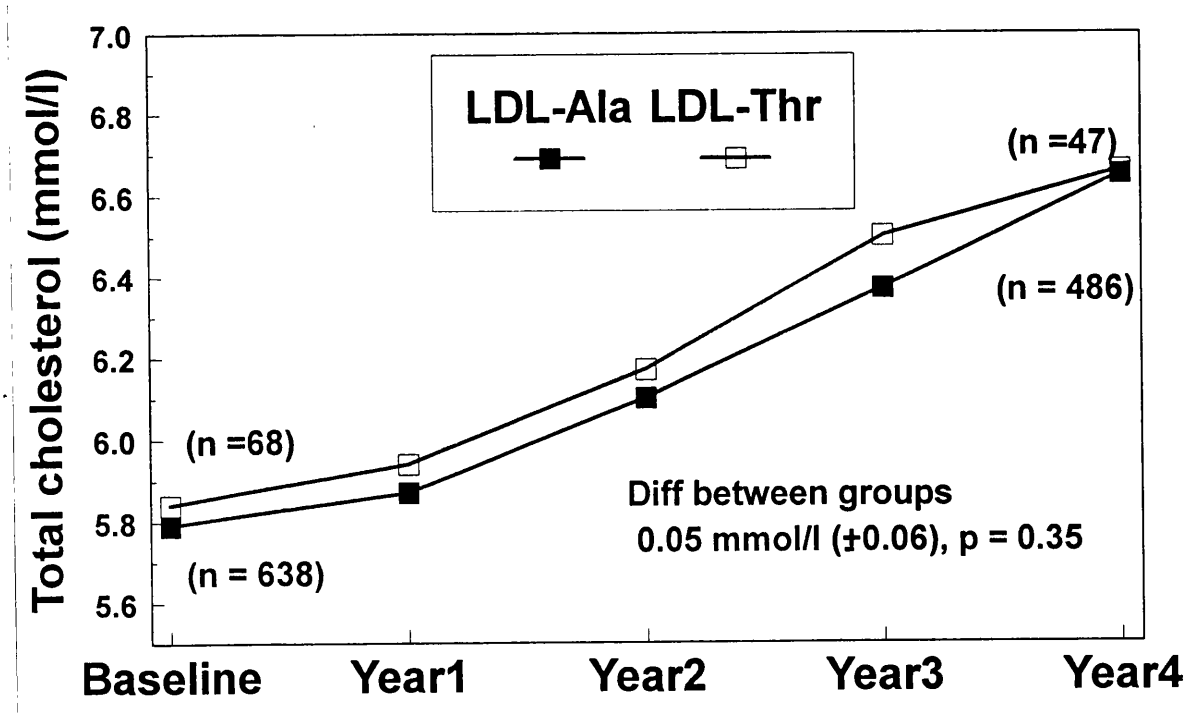

Figure 4.5.1. Changes in mean total plasma cholesterol concentration in men with different A370T genotype. Measurements at baseline and over the four years period in all are shown on this graph.

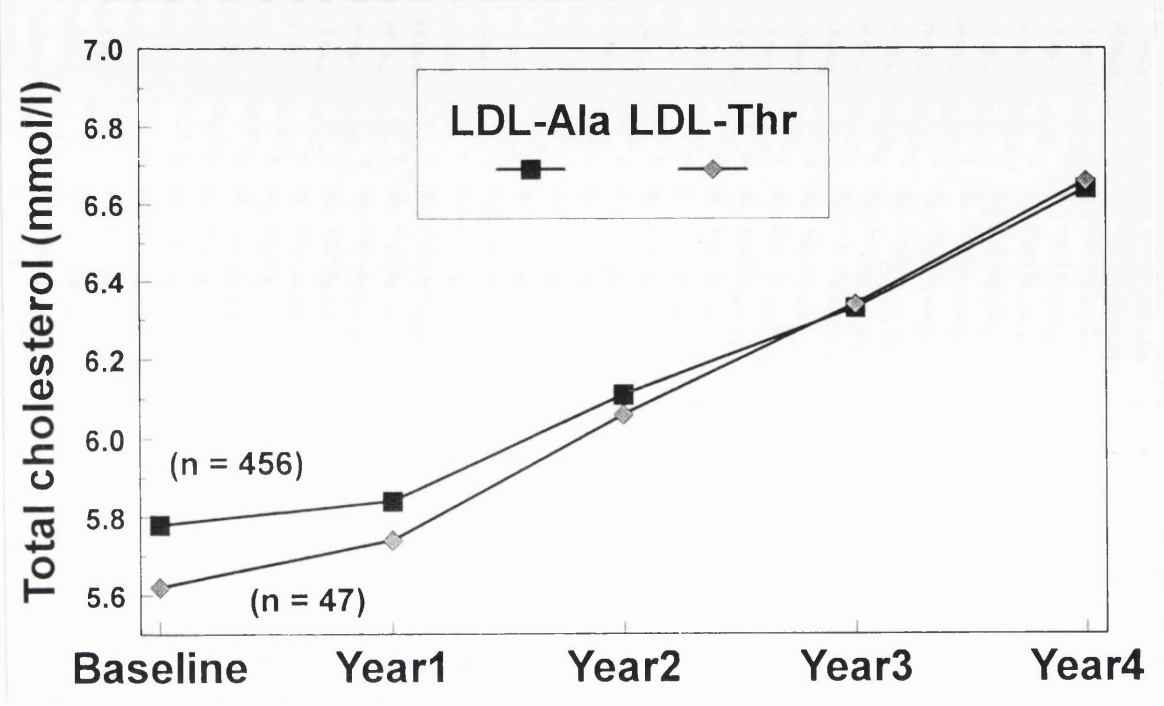

Figure 4.5.2. Changes in mean total plasma cholesterol concentration in men with different A370T genotype. Measurements at baseline and over the four years period in those individuals with complete data are shown on this graph.

Table 4.5.1. shows some of the baseline data in those individuals that have complete data (n = 503) compared to those that have been lost to follow up (n = 204) with respect to genotype. Those individuals that are lost to follow up and have the genotype A370T (n = 21) have significantly higher total plasma cholesterol concentration than any other group of individuals (6.28 mmol/l) (p = 0.01). By contrast there is no difference in total plasma cholesterol concentration between those lost to follow up and those seen at all visits having the A370 genotype only, 5.80 mmol/l and 5.79 mmol/l respectively. Of those individuals with the A370T genotype lost to follow up there were twice as many smokers, 10/22 (45%) compared to 10/47 (21%) in the group with complete data (p = 0.08), but the frequency of smoking was not different in the different genotype groups.

Table 4.5.1. Total plasma cholesterol concentration in all individuals according to follow up status and genotype.

Genotype	number		TC mmol/l (±SD)	
	C†	IC‡	C	IC
AA	456	183	5.79 (1.07)	5.80 (1.02)
AT	47	21	5.63 (0.99)	6.28*(0.76)

†number of individuals with complete data (C), ‡number of individuals with incomplete data (IC), *p = 0.01

4.5.3. Discussion

The frequency of the T370 allele was 0.053 in this sample of UK men which is similar to what has been observed elsewhere (Kotze et al 1989b, Taylor et al 1988, Leitersdorf et al 1989a, Gudnason et al 1995a in Section 4.1. and Section 4.3.).

The men in this study have been seen at baseline and at annual follow for four years, a total of five visits. In these men, the mean total plasma cholesterol concentration rises with time, as expected, and this occurred to the same extent in both genotype groups when examined for all the visits. However, when looked at cross sectionally in the groups, the mean total plasma cholesterol concentration drops slightly with age. The reason for this may lie in the age composition of the group (from 50 to 61 years old), and when other British studies are compared, this age range shows almost no increase in total plasma cholesterol (Tunstall Pedoe et al 1989).

The most significant finding of this study is that at baseline, those with the T370 allele who

are lost to follow up by the 4th year of the study have 7.7% higher mean total plasma cholesterol than those with only the A370 allele (either those A370 with complete data or those lost to follow up). In the whole group the consequence of this is that although at baseline those with the T370 allele have 7.7% higher mean total plasma cholesterol than those with the A370 allele only, those carriers of the T370 allele with data available for all the 4 years, have 2.8% lower mean total plasma cholesterol concentration compared with those with the A370 allele only. Therefore a subset of A370T heterozygotes have significantly higher average total plasma cholesterol concentration, and this subset of men seems to be lost to follow up. Since high plasma cholesterol is a risk factor for MI it is possible that the reason for this loss could be because of the development of IHD, but there are no data currently available to examine this. This observation is however compatible with that of an increased frequency of carriers for the T370 allele in the offspring of MI sufferers before the age of 55 years in the EARS group (see Section 4.3.).

CHAPTER 5 - FINAL DISCUSSION AND FUTURE DIRECTIONS

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5.1. Final discussion and future directions.

In this section the results from the studies performed on exon 4 in the FH patients and on the A370T polymorphism in the general population and the direction of future experiments will be discussed. Finally, concluding remarks will be made in the light of the results obtained in the studies described in this thesis.

5.1.1. Exon 4

The three main observations made in the studies on the FH patients, described in this thesis are: firstly the high number of mutations found in the 3' part of exon 4, secondly the high plasma cholesterol level associated with mutations in this part of the gene and thirdly the association of plasma cholesterol concentration with the different classes of mutation.

The high number of individuals with, and the frequent occurrence of mutations in the 3' part of exon 4 of the LDL receptor gene, both in the samples investigated in the studies described here and in other reports (Hobbs et al 1992), is an intriguing phenomenon. There are a number of possible reasons for this.

1) DNA structure-specific reasons.

2) Protein structure- or function-specific reasons.

3) Clinical bias.

4) Sample bias.

All these factors could contribute to the high number of mutations in this part of the LDL receptor gene, either on their own, but more likely in combination, and will be discussed in turn.

1) DNA structure-specific reasons.

The first possible effect of DNA structure on the occurrence of mutations is the presence of CpG dinucleotides. Of the described single base pair substitutions causing human disease the CpG dinucleotide is involved most frequently, accounting for about 33% of reported mutations (Cooper and Krawczak 1990), and there is evidence that many of them have occurred independently at the same site in different individuals (Cooper and Krawczak 1990). CpG dinucleotides are known hotspots for mutations through a mechanism involving deamination of methylated cytosine to thymidine (Lindahl and Nyberg 1974, Bird 1980, Duncan and Miller 1980). Transversions are also known to occur at CpG dinucleotides in higher frequencies than expected (Cooper and Krawczak 1990).

Of the mutations in the LDL receptor gene, identified in the Dallas collection of fibroblasts from homozygous FH patients, 16% have a mutation involving a transition at a CpG dinucleotide (Hobbs et al 1992). There are however a number of transversions reported at CpG sites in the LDL receptor gene.

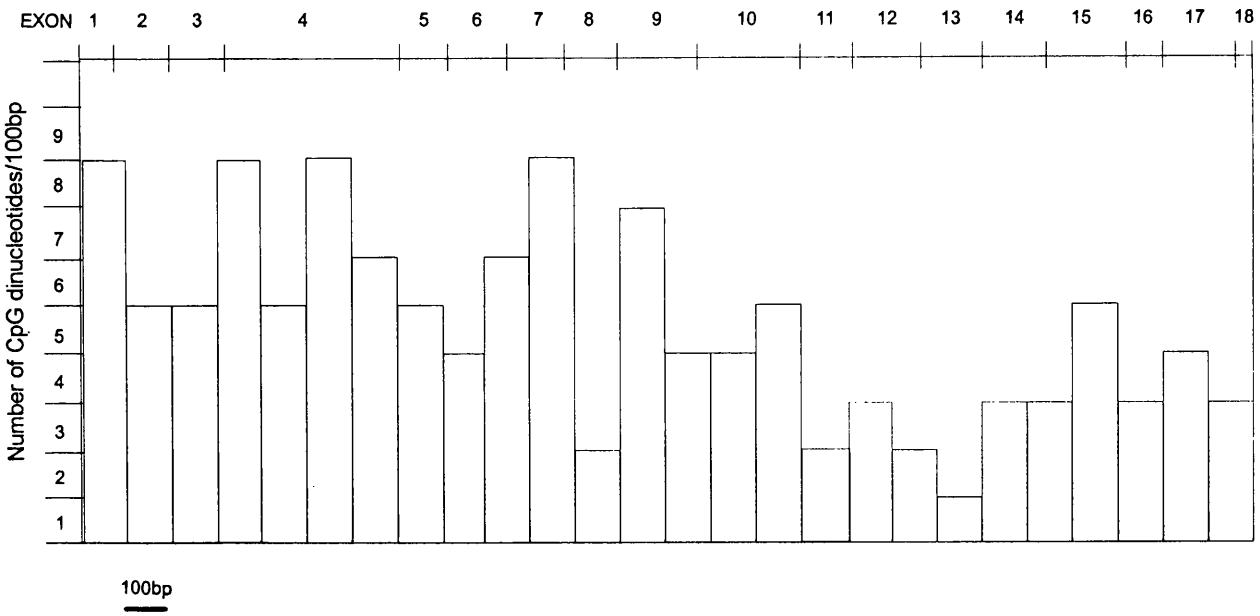

Figure 5.1.1. The distribution of CpG dinucleotides in the LDL receptor gene. The data are presented as number of CpG dinucleotides per 100 bases, each bos spanning 100 bp as indicated by the size bar. The exon-exon junctions in the mRNA are indicated my vertical lines at the top of the Figure.

Figure 5.1.1. shows the distribution of CpG dinucleotides in the LDL receptor gene. It is clear from the figure that there are more CpG dinucleotides in the 5' part of the gene. This is common for many genes and is thought to represent non-methylated CpG islands, frequently seen in the 5' end of genes (Bird 1987). The high C + G content of these CpG islands are thought to be mainly attributable to the absence of the CpG deficiency which is seen in methylated regions of the genome (Tykocinski and Max 1984, Bird et al 1985). However, there is no evidence that the 5' part of the LDL receptor gene is un-methylated, and there are a number of mutations that have occurred by a transition at a CpG in the first part of the LDL receptor gene, such as E80K, E119K, S156L E187K, E207K (Hobbs et al

1992). Furthermore, the CpG dinucleotide involving codons 206 and 207 has been involved in mutations in several different patients. The C in codon 206 is mutated by a transversion in the D206E South Afrikaner-1 mutation (Kotze et al 1989a. Leitersdorf et al 1989b), while the first G in codon 207 has mutated to all possible combinations in different patients. The transition causing E207K has occurred at least twice independently, in a French-Canadian and in an Mexican American (Leitersdorf et al 1990, Hobbs et al 1990). The transversion G to C, causing E207Q has occurred in an African American (Hobbs et al 1992) and the transversion G to T, causing E207X has been found in an Moroccan American (Hobbs et al 1992). This mutation is also frequently found in our sample of English individuals (1.6%). Even though detailed information is not available on the haplotypes of the mutations, it is not unlikely, in the light of the different ethnicity, that this mutation has occurred independently more than once.

Recurrent mutations in the LDL receptor gene have also been reported in other parts of the gene. It has for example been shown that a mutation at a CpG dinucleotide in the codon for P664L in exon 14 of the LDL receptor gene (Soutar et al 1989) has occurred independently at least twice and probably three times according to haplotype analysis (King-Underwood et al 1991). This mutation has also been found in Dutch FH patients (Defesche et al 1992), in French Canadians (Defesche et al 1992) and in Chinese FH patients in Hong Kong (Wong K. K. personal communication). The haplotype on the Dutch and the French Canadians is compatible with that on the Indian/Norwegian described by King-Underwood et al (1991). The Chinese had not determined the associated haplotype. Although recombination at meiosis between chromosomes could, over long periods of time, result in such a mutation being spread onto several haplotypes, analysis using multiple variable sites

flanking the mutation, suggest this explanation is unlikely.

In addition to the point mutations in the 3' part of the LDL receptor gene a number of small deletions and insertions have been described in this part of the gene (Gudnason et al 1993a, Meiner et al 1991, Hobbs et al 1992, Sun et al 1994, Peeters et al 1995, Kotze et al 1995, Theart et al 1995). Multiple mechanisms have been suggested to be responsible for deletions and insertions of less than 20 base pairs (Krawczak and Cooper 1991, Cooper and Krawczak 1991). In 93% of the genes with such deletions the DNA sequence involved contained direct repeats of between 2 bp and 8 bp, that either included or partially overlapped the deleted bases. In the case of the 3 bp deletion in the LDL-receptor gene, there is a repeat sequence of TGG-TGG with the internal GGT being deleted (see figure 5.1.2.). The 2bp deletion has a dinucleotide repeat GA-C-GA overlapping the CG deletion. In either case, the exact mechanism of the deletion itself is not obvious. The 2bp deletion (Fs206) is contained within a hexanucleotide with homology to a reported hotspot for deletions (consensus TGAGGA) (Krawczak and Cooper 1991). This sequence has similarity to DNA polymerase-alpha "arrest" sequences (Weaver and DePamphilis 1982) suggesting that arrest of synthesis may be involved in the mechanism of the deletion. The 2bp deletion involves a CpG dinucleotide, which is also the site of the mutations D206E and E207K.

In the case of small insertions, very similar mechanisms with slight modifications have been proposed (Cooper and Krawczak 1991). In 17 out of 20 insertions analyzed in the report by Cooper and Krawczak (1991) a sequence homolog to the hotspot sequence for deletions reported by Krawczak and Cooper (1991) was found in the vicinity of the insertion, further indicating a sequence specific mechanism for these insertions as well as for the deletions.

Furthermore, there is a homology with the TGGCGAA motif postulated to potentiate non-random retroviral integration (Wilson and Cohen 1988). The 3' part of exon 4 can thus been said to contain sequence that promotes both small insertions and small deletions.

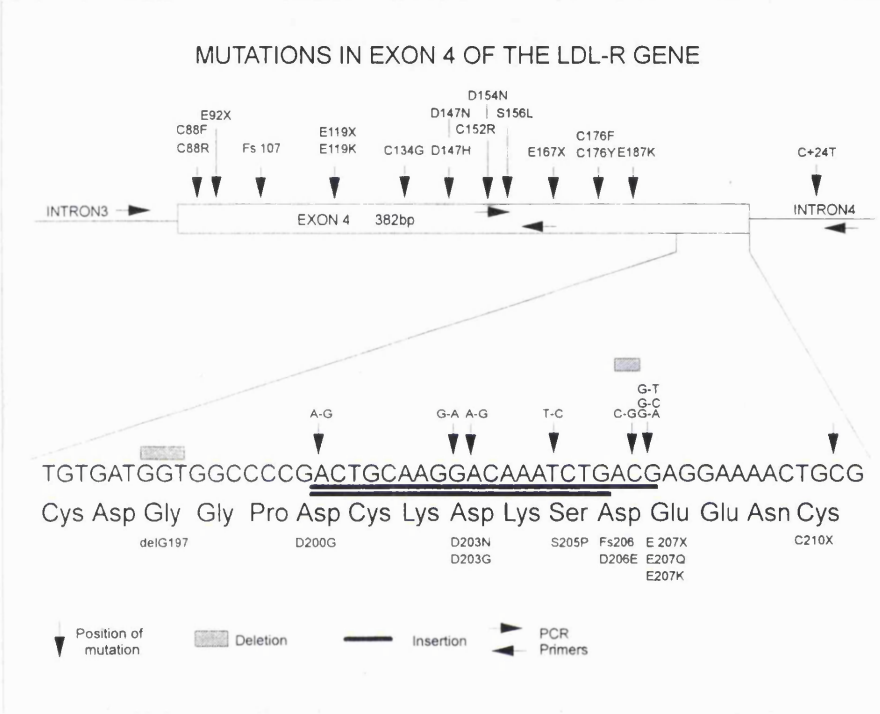

Figure 5.1.2. A diagram of the of exon 4 with flanking introns. Detail of 50 bases of the 3' end is shown with the positions of the described mutations.

The mutations identified in the 3' part of the LDL receptor gene are concentrated in a region of less than 50 bases (Figure 5.1.2.), containing close to 20 published mutations; single base substitutions and small insertions and deletions. (Hobbs et al 1992, Gudnason et al 1993a, Gudnason et al 1993c, Meiner et al 1991, Sun et al 1994, Kotze et al 1995, Peeters et al 1995, Theart et al 1995) which may further support a DNA-sequence-specific underlying mechanism.

2) Protein structure or function specific reasons.

The second reason for the high number of mutations encountered in this part of the LDL receptor gene might be due to functional reasons. It has been shown that there is a strong homology between the cysteine rich repeats of the ligand binding region in the 3' part of each repeat, and that part of the repeats have been suggested to be crucial to the binding of basic residues of the ligands to the receptor (Goldstein et al 1985). The sequence CDXXXDCXDXSDE harbours most of the mutations in repeat 5, and missense mutations have been described in this region in all the other repeats (Hobbs et al 1992). Furthermore, when the distribution of mutations in the cysteine rich repeats of the ligand binding domain is examined, it is clearly seen that they tend to cluster in the 3' part, further supporting the functional importance of this part of these repeats (see Figure 3.2.17. in Chapter 3).

From mutational analysis (Esser et al 1988, Russell et al 1989) it has been suggested that there is a 2-fold symmetry in the binding site of the receptor. The existence of such a symmetry is supported, both by the fact that repeat 1 is not essential for binding of the ligand and that there is an 8 amino acid linker between repeats 4 and 5 (see Figure 5.1.3.). A linker of the identical length but of divergent sequence is also found in the rabbit and the hamster receptor protein (Yamamoto et al 1986, Hoffer et al 1993). Interestingly, in the VLDL receptor, which has extremely strong homology to the LDL receptor, the linker between the repeats equivalent to repeats 4 and 5 is one amino acid shorter. In addition it is lacking the first three amino acids of repeat 5 equivalent (Sakai et al 1994), but whether this is the reason for the VLDL receptor not binding LDL remains to be elucidated. It has been speculated that such a symmetrical structure in the LDL receptor, centering about

repeats 4 and 5, would suggest that repeats 2 and 3, and 6 and 7 might be functionally analogous. Mutational analysis suggest this is the case. Similarly it has been suggested that repeat 4 and 5 are functionally homologous (Soutar & Knight 1990). Repeat 5 is essential for the binding of β-VLDL as shown by both mutational analysis of introduced sequence changes (Esser et al 1988, Russell et al 1989) and from the analysis of naturally occurring mutations (Hobbs et al 1992). On the other hand no analysis of artificially introduced mutations have been carried out, but a naturally occurring mutation in the highly conserved part of repeat 4, the S156L mutation has full ability to bind β-VLDL.

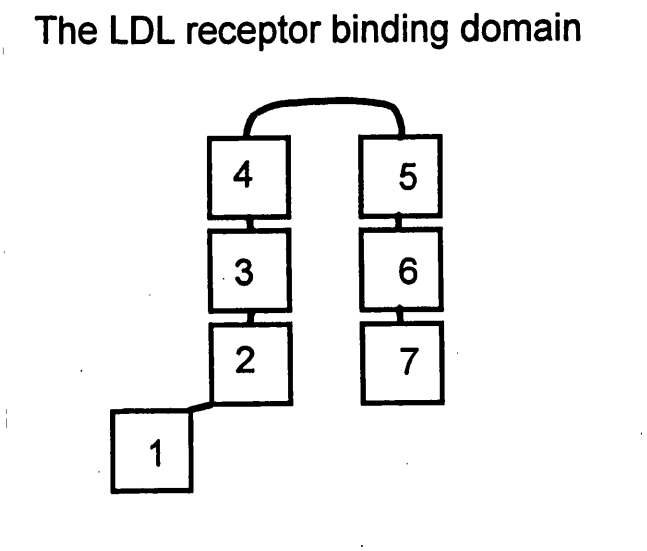

Figure 5.1.3. Schematic drawing of the ligand binding domain of the LDL receptor. The boxes denote the repeats of the ligand binding domain and are labelled accordingly. Emphasis is put on the symmetry around the linker between repeats 4 and 5 and is highly speculative.

Repeat 5 which is required for the binding of apoE-rich β-VLDL has three negatively charged amino acids, whereas the other repeats only have two. Goldstein et al (1985) proposed that multiple copies of apoE on one particle can arrange themselves to make contact with the repeat sequences of the LDL receptor. Dyer et al (1995), working with the fragments of apoE that are responsible for the binding, propose that the dimer apoE peptides

bind the LDL receptor by making contact with more than one of the negatively charged repeat sequences. They propose that the dimer apoE bind the LDL receptor because they display at least two appropriately spaced clusters of positively charged residues on an amphipathic α-helix that mimics the binding of more than one apoE molecule per LDL receptor (Dyer et al 1995). The organisation of the structure of the repeats of the binding domain of the LDL receptor will not be solved until crystallographic data are available. Whatever the reason for differences in the ability of the LDL receptor to bind apoE containing ligands, it is clear that repeat 5 is responsible for binding of apoE to the LDL receptor.

3) Clinical bias.

The third possible reason for the high number of mutations observed in repeat 5 of the ligand binding domain is a clinical bias, because individuals with mutations in this part of the gene may be more severely affected than individuals with other mutations and are thus more frequently found in lipid clinics, as suggested by Hobbs et al (1992).

It is of relevance in this regard that repeat five has been shown to be essential for apoE binding to the LDL receptor as well as apoB binding (Esser et al 1988, Russell et al 1989), and thus mutations in repeat five will affect binding of both remnants and LDL. In support of this idea, the data from this study show that individuals with mutations in repeat five have significantly higher total and LDL cholesterol than the rest of the FH patients. Furthermore, this is supported by the observations that individuals with mutations causing a null phenotype, with no residual LDL receptor activity, have significantly higher plasma

total and LDL cholesterol concentration than FH individuals with either an identified mutation causing a receptor defective phenotype or with no identified mutation. However, these two groups of mutations (those in repeat 5 and the null alleles) only account for about 20% of the FH samples, so this conclusion may be premature. The possible impact of differences in the plasma cholesterol concentration on atherosclerosis and coronary disease is discussed in chapter 3 section 3.3.2.

As pointed out in that section there is clear evidence in homozygous FH, that those with lower levels of receptor activity have higher LDL cholesterol (Sprecher et al 1985) and a more rapid progress of atherosclerosis (Goldstein and Brown 1989). However, although there is some evidence of a relationship between serum cholesterol levels and age of onset of CAD in heterozygous FH individuals, a clear correlation has not been demonstrated between the level of cholesterol and CAD (Jensen et al 1967, Beaumont et al 1976, Gagné et al 1979, Hill et al 1991). It has also been shown that in patients with FH that there is a significant positive correlation between intima-media thickening in the femoral artery and total serum cholesterol levels (Wendelhag et al 1993). In addition, in a recent study of the Afrikaner population FH patients with CAD had 8% higher cholesterol than those without CAD (Kotze et al 1993).

4) Sample bias.

The fourth possible reason for the high number of mutations in the 3' part of repeat 5 of the LDL receptor could be due to the fact that this part has been the subject of extensive molecular analysis and may have biased the frequency of mutations identified. However,

the fact that many fewer mutations were identified in the rest of exon 4 of the LDL receptor gene, coding for repeat 4 and repeat 3 of the ligand binding domain, does not support this contention, since a similar amount of analysis was carried out there. In addition, the high number of mutations in the 3' part of exon 4 in the Dallas collection of fibroblasts from homozygous FH patients, suggests that this may be a genuine observation. This will not be possible to answer finally until all the underlying mutations for FH in the sample have been identified.

From this discussion future work needs to be aimed at;

1) Increasing the sample size, or looking at other samples to confirm or reject what has been observed in this study

2) Identification of the rest of the mutations, responsible for FH in this sample.

3) Further analysis of the DNA sequence in the 3' part of exon 4 as potential mutational hotspot.

4) Further evaluating the relationship between mutation type or the functional class into which a mutation falls, by more accurate clinical evaluation of atherosclerosis using techniques such as ultrasound.

5.1.2. A370T

The studies described here on the alanine to threonine change in codon 370 of the LDL receptor suggest an association of this amino acid change with raised plasma concentration of total cholesterol, LDL cholesterol and apoB concentration in the Icelandic male population. There is a trend in the same direction in both the male offspring of men that had had a MI at an early age from across Europe (4.50 mmol/l vs 4.75 mmol/l) as well as in the sample of healthy men from the UK, though the differences are not statistically significant.

Population studies have shown that a 1% rise in cholesterol levels above 5.2 mmol/l (0.052 mmol/l), is associated with 2% increase in risk of CAD (Grundy 1986), and thus it can be inferred that in the Icelandic study, men with the T370 allele would have approximately 20% greater risk of developing CAD compared with individuals homozygous for the A370 allele. This higher risk associated with the T370 allele is supported by its higher frequency in the EARS offspring of men that had had a MI at an early age, corresponding to a risk of 1.49 of having a father with an MI under the age of 55 in that sample. In addition, in the sample of healthy men participating in the NPHS II over a period of 4 years, those men that were lost to follow up and were carriers for the T370 allele had significantly higher total plasma cholesterol concentration than those lost to follow up who were homozygous for the A370 allele (6.28 mmol/l vs 5.80 mmol/l, $p = 0.01$). The reason why these individuals were lost to follow up is not known and in particular there is no information available on whether they had MI, although there was no difference in the frequency of the T370 allele in those lost to follow up. To answer the question as to whether the T370 allele

contributes to an increased risk for developing atherosclerosis and CHD would require a prospective study.

In the EARS study the female offspring of MI sufferers also presented an increased frequency of the T370 allele, although they did not show higher plasma cholesterol concentration. On the contrary they had a significantly **lower** total plasma cholesterol, LDL cholesterol and apoB concentration than was seen in the controls. The same lowering effect associated with the T allele was seen in the sample of Icelandic women, although here it did not reach statistical significance. Interestingly, the lower plasma lipid levels in the female offspring of cases was not seen in those taking the steroid contraceptive pill, but unfortunately there were no information available on the use of the contraceptive pill in the Icelandic women sample. This observation may have some bearing on the mechanism of the different levels of plasma lipids observed in men and women, where the role of hormones is clearly involved. There is some evidence, in mice, for differences between male and female in the regulation of the LDL receptor gene mRNA in response to oestrogen (Tang et al 1991). In those studies the male mice responded to a dose of oestrogen by increasing the mRNA for the LDL receptor in liver cells but without an increase in the LDL receptor activity (Tang et al 1991), whereas no increase in mRNA or activity was seen in the female mice. In both rats and rabbits, oestradiol increases the LDL receptor mRNA and the LDL receptor activity (Kovanen et al 1979, Ma et al 1986), but this was not examined separately in males and female animals.

Pharmacological doses of oestrogen have been reported to increase hepatic catabolism of LDL by the LDL receptor pathway (Eriksson et al 1989, Kushwaha et al 1990, Ma et al

1986). Regulation of the LDL receptor by physiological concentration of the hormone is though not worked out yet.

The cell studies described in this thesis showed no difference on LDL receptor function of the A370 and the T370 alleles. These studies are not capable of demonstrating a small effect of defects in the recycling or in the stability of the LDL receptor. Cell experiments, that would examine recycling and receptor stability would have to be carried out before any conclusion can be drawn.

To measure the recycling of the receptor, experiments such as described by Davis et al (1987a) would have to be used. There, cells were incubated with 125 β-VLDL at 37°C for different periods of time, chilled at 4°C and washed, and both the internalized and degraded LDL measured. In normal cells these would rise linearly with time, but would reach a peak after a certain time in the mutated cells, and then fall gradually (depending on the severity of the recycling defect, and degradation of the abnormal receptor). The loss of receptors could also be examined by the incubation of unlabelled LDL at 37°C for different periods of time and then washing of the cells and incubation of labelled LDL at 4°C to measure the number of receptors. Furthermore, by pulse-labelling the receptors with ^{35}S-methionine and then incubation with LDL for different length of time, the disappearance or degradation of the receptor could be measured. It is thus evident that the experiments performed here are not the most likely ones to answer the question about a possible subtle functional defect resulting from the A370T change, and further experiments will be needed.

The regulation of the LDL receptor by sterols is mostly explained by the action of the sterol

regulatory element binding protein (SREBP) (Wang et al 1994). There is some evidence for this pathway being involved in the regulation of LDL receptor activity by steroid hormone, such as oestrogen, which may deplete the cells of cholesterol (Angelin et al 1992, Colvin et al 1993) and thus activate the release of SREBP (Wang et al 1994) to upregulate the LDL receptor. However, there is some evidence for a non SRE sterol regulation of the LDL receptor (Sharkey et al 1990).

It is thus clear that the control of LDL receptor activity regulation has not been fully worked out, and that important aspect, which may explain a large proportion of the normal variation of LDL receptor activity in the general population, still has to be elucidated. This may also be of importance in the FH patients, where the role of the apparently normal LDL receptor has been implemented in the determination of plasma cholesterol concentration on the top of the effect of the mutated LDL receptor gene (Vuorio et al 1995). In this study, individuals who lacked the cutting site for *Pvu*II polymorphism in intron 15 of the LDL receptor gene (*Pvu*II -) had somewhat higher plasma cholesterol concentration than those FH patients with the presence of the cutting site (*Pvu*II +). Confirmation of this effect in a second sample would be useful for estimating the role of the apparently normal LDL receptor in FH patients.

In the general population, the A370T change could be reflecting the *Pvu*II effect described by various researchers (Pedersen & Berg 1988, Schuster et al 1990b, Humphries et al 1991). In these studies the cutting allele of the *Pvu*II (*Pvu*II+) was associated with lower plasma cholesterol concentration. In the study of LDL receptor haplotypes described by Leitersdorf et al (1989a) the T370 allele (*Stu*I-), is in all cases on a *Pvu*II+ allele, and in

other studies in most cases (Berkman et al 1992, Kotze et al 1989c, Miserez et al 1993, Rodningen et al 1993, Schuster et al 1990b). It is thus possible that the T370 allele is on the same chromosome as a functional change causing the cholesterol lowering effect of *Pvu*II+, and T370 could be that functional change itself. None of the aforementioned studies analyzed the effect of the *Pvu*II change in men and women separately. An obvious step to resolve this is to repeat the analysis in the Icelandic group with the *Pvu*II restriction endonuclease. Such a study has been hampered by the lack of a PCR based technique to detect this polymorphism, which as present has to be done by Southern blotting.

Any variation in the apparent normal LDL receptor gene that influences the plasma cholesterol concentration has potential epidemiological and clinical importance. Approximately 10% of the general population are carriers of the T370 allele, (which is similar to the frequency of the apoE2 allele), and even if it were associated with a small effect on lipid levels it would be of epidemiological and clinical importance. It is thus of great relevance to elucidate these effects and there are four areas of immediate further study:

1) Repeat the study on another group of offspring of young MI patients.

2) Examine the impact of the A370T polymorphism in a prospective study.

3) Do further cell studies with respect to the recycling of the LDL receptor.

4) Examine the effect of the *Pvu*II polymorphism in intron 15 in the same samples as described in this study.

5.1.3. Concluding remarks

The LDL receptor and variations in its gene are clearly contributing both to the plasma level as well as to the variation in plasma cholesterol concentration in the population. It has a large effect on plasma level of cholesterol in patients with FH, in addition to having an effect on the variation of plasma cholesterol concentration in FH according to the type of mutation in the receptor as shown in this thesis. Furthermore, there is a clear evidence for the effect of the LDL receptor gene on the variation in plasma cholesterol in the general population, within the "normal" range of plasma cholesterol levels, both as shown in this thesis and shown in other studies (Pedersen & Berg 1988, Schuster et al 1990b, Humphries et al 1991). The importance of variation in the plasma cholesterol concentration in the general population is of significant clinical importance as a 1% increase in plasma cholesterol increases the risk for CAD for 2%, for cholesterol levels above 5.2 mmol/l (Grundy 1986). This means that an increase of 10% or 0.5 mmol/l in plasma cholesterol concentration would be reflected in 20% increased risk for CAD.

There is thus no doubt about the importance to work out the relevant genetic variation in the LDL receptor gene that may contribute to this risk. For identification of the genetic variation two approaches could be used:

1) Searching for mutations in the coding sequences of the LDL receptor gene in patients with FH, using such techniques as SSCP or DGGE. This would allow the detection of many if not most mutations with a large effect on plasma cholesterol. In addition a large number of polymorphism could be identified as has been reported (Leren et al 1993b).

Some of these polymorphisms might transpire to be of significance with respect to the effect on plasma cholesterol concentration. Some might even be due to amino acid changes like the A370T polymorphism in exon 8 which is described in the thesis. However, as a direct approach in the general population for the search of a genetic variation in the LDL receptor gene with an effect on plasma cholesterol concentration, it is not feasible.

2) Use the known polymorphisms in the LDL receptor gene to look for allelic association with plasma cholesterol concentration, so as to be able to identify a subset of individuals to examine further with respect to functional sequence variation. This could be done using a polymorphism such as the *Pvu*II or any of the other polymorphisms in the LDL receptor gene previously identified, or those that would be identified when screening the FH patients for mutations in the LDL receptor gene.

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PCR-Based Method to Create a Specific Mutation as a Reference for Oligomelting

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In screening selected populations for known mutations in genes relevant to risk for hyperlipidemia, atherosclerosis, and coronary artery disease, one gene we have examined is the low-density-lipoprotein receptor (LDLR) gene in patients with familial hypercholesterolemia (FH), which has a frequency of 1:500 in most populations (*1*). More than 30 mutations in this gene have been described, with gross alterations accounting for about 2–6% of defects in the populations investigated (*2*), leaving the majority to be point mutations. Some mutations create a change of a recognition site for a restriction enzyme, allowing direct detection; the remainder can be detected by the oligomelting technique with allele-specific oligonucleotides (ASOs) (*3*). One problem in using ASOs to screen for mutations is the unavailability of a DNA sample from a patient with the mutation to use as a positive control in the test. To overcome this problem, we have used a polymerase chain reaction (PCR)-based method to create mutations of interest, using primers containing the mutated DNA sequence [this method is frequently used to create new restriction sites in PCR fragments via 3′ mutated primers (*4*)].

As an example, we describe the creation of a mutation in exon 4 of the LDLR gene involving substitution of T for C in base position 543 of the gene, changing the amino acid Ser_{156} to Leu. This mutation has been described as the cause for FH in a family that is a Puerto Rican kindred living in the United States (*5*).

First fragment A, from the beginning of exon 4 into intron 4, was amplified by using primers 1 and 2 (Figure 1*a*). The PCR cycles were 95 °C for 5 min and 68 °C for 6 min once; a subsequent 30 cycles of 95 °C for 1 min and 68 °C for 6 min; and finally 15 min at 68 °C. (We have found it easier and more reliable to amplify from a PCR fragment than genomic DNA when using a primer with a central mismatch, possibly because of the decreased stability of such a primer.) Fragment A was then electrophoresed on 1% agarose gel, excised from the gel, and frozen at −20 °C. After being thawed, 1 μL was taken and diluted 100-fold in sterile distilled water, and 1 μL of that was used with primers 2 and 3 to generate fragment B, which contained the mutation (Figure 1*a*). The PCR cycles were one cycle of 95 °C for 5 min, 45 °C for 3 min, and 72 °C for 3 min; followed by 30 cycles of 95 °C for 1 min, 45 °C for 1 min, and 72 °C for 2 min; and finally 72 °C for 10 min. Fragment C

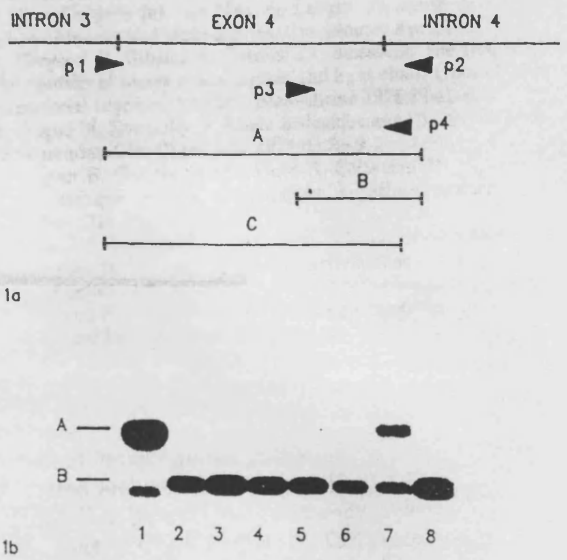

Fig. 1. (*a*): Relative positions of the PCR primers and a schematic of the fragments amplified as described in the text; (*b*): autoradiograph of a normal and mutated allele detected with radiolabeled ASO1 and ASO2

(*a*) *P1*: 5′CATCCATCCCTGCAGCCC3′ is a positive strand from the junction of intron 3 and exon 4. *P2*: 5′GGGACCCAGGGACAGGTGATAGGAC3′ is a negative strand from intron 4 (*7*). *P3*: the mutated allele from exon 4 (*5*) thus is ASO2 (see below). *P4*: 5′CCTTTTGACGCCATACCCGC3′ is from the exon 4–intron 4 junction. (*b*) Amplified samples (10 μL) were electrophoresed and double-blotted onto a Hybond-N filter (*5*). The filters were prehybridized and then hybridized separately with radiolabeled ASO1 [5′AAGATGGCTCGGATGAGTG3′ (the normal allele)] or ASO2 [5′AAGATGGCTTGGATGAGTG3′ (the mutated allele)] for 1 h. The filters were washed and autoradiographed at −70 °C for 3 h. *Lane B* is hybridized with the normal allele probe (ASO1), and *lane A* with the "mutant" probe (ASO2). Sample *1* is the mutated PCR product, showing a different electrophoretic mobility from sample *7*, which is from a patient with the Ser_{156}→Leu mutation in the LDLR gene. *2, 3, 4, 5, 6,* and *8*: samples from FH patients who do not have this mutation

containing the normal allele was amplified with use of primers 1 and 4 (Figure 1*a*), and PCR conditions were as for fragment A. Fragments B and C were then electrophoresed in 1.2% agarose, double-blotted onto Hybond-N filters as described (*6*), and hybridized with ASO1 and ASO2 (Figure 1*b*), which detect the normal and mutated alleles, respectively (ASO2 being primer 3). The results (Figure 1*b*) show that, under appropriate washing conditions, ASO2 hybridizes only to the mutant allele.

Using the modifications that we describe here for reported methods (*4*), we can quickly create positive controls for known mutations in a gene to use for oligomelting techniques. This ASO approach is very useful when screening many samples for different rare mutations in a particular part of a gene: once the amplified samples have been bound to a filter, they can be investigated repeatedly by using ASOs that detect different mutations.

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Identification of Recurrent and Novel Mutations in Exon 4 of the LDL Receptor Gene in Patients With Familial Hypercholesterolemia in the United Kingdom

V. Gudnason, L. King-Underwood, M. Seed, X.-M. Sun, A.K. Soutar, and S.E. Humphries

A group of 200 patients with familial hypercholesterolemia (FH) who were attending lipid clinics in the London area have been screened for four known point mutations and a microdeletion in exon 4 of the low density lipoprotein receptor gene by polymerase chain reaction (PCR) amplification of genomic DNA and either enzyme digestion of the product or hybridization with allele-specific oligonucleotides. A point mutation of Ser_{156}→Leu that was initially described in a Puerto Rican family was found in one patient of Polish origin on a different haplotype from that described originally and thus is likely to have occurred independently. A 3-bp deletion that causes deletion of amino acid Gly_{197} was found in six of the patients, who were all of Jewish origin and who shared the same haplotype for the mutant allele. A mutation of Asp_{206}→Glu that has been described in the Afrikaner population was found in three patients, two of UK origin and one a recent immigrant from South Africa. In all cases the haplotype of the mutant allele was compatible with that described in the original patient. The mutations at Asp_{154} reported in South African patients and at Glu_{207} reported in French Canadian patients were not detected in this sample. However, two additional mutations have been identified in this sample: the first, a 2-bp deletion in codon 206 that was found in five patients, all of British ancestry, and the second, a point mutation in a single patient of Irish origin that creates a stop codon at residue Cys_{210}. Of the 200 FH patients in the sample, the molecular defect in 16 (8%) could be detected by PCR using three different tests. Thus, based on the results from analysis of exon 4, the data suggest that during screening of FH patients who have been selected from a population of heterogeneous origin, only a limited spectrum of reported mutations will be found and the occurrence of the same mutation in different patients does not necessarily imply that they share a common ancestor, especially for those mutations occurring at 5′-CpG-3′ dinucleotides. (*Arteriosclerosis and Thrombosis* 1993;13:56–63)

KEY WORDS • LDL receptor gene • familial hypercholesterolemia • mutations

Familial hypercholesterolemia (FH) is a monogenic disorder[1] that is inherited as an autosomal dominant disease. In the classical clinical descriptions of the disorder more than 50 years ago,[2,3] it was shown to be associated with a twofold to threefold elevation of serum cholesterol together with tendinous xanthomas, arcus cornea, and premature atherosclerosis in many of the affected heterozygotes. The frequency of the disease in most communities is one in 500, making FH one of the most common monogenic diseases known.[4] In some individuals with the clinical diagnosis of FH, the disorder is caused by a mutation in the apolipoprotein B (apoB) gene,[5] but in the majority of patients, the disorder is caused by different mutations in the low density lipoprotein (LDL) receptor. Because of founder effects, the frequency of the disease and of particular mutations is much higher in some countries with populations that have been isolated by cultural or geographic boundaries. For example, in French Canadians the same deletion is found in 60% of FH patients,[6] and in the Christian Lebanese one mutation is responsible for the disorder in 98% of all FH patients,[7] while in Afrikaners in South Africa three mutations are responsible in more than 95% of FH patients,[8,9] with the most common mutation being found in two thirds of all patients.

A number of mutations in the LDL receptor gene in FH patients have now been described.[10] Of these mutations, more than 30 are deletions, and, where characterized in detail, many of these are thought to be caused by recombination between Alu sequences.[10] The observed frequency of deletions in studies of FH patients who have been screened has been between 2% and 6%.[10–13] In addition, more than 10 single-base-pair (bp) changes have been reported, but overall the known mutations account for a very small number of all

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patients, leaving the majority of defects responsible for FH uncharacterized. Thus, in a genetically heterogeneous population, the number of different mutations is likely to be high, even though each particular mutation may be common to several families. The frequency of any particular mutation will be largely dependent on its time of occurrence in a given population and on fluctuations in population size and migration patterns.

In recent years we have collected samples from more than 200 FH patients who were attending lipid clinics in London and the south of England in order to investigate the spectrum of mutations in the LDL receptor gene and to determine their approximate frequencies as the initial step in identifying and characterizing all patients with FH in this area of the United Kingdom. All of the patients have FH as defined by classical clinical and biochemical criteria. This paper describes the results of screening the 200 patients for five of the reported mutations in exon 4 of the LDL receptor gene. Four of the 1-bp substitutions that cause FH that have been described in this exon are a G→A that changes Asp_{154} to Asn,[14] a G→T that changes Ser_{156} to Leu,[15] a C→G that changes Asp_{206} to Glu,[8] and a G→A that changes Glu_{207} to Lys,[16] the three last occurring at a 5′-CpG-3′ (CpG) dinucleotide. In addition, a 3-bp deletion in exon 4 has been described that causes the deletion of amino acid Gly_{197}.[10,17]

Methods

Patients

Complete details of the patient selection have been described elsewhere.[13] In brief, the patient sample consisted of 189 heterozygous and 11 homozygous patients (all apparently unrelated) with the diagnosis of FH who were attending lipid clinics in the London area. Previously defined standard diagnostic criteria for FH were applied, including a total serum cholesterol level of more than 7.5 mmol/l and plasma level of LDL cholesterol above 4.9 mmol/l, with tendon xanthomas and/or premature coronary artery disease in the patient or a first-degree relative. Of the 200, 35% have tendon xanthomas, and in the families of 44 patients, the involvement of a mutation in the LDL receptor gene has been confirmed by cosegregation of restriction fragment length polymorphisms (RFLPs) of the gene with hypercholesterolemia. Patients with the mutation in the gene for apoB causing familial defective apoB were excluded.[5]

Amplification of Genomic DNA

Exon 4 of the LDL receptor gene was amplified by polymerase chain reaction (PCR)[18] with two sets of oligonucleotide primers: primer 1, 5′CATCCATCCCTGCAGCCC3′ from the intron 3–exon 4 boundary, and primer 2, 5′CGCCCATACCGCAGTTTTCC3′ from the exon 4–intron 4 boundary, together amplifying a 405-bp fragment designated as fragment I; and primer 3, from bp 528 to bp 547 in exon 4 (5′CGACTGCGAAGATGGCTCGGA3′) and primer 4 in intron 4 (5′GGGACCCAGGGACAGGTGATAGGAC3′), giving a 242-bp fragment designated as fragment II. Oligonucleotides were obtained from Severn Biotech Ltd., Kidderminster, UK. The amplifications were performed in an automated thermal cycler (Cambio, Cambridge, UK) with *Thermus aquaticus (Taq)* DNA polymerase (Perkin Elmer-Cetus, Norwalk, Conn.) in the buffer as recommended by the manufacturer and a total volume of 50 μl. The conditions were 95°C for 5 minutes and 68°C for 6 minutes once and subsequently at 95°C for 1 minute and 68°C for 6 minutes for 30 cycles.

Identification of Mutations

To detect the Asp_{154}→Asn mutation, PCR fragment I (20 μl) was digested with 10 units *Mbo* II (Anglian Biotec Ltd.) for 16 hours in a total volume of 30 μl. The digestion mixture was then analyzed by electrophoresis through a 1.2% agarose gel (Ultrapure Agarose, BRL). To detect the Asp_{206}→Glu mutation, PCR product II (20 μl) was digested with 12 units *Dde* I (Anglian Biotec Ltd.) for 16 hours in a volume of 30 μl in the supplied buffer. The digested fragments were then analyzed by electrophoresis on a 7.5% polyacrylamide gel. DNA bands were visualized by UV transillumination of ethidium bromide–stained gels.

Blotting and Hybridization With Allele-Specific Oligonucleotides (ASOs)

To detect the Ser_{156}→Leu and the Glu_{207}→Lys mutations, PCR fragment II was fractionated by electrophoresis for 3 hours on a 1% agarose gel, which was then denatured in 1.5 M NaCl/0.5 M NaOH for 30 minutes at room temperature and then "double blotted" (10 μl) between two pieces of nylon membrane (Hybond-N+, Amersham, UK), using the denaturing solution as the transfer buffer. The DNA was bound to the filters by UV light from a transilluminator for 1 minute. Three pairs of ASOs were used: for the codon 156 mutation, ASO 1 (5′AAGATGGCTTGGATGAGTG3′) and its normal allele ASO 2 (5′AAGATGGCTCGGATGAGTG3′)[15]; for the codon 207 mutation, ASO 3 (5′CAAATCTGACAAGGAAAACT3′→) and its normal allele ASO 4 (5′CAAATCTGACGAGGAAAACT3′[16]); and for the codon 197 deletion, ASO 5 (5′TGTGATGGCCCC3′→) and ASO 6 (5′GTGATGGTGGCC3′→), the respective normal allele. Positive controls for the mutations were synthesized as described previously by PCR amplification with oligonucleotide primers that introduced the specific mutations.[19] All ASOs were labeled at the 5′ end with T4 polynucleotide kinase (BRL, Paisley, UK) and adenosine 5′-[α-^{32}P]triphosphate (Amersham, UK) to a specific activity of approximately 0.1 μCi/pmol. The filters were hybridized for 1 hour in 5× saline–sodium phosphate–EDTA buffer ([SSPE]) 1× SSPE is 0.9% NaCl, 50 mM sodium phosphate, and 5 mM EDTA)/0.5% sodium dodecyl sulfate (SDS)/5× Denhardt's solution at 42°C for ASOs 1, 2, 3, and 4 and at 33°C for ASOs 5 and 6. The filters were then washed for 3 minutes at room temperature in 2× SSPE and 0.2% SDS for all ASOs and subsequently for 10 minutes in 0.2× SSPE and 0.1% SDS at 42°C for ASOs 1, 2, 3, and 4 and at 39°C for ASOs 5 and 6. An additional 10-minute wash in 0.1× SSPE and 0.1% SDS was done for ASOs 1, 2, 3, and 4. Exposure time for autoradiography was 2–16 hours.

Haplotype Analysis

Genotypes for six LDL receptor gene RFLPs were determined either by restriction digestion of genomic DNA followed by Southern blotting as described for

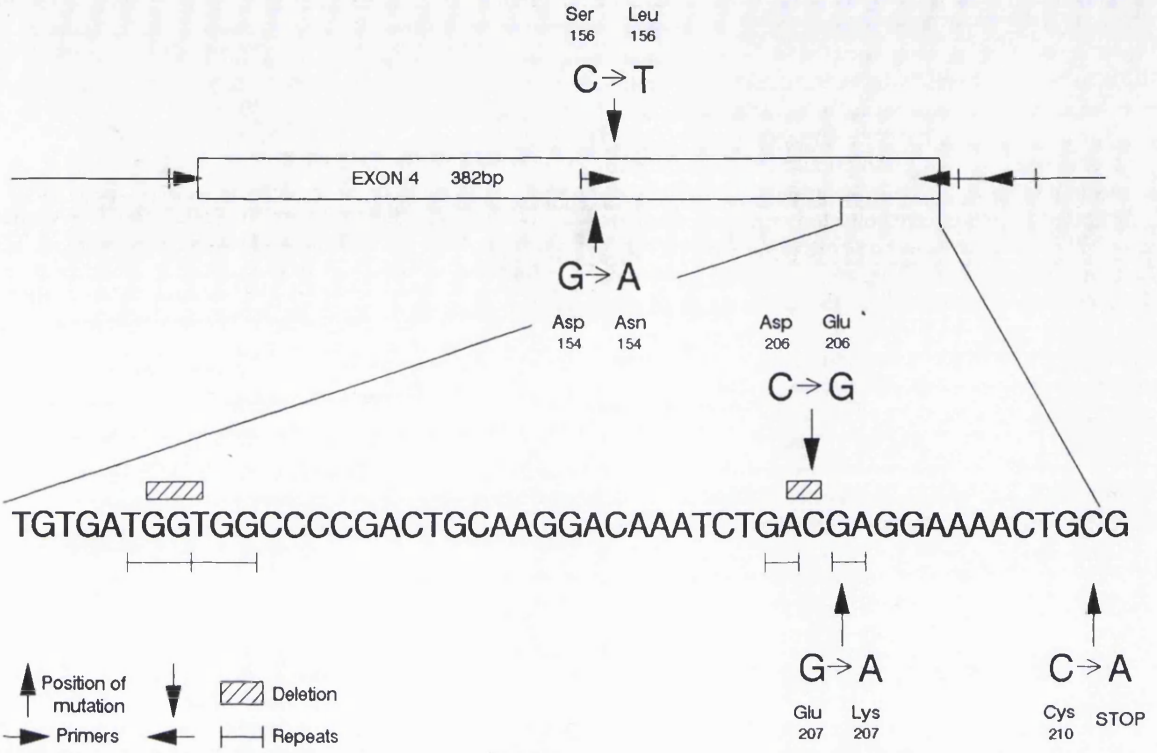

FIGURE 1. *Mutations in exon 4 of the low density lipoprotein receptor (LDL-R) gene. Diagram of the 3' end of exon 4 of the LDL-R gene and its flanking intron shows the positions of four previously described mutations[7] and the two new mutations described in this paper. Deleted bases are indicated by hatched boxes over the sequences, and single-base changes are indicated by vertical arrows (↑) together with the identity of the codon(s) concerned. Oligonucleotide primers used for amplification are shown by horizontal arrows (5'→3'). Occurrence of mutations in the sample of patients from the United Kingdom is summarized in Table 1.*

Pvu II[20] or by PCR amplification with oligonucleotides flanking each of the variable restriction sites for *Taq* I, *Stu* I,[21] *Hinc* II,[22] *Ava* II, and *Nco* I.[23] Alleles were designated as "+" or "−" indicating the presence or absence, respectively, of the cutting site.

Direct Sequencing

PCR products were purified by electroelution of the appropriate bands from 1% agarose[24] and ethanol precipitation. The fragments were sequenced directly as described.[23]

Results

A total of 211 LDL receptor–defective alleles (11 homozygous and 189 heterozygous patients) were screened for the known mutations in exon 4 of the LDL receptor gene shown in Figure 1, and the results are summarized in Table 1. All patients in whom a mutation was found were heterozygous for FH. In this sample, no patients with the Asp_{154}→Asn or the Glu_{207}→Lys mutation were found. In one patient the Ser_{156}→Leu mutation was detected (Figure 2), and the Asp_{206}→Glu mutation was found in three (Figure 3). The sequence of both of these mutations was confirmed by DNA sequencing of the amplified fragments (Figures 4a and 4b). In one patient a different abnormal restriction fragment pattern was observed when fragment II was digested with *Dde* I, during which the 134-bp fragment was cut into 80- and 54-bp fragments. Sequencing amplified fragment II from this patient revealed a single base substitution (C→A) that changed codon Cys_{210} to stop (Figure 4b). In addition, extra bands were observed in 11 of the patients after polyacrylamide gel electrophoresis of *Dde* I–digested fragment II. These bands migrated more slowly than the normal bands and behaved like heteroduplexes, which are characteristic of insertions or deletions of a few bases.[25] These extra bands were observed in two different patterns, one of which was found in six patients and the other in five patients (Figure 3). Hybridization of the PCR fragments with ASOs strongly suggested that one of these abnormal patterns, i.e., the one observed in six patients, was due to the presence of the 3-bp deletion of the codon for Gly_{197} in one allele (Figure 2). This was confirmed by direct sequencing of the PCR product (Figure 4c). Direct sequencing of the PCR product from patients with the other abnormal pattern revealed the presence of a deletion of the last two bases of codon 206 in one allele (Figure 4c). In two clinically homozygous patients known to have one allele in which exon 4 was deleted, PCR fragments comprising the other allele of exon 4 were sequenced.

The genotypes of patients with detected mutations were determined at six polymorphic sites within the LDL receptor gene. In instances where a patient or a relative was homozygous for the RFLP, the haplotype of the defective allele could be defined unambiguously. In instances where the patient was heterozygous for the

TABLE 1. Frequency of Mutations in Exon 4 of the LDL Receptor Gene

Mutation	No. in this study*	Ethnic origin of patients	Haplotype of the defective LDL receptor gene†						Previous description
			T	S	H	A	V	N	14
Asp_{154}→Asn	0								
Ser_{156}→Leu	1	Poland (1)	−	+	−	+	−	+	15
		Afrikaner (1)	−	+	+	−	−	−‡	8
Asp_{206}→Glu	3								
		British (2)							
Glu_{207}→Lys	0								16
3-bp deletion (Gly_{197})	6	Jewish (6)	−	+	−	+	−	−	17
Cys_{210}→stop	1	British (1)	−	+	+/−	X	+/−	+	None
2-bp deletion (bases 694 and 695)	5	British (5)	−	+	+	−	+/−	+	None

*Number found in the group of 211 low density lipoprotein receptor (LDL-R) defective alleles.

†Genotype as determined by haplotype; "+" or "−" indicates the presence or absence, respectively, of the cutting site. +/−, Unable to determine unambiguously; T, *Taq* I; S, *Stu* I; H, *Hinc* II; A, *Ava* II; V, *Pvu* II; N, *Nco* I; X, not determined.

‡Additional polymorphisms in the haplotype for this mutation are *Sph* I− and *Apa* LI-5′−.

RFLP, the haplotype was deduced by using information from available relatives and assuming that no recombination had occurred. An example of this is presented in Figure 5 for the Asp_{206}→Glu mutation. As shown in Table 1, the other two patients with this mutation have a haplotype compatible with that observed in the family, although it cannot be determined unambiguously. For the Gly_{197} deletion, all six patients have the same (or compatible) haplotype for the defective allele, which differs from the haplotype that is deduced as common for all 2-bp-deletion patients (Table 1).

The biochemical and clinical characteristics of the patients in whom mutations in exon 4 were detected are presented in Table 2. The untreated total plasma cholesterol and LDL cholesterol levels varied widely, even within groups of patients with the same mutation.

Discussion

The general population in London is very mixed with regard to racial and ethnic origin, and therefore, FH patients will also vary widely in their geographic background and exhibit the spectrum of mutations found elsewhere in the world. As part of a systematic approach to identify mutations that cause FH in the United Kingdom, this paper describes the results of screening for known mutations in exon 4 of the LDL receptor gene in 211 alleles in patients with a clinical diagnosis of FH who were attending lipid clinics in the London area. In this sample we have previously identified nine patients with gross deletions of the LDL receptor gene.[13] Of the five previously reported mutations in this exon, three were represented in the London sample. No patients were found with the Asp_{154}→Asn mutation

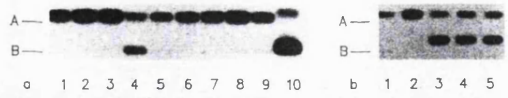

FIGURE 2. *Detection of mutations in exon 4 by hybridization with allele-specific oligonucleotides (ASOs). Amplified fragment I (see text) was fractionated by electrophoresis on an agarose gel and transferred to nylon membranes (blots) as described in "Methods."* Panel a: *Detection of Ser_{156}→Leu mutation. Blot A was hybridized to a normal-sequence ASO and blot B to the ASO specific for the mutated sequence. Lane 10 is a positive control whose size is different from that of the exon 4 polymerase chain reaction product.[19] DNA fragments in lanes 1, 2, 3, 5, 7, 8, and 9 are negative for the Ser_{156}→Leu mutation. Sample in lane 4 is positive for that mutation.* Panel b: *Detection of the Gly_{197} deletion alleles. Blot A was hybridized to an ASO specific for the normal sequence. Blot B was hybridized to an ASO containing the deleted sequence. Lanes 3, 4, and 5 are positive for that mutation, and lanes 1 and 2 are negative.*

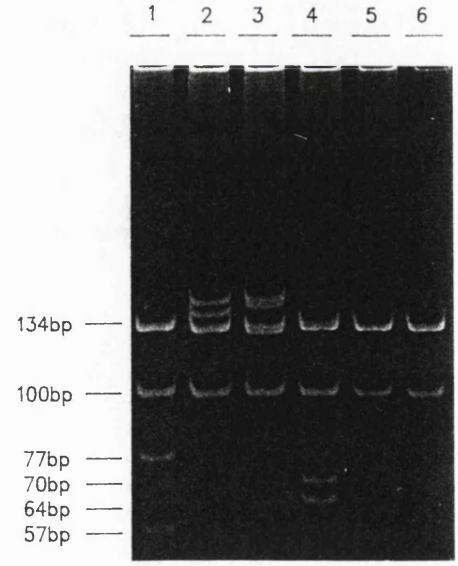

FIGURE 3. *Detection of mutations in exon 4 by polyacrylamide gel electrophoresis. Fractionation of polymerase chain reaction fragment II by polyacrylamide gel electrophoresis after digestion with* Dde *I and staining with ethidium bromide as described in "Methods." Each lane contains* Dde *I–digested amplified DNA from patients heterozygous for the following mutations: Cys_{210}→stop (lane 1), 2-bp deletion (lane 2), Gly_{197} deletion (lane 3), and Asp_{206}→Glu (lane 4); lanes 5 and 6 show the normal restriction fragment pattern.*

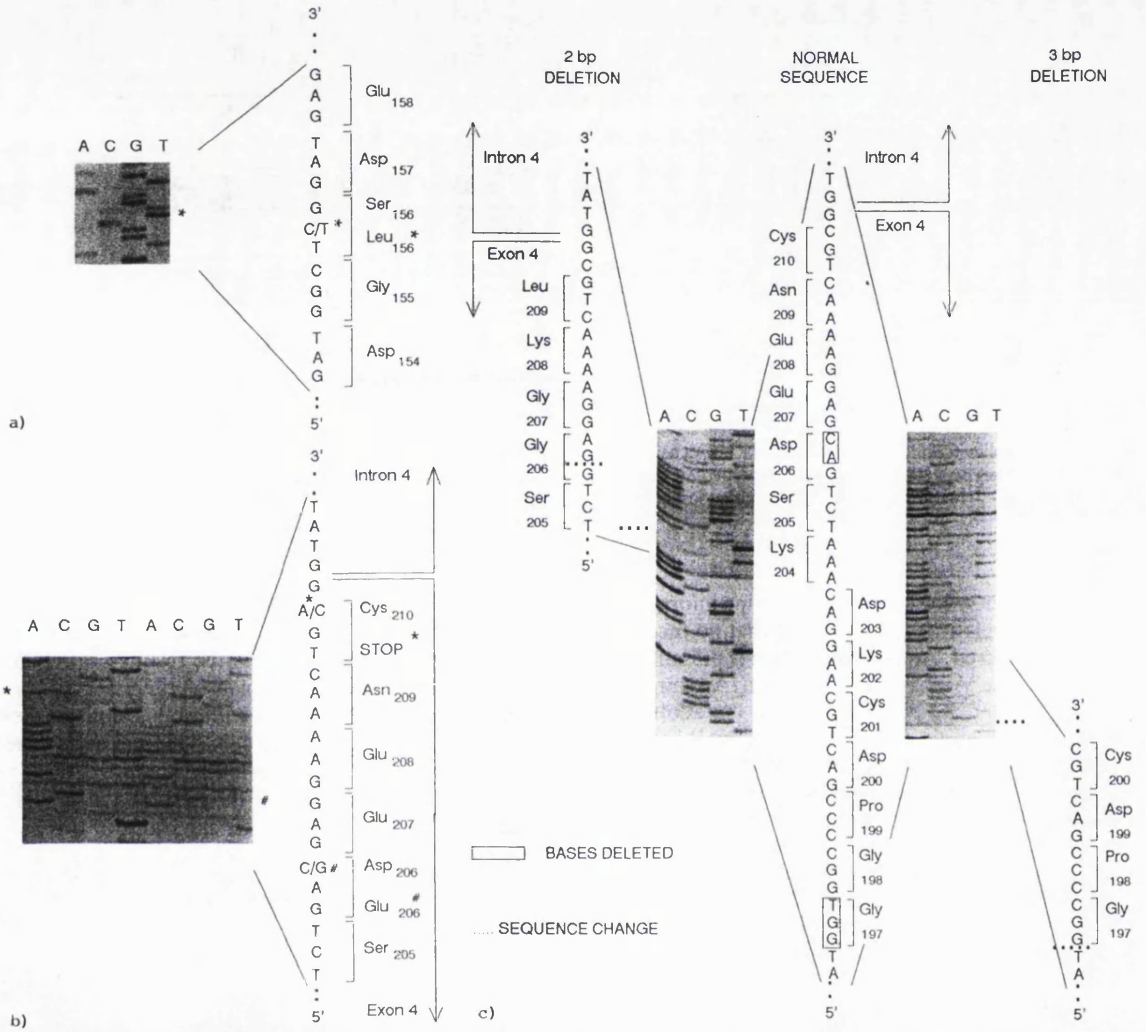

FIGURE 4. *DNA sequence of the amplified fragments of exon 4 from patients in whom mutations were detected. Polymerase chain reaction–amplified fragment of DNA was purified by agarose gel electrophoresis and sequenced directly with one of the pair of oligonucleotide primers used for amplification as described in "Methods."* Panel a *shows the sequence of the* Ser_{156}*→Leu mutation, with fragment I and an oligonucleotide primer spanning bases 427–448 of the low density lipoprotein receptor gene.* Panel b: *Sequence of the fragment from patients heterozygous for the* Asp_{206}*→Glu (right portion) and the* Cys_{210}*→stop (left portion).* Panel c *shows the sequence of the fragment from a patient heterozygous for the 3-bp deletion of* Gly_{197} *at the right and the 2-bp deletion of bases 694 and 695 at the left.*

reported in Afrikaners in South Africa[14] or the Glu_{207}→Lys found in a French Canadian and a Mexican patient.[16] However, the other two previously described point mutations were found in the London sample. The Ser_{156}→Leu change that has been described in a Puerto Rican family living in the United States[15] was identified in one patient from London, who is a second-generation immigrant from Poland. The haplotype of the Leu allele in this patient differs in the *Nco* I and *Ava* II polymorphisms from the haplotype reported in the initial description of this mutation,[15] and thus, it is likely to have arisen independently. A previously unreported 1-bp change was also found in one patient. This was a C→A transversion at a CpG dinucleotide that created a termination codon at amino acid Cys_{210}. This truncated protein is likely to be degraded intracellularly, and experiments are under way to investigate this possibility.

The Asp_{206}→Glu mutation, which occurs in 65% of patients with FH in the Afrikaner population in South Africa,[9] was found in three patients in this sample. One of the patients identified in the London sample is from South Africa but is not of Afrikaner origin, while the other two have lived in England for many generations with no evidence of recent migration. For all three patients the genotypes were consistent with the Glu mutation being carried on the same six-polymorphism haplotype (determined unequivocally in one family), which was identical with the haplotype for this mutation reported in the Afrikaner population.[8] This mutation has also been reported in an FH patient of English ancestry in North America, in whom the mutation is on a haplotype differing from that in the South African patient only at the 3′*Apa* LI polymorphism, which is downstream of the LDL receptor gene.[10] The majority of the Afrikaner population is of Dutch origin,[26] but the

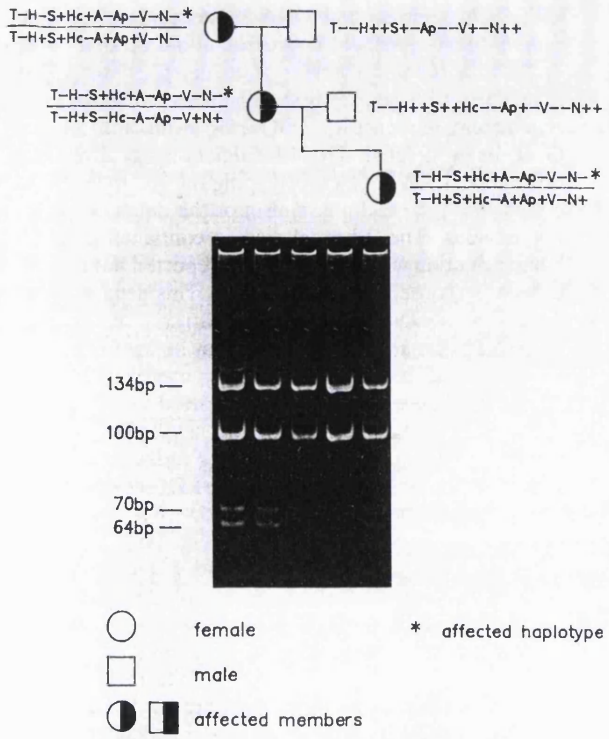

FIGURE 5. *Pedigree of one patient heterozygous for the Asp_{206}→Glu mutation in the LDL receptor gene. Those heterozygous for the mutation are designated by half-filled symbols, and unaffected relatives are designated by open symbols. T,* Taq *I; H,* Sph *I; S,* Stu *I; Hc,* Hinc *II; A,* Ava *II; Ap,* Apa *LI-5'; V,* Pvu *II,* N, Nco *I. "+" and "–" refer to the presence and absence, respectively, of the cutting site. Deduced haplotype for the affected allele is designated with an asterisk.*

Asp_{206}→Glu mutation has not been found in FH patients in The Netherlands despite an extensive search (J. Kastelein, personal communication). However, an estimated 5% of the Afrikaans-speaking population in 1867 were of English descent,[26] and therefore, it is possible that this mutation occurred originally in an individual in England.

Of the point mutations that have been described in the LDL receptor gene, almost half have occurred at a CpG dinucleotide "hot spot." Indeed, of the described 1-bp substitutions that cause human disease, the CpG dinucleotide is involved most frequently, accounting for about 35% of reported mutations,[27] and there is evidence that many of them have occurred independently

TABLE 2. Clinical Characteristics of Individuals With Various Mutations in Exon 4 of the LDL Receptor Gene

Mutation	FH number*	Sex	Age (years)†	TC (mmol/l)‡	TG (mmol/l)‡	HDL-C (mmol/l)‡	LDL-C (mmol/l)§	TX	CAD‖
Ser_{156}→Leu	FH 181	M	47	11.30	1.99	1.25	9.15	Y	N
Asp_{206}→Glu	FH 104	F	29	7.90	0.90	1.24	6.25	Y	N
	FH 105	M	50	11.20	0.90	0.83	9.96	Y	N
	FH 154	F	61	13.30	1.70	1.70	10.83	Y	Y
Cys_{210}→stop	FH 40	F	43	8.90	1.13	1.45	6.93	Y	Y
3-bp deletion (Gly_{197})	FH 13	F	39	11.00	0.80	0.96	9.67	Y	N
	FH 21	M	52	10.70	1.50	1.29	8.62	Y	Y
	FH 41	F	58	14.70	2.20	1.10	12.60	Y	Y
	FH 46	M	36	10.40	0.73	1.24	8.80	Y	N
	FH 47	M	49	12.40	1.45	1.14	10.60	Y	Y
	FH 118	M	36	9.30	0.77	1.11	6.85	Y	N
2-bp deletion of bases 694 and 695	FH 9	M	40	8.90	0.69	0.98	6.60	Y	N
	FH 36	M	49	10.60	1.10	0.96	9.10	Y	Y
	FH 69	M	43	12.80	1.15	1.48	10.79	Y	N
	FH 53	M	46	9.60	1.20	1.10	7.95	Y	Y
	FH 186	F	34	9.70	0.90	1.40	7.77	Y	N

LDL, low density lipoprotein; FH, familial hypercholesterolemia; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TX, tendinous xanthomas; CAD, coronary artery disease.

*The number is an arbitrary identification number assigned to the patient for this study.

†Age at the time of cholesterol measurement.

‡Untreated levels.

§Calculated by Friedewald's formula: LDL-C=TC−HDL-C−TG/2.2 mmol/l.

‖Positive diagnosis of CAD as myocardial infarct, coronary artery bypass graft, or angina pectoris diagnosed by coronary angiography or positive exercise test.

at the same site in different individuals.[27] There have been similar examples in the LDL receptor gene, and Leitersdorf et al[16] have reported that a mutation at a CpG in codon Glu_{207} has occurred on a gene with a different haplotype in a French Canadian patient and in a Mexican FH homozygote. We have recently shown that a mutation at a CpG dinucleotide in the codon for Pro_{664} in exon 14 of the LDL receptor gene[28] has occurred independently at least twice and probably three times according to haplotype analysis.[23] Although recombination between chromosomes during meiosis could, over long periods of time, result in such a mutation being "spread" onto several haplotypes, analysis with multiple variable sites that flank the mutation suggests that this explanation is unlikely. CpG dinucleotides are hot spots for mutations through a mechanism that involves deamination of methylated cytosine to thymidine.[29-31] Transversions are also known to occur at CpG dinucleotides at higher frequencies than expected.[27] Of the five known point mutations in exon 4 of the LDL receptor gene, four occur at a CpG dinucleotide, of which two are transitions and two are transversions. Two of the mutations have occurred at the same CpG dinucleotide: the Asp_{206}→Glu, which is a transversion, and the Glu_{207}→Lys, which is a transition. There are 26 CpG dinucleotides in this 384-bp exon, which is approximately one in 15 bases, compared with one in 22 bases on average in the remainder of the coding region of the LDL receptor gene.

The other mutations found in this exon are two small deletions, both of which are relatively common in our sample. The Gly_{197} deletion occurs in six of the FH patients in London, who are all of Jewish origin and one of whom is a recent immigrant from South Africa. All alleles carrying the deletion have or are consistent with the same six RFLP haplotypes. This mutation was originally described in an FH homozygous patient living in the United States,[10] and it causes a reduced rate of intracellular processing of the receptor protein, leading to a reduced number of receptors on the cell surface.[10] This deletion has recently been identified at a high frequency in patients of Lithuanian Jewish origin,[17] with the reported haplotype of the chromosome carrying the deletion being compatible with that observed in the patients from London. The other small deletion is the next most frequent mutation in our sample, occurring in five of the patients, all of which are consistent with the mutations being on the same haplotype. All of these patients are of English descent. If the RNA is spliced normally, the deletion would lead to a frameshift and stop codon and create a truncated protein of the first 205 residues of the LDL receptor with an additional 11 abnormal residues. By comparison with other mutant forms of receptor protein,[10] it is likely that this protein would be rapidly degraded in the cell. Taken together, these two deletions were found in 11 of 211 alleles, accounting for approximately 5% of all FH patients in the London sample, although because of differences in ethnic makeup it is likely that the frequency of these deletions will vary in other regions of the United Kingdom.

The finding of two small deletions within such a small region of the LDL receptor gene is noteworthy. Multiple mechanisms have been suggested to be responsible for deletions of fewer than 20 bp.[32] In 93% of the genes with such deletions the DNA sequence involved contained direct repeats of between 2 and 8 bp that either included or partially overlapped the deleted bases. In the case of the 3-bp deletion in the LDL receptor gene, there is a repeat sequence of TGG-TGG, with the internal GGT being deleted. The 2-bp deletion has a dinucleotide repeat GA-C-GA overlapping the AC deletion. In either case the exact mechanism of the deletion itself is not obvious. The 2-bp deletion is contained within a hexanucleotide with homology to a reported hot spot for deletions (consensus TGAGGA).[32] This sequence has similarity to DNA polymerase-α "arrest" sequences,[33] suggesting that arrest of synthesis may be involved in the mechanism of the deletion. Currently, exon 4 of the LDL receptor gene has eight different reported mutations that cause FH,[10,34] and because of the sequence, the high CpG content, and the fact that exon 4 codes for a critical region in the binding domain of the LDL receptor, it is likely that others remain to be found.

In each case where several patients with the same mutation were identified, we found a wide range of biochemical and clinical expression of the mutant alleles. Because of the small number of individuals involved, it is not possible at present to draw any conclusions about the relation between a specific mutation and the biochemical and clinical parameters, but this will become possible as more patients with defined mutations in the LDL receptor gene become available.

Our study confirms the large number of different mutations of the LDL receptor gene present in an urban population of mixed origin. In the sample of 200 patients, large deletions or rearrangements of the LDL receptor gene were detected in nine individuals,[13] while five individuals with the Pro_{664}→Leu mutation[23] and five with the Glu_{80}→Lys mutation[35] have previously been identified. We have now detected specific mutations in exon 4 in an additional 16 patients. Since the apoB $Arg_{3,500}$→Gln mutation has been detected in 3% of FH patients in London,[5] in total the molecular defect has been identified in more than 20% of these patients by using techniques that could be routinely applied in any laboratory.

Acknowledgments

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Molecular Medicine Original Article

The Clinical Investigator

Use of the single-strand conformational polymorphism method to detect recurrent and novel mutations in the low-density lipoprotein receptor gene in patients with familial hypercholesterolaemia: detection of a novel mutation $Asp^{200} \rightarrow Gly$ *

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Summary. The single-strand conformational polymorphism (SSCP) method was used to look for mutations in the 3' half of exon 4 of the low-density lipoprotein receptor gene in patients with familial hypercholesterolaemia (FH). One set of conditions were found which allowed the detection of four of the mutations that have previously been reported in this part of the gene and detected in patients in the United Kingdom: the 3-bp deletion (del Gly^{197}) the 2-bp deletion (STOP 216), the $Asp^{206} \rightarrow Glu$ mutation and the $Cys^{210} \rightarrow STOP$. The method was used to screen 50 patients with definite or probable FH from London. Two were identified who were carriers of the 3-bp deletion of Gly^{197}, one who was a carrier of the $Asp^{206} \rightarrow Glu$ mutation and one who was a carrier of a novel mutation that alters $Asp^{200} \rightarrow Gly$. This mutation creates a cutting site for the restriction enzyme *Msp*I. In a further sample of 200 patients from London with FH one additional apparently unrelated individual was detected who was a carrier of this defect. Thus in the sample of 50 patients, four (8%) had a mutation in this part of exon 4 that could be readily detected using the SSCP method, suggesting that this approach will be useful for rapid screening for mutations in patients with FH.

Key words: Familial hypercholesterolaemia – Single-strand conformational polymorphism

Familial hypercholesterolaemia (FH) is a common inherited disease showing an autosomal dominant pattern of inheritance [4]. It is characterized clinically by elevation in the concentration of low-density lipoprotein (LDL) cholesterol in blood, tendon xanthomata and an increased risk of myocardial infarction. FH is present in 5–10% of individuals who develop coronary artery disease (CAD) under the age of 55 years [8, 31] and is therefore the best understood single-gene cause of hyperlipidaemia and thus atherosclerosis risk. Once identified, the hyperlipidaemia of these patients is responsive to treatment by diet and drugs [3, 36], and since such treatment reduces subsequent CAD morbidity and mortality in hyperlipidaemic individuals in the general population [36], it is reasonable to predict that such therapeutic intervention will be of benefit to patients with FH.

Cloning of the human LDL receptor gene [38] has made it possible to study FH using DNA technology, and there have been at least 40 different mutations of the LDL receptor gene characterized at the DNA level [11]. Many more are likely to be found, and Brown and Goldstein [4] have postulated from the number of homozygous FH patients whom they have investigated that in the Dallas collection alone there could be as many as 183 mutant alleles, although only a minority have been characterized at the DNA level so far [11]. Several studies have been published demonstrating that within a geographically or culturally isolated population, or where a large proportion of persons are related by descent because of migration, there may be a single mutation causing FH in many of the patients [1, 10, 18, 19, 21, 33]. In most countries in Europe where there is a very heterogeneous population, it is unlikely that any mutations are present at a frequency in FH patients of greater than 2–5%. In a group of 200 FH patients in London we have

Abbreviations: FH = familial hypercholesterolaemia; LDL = low-density lipoprotein; CAD = coronary artery disease; SSCP = single-strand conformational polymorphism; PCR = polymerase chain reaction

* Dedicated to Prof. Dr. N. Zöllner on the occasion of his 70th birthday

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recently reported that 5% of patients had a gross deletion [32], while in a further 12% a small deletion or a single base mutation was detected in exon 3, 4 or 14 [9, 14, 37], and thus the specific defect causing FH can be determined in 17% of patients from our London sample. Even in a heterogeneous population such as in that in the United Kingdom it has also been reported that the frequency of some mutations may be high in certain local areas [37]. However, either many further mutations must be found, or a different strategy must be developed if DNA methods are ever to become a useful adjunct to classical screening methods.

One method recently described for detecting mutations is called single-strand conformational polymorphism (SSCP) and is based on the fact that single base changes result in conformational changes in single-stranded DNA which can be detected as a different pattern of migration on a polyacrylamide gel [27]. The advantage of this method is that it is rapid and does not use toxic chemicals or require a hybridization step, but it may not detect all single base changes, and to attempt to overcome this problem gels are run in varying conditions of temperature and gel porosity. We used this method to screen part of exon 4 of the LDL receptor gene for mutations in a group of 50 FH patients from London.

Materials and methods

Patients

The patient sample consisted of 50 heterozygous patients (all apparently unrelated) with the diagnosis of definite ($n=35$) or probable ($n=15$) FH, attending the Lipid Clinic in the Department of Medicine, University College London Medical School. Standard diagnostic criteria for FH were used [4, 32], including a total serum cholesterol of more than 7.5 mmol/l and plasma level of LDL cholesterol above 4.9 mmol/l, with tendon xanthomas in the patient, and a first- or second-degree relative with tendon xanthomas and/or definite myocardial infarction under the age of 55 years. Of the 50, 48% had tendon xanthomas. Patients with the mutation in the gene for apolipoprotein B causing familial defective apolipoprotein B were excluded [35].

Amplification of genomic DNA

The 3′ half of exon 4 of the LDL receptor gene was amplified by polymerase chain reaction (PCR) [28] with a pair of oligonucleotide primers; from bp 528 to bp 547 in exon 4: 5′CGACTGCGAAGATGGCTCGGA 3′ and primer in the intron 4: 5′GGGACCCAGGGACAGGTGATAGGAC 3′, giving a 242-bp fragment. Oligonucleotides were obtained from Severn Biotech (Kidderminster UK). The amplifications were performed in an automated thermal cycler (Cambio, Cambridge, UK) using *Thermus aquaticus* DNA polymerase (Perkin Elmer/Cetus, CT, USA) in the buffer recommended by the manufacturer and a total volume of 50 µl. The conditions were 95°C for 5 min and 68°C for 6 min once and subsequently at 95°C for 1 min and 68°C for 6 min for 30 cycles.

SSCP

The 242-bp fragment was labelled by PCR amplification using conditions as above, with the addition of 0.1 µl per reaction mixture of [α ^{32}P] 2′deoxycytosine triphosphate (800 Ci/mmol, 10 µC/µl; Amersham UK). A quantity of 5 µl of the PCR mixture was diluted with 25 µl of 0.1% sodium dodecyl sulphate and 10 mM ethylene diamine tetra-acetic acid, and 5 µl of this dilution was mixed with 5 µl formamide dye (95% formamide, 20 mM ethylene diamine tetra-acetic acid, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The DNA was denatured by boiling at 90°C for 5 min and snap-cooled on ice. Samples (4 µl) were loaded onto a 4.5% polyacrylamide non-denaturing gel (ratio of acrylamide to bisacrylamide 49: 1) in TBE buffer with or without 10% glycerol. The gel was run either at 20 mA for 16 h at room temperature or at 45 mA for 3.5 h at 4°C. The gel was dried and exposed to hyperfilm β max (Amersham, UK) for 12–24 h at −70°C before developing.

Direct sequencing

PCR products were purified by electroelution of the appropriate bands from 1% agarose and ethanol precipitation. The fragments were sequenced directly as described [9, 14].

Screening for Asp^{200}→Gly

Genotype for the Asp^{200}→Gly mutation was determined by *Msp*I restriction digestion. Amplified DNA was incubated with 10 U of *Msp*I in buffer recommended by the manufacturer (Boehringer Mannheim, FRG) overnight at 37°C. DNA was loaded onto a 1.8% agarose gel in TAE buffer (0.15 M TRIS, 0.05 M ethylene diamine tetra-acetic acid, ph 7.7), and fragments were separated by electrophoresis. The gel was stained with ethidium bro-

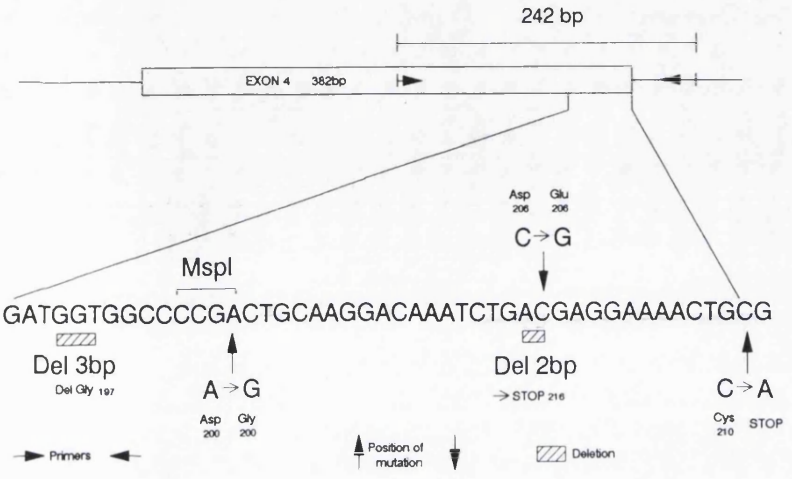

Fig. 1. Mutations in exon 4 of the LDL receptor gene. A diagram of the 3′ end of exon 4 of the LDL receptor gene and the flanking intron showing the positions of the four previously described mutations [9] and the new mutation described in this paper. *Hatched Boxes* (under the sequences), deleted bases; *vertical arrows*, single base changes, together with the identity of the codon(s) concerned; *horizontal arrows*, oligonucleotide primers used for amplification (5′→3′)

mide and photographed. The normal pattern is of a single fragment of 242 bp; the mutation creates an *Msp*I site and fragments of 148 and 96 bp.

Results

Samples were available from FH patients with four different mutations (previously confirmed by sequencing) in the 3′ half of exon 4 of the LDL receptor gene [9]. These were the 3-bp deletion (del Gly^{197}), the 2-bp deletion that results in an in-frame STOP at codon 216, the Asp^{206}→Glu mutation found commonly in Afrikaner patients and the Cys^{210}→STOP (Fig. 1). DNA from these patients was amplified and run on non-denaturing gels either at room temperature or at 4°C with or without glycerol. Only one set of conditions allowed detection of all these mutations in a single run (Fig. 2, left), and these were subjected to electrophoresis for 16 h at room temperature with 10% glycerol. The 3-bp deletion was easily detected as a doublet of the upper single strand and as a well-resolved doublet in the lower single strand. The 2-bp deletion had a small effect on increasing the migration of the upper band, and a large effect on the lower band, which showed a much faster migration rate. The Cys^{210}→STOP mutation showed the smallest difference in migration pattern in these conditions, with a broadening of both the upper and lower bands, while the mutation creating Asp^{206}→Glu caused the upper single strand to migrate more slowly that the control DNA, with no detectable change in the lower band. In addition, in all samples a double-stranded heteroduplex band or bands could be seen, most clearly under conditions of a gel without glycerol and run at 4°C (Fig. 2, right), which clearly distinguished all mutations from the migration pattern of normal DNA.

Samples from 50 apparently unrelated FH patients were amplified and run on non-denaturing gels at 4°C with glycerol, and the results from 16 patients is shown in Fig. 2b. In all samples there are the expected two major single-strand bands plus a more rapidly migrating double strand band. The intensity of the bands varied among samples, presumably reflecting differences in PCR efficiency. In four patients a different pattern was observed: in one (patient YT-19) the typical pattern of the 3-bp deletion, in a second (YT-22) a more slowly migrating upper band similar to that observed with the Asp^{206}→Glu, and in a third (YT-27) a novel pattern of a more rapidly migrating upper band, with an additional "heteroduplex" band being visible slightly above the double strand. In one other patient (YT-4, not shown) the pattern typical of the 3-bp deletion was seen.

DNA from these four individuals was used to amplify the exon 4 fragment, and direct sequencing was carried out. For the samples from patients YT-4 and YT-19 the presence of the 3-bp deletion was confirmed (not shown), and results from the other samples are shown in Fig. 3. Patient YT-22 was heterozygous for C and G in codon 206 as predicted, which changes the amino acid from Asp to Glu. Patient YT-27 was heterozygous for A and G in codon 200, which would change Asp (GAC) to Gly (GGC). This A→G change creates a site for the enzyme *Msp*I (normal sequence CCGA, mutant CCGG; Fig. 1), and the mutation can easily be identified by *Msp*I digestion of amplified DNA (Fig. 4). DNA from a previously described [9, 32] additional 200 apparently unrelated FH patients

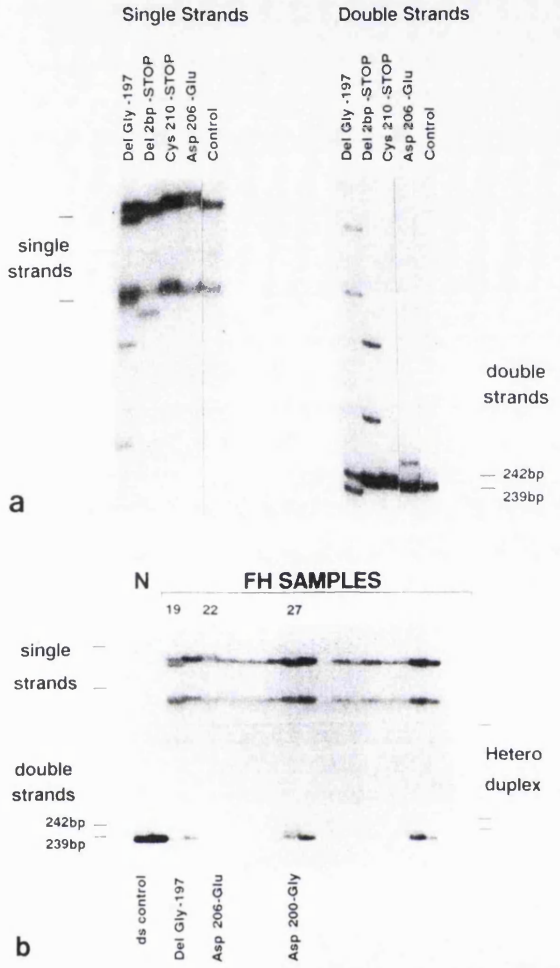

Fig. 2a,b SSCP gel to detect mutations in exon 4. Separation of single-stranded DNA of PCR-amplified radiolabelled DNA. **a** *Left*, from patients with known mutations in exon 4: *lane 1*, 3-bp deletion; *lane 2*, 2-bp deletion; *lane 3*, Cys^{210}→STOP; *lane 4*, Asp^{206}→Glu. Conditions: 16 h at 20 mA, room temperature, 10% glycerol. *Right*, heteroduplexes observed in double-strand DNA. (Same order of samples). Gel run 3.5 h 4°C 45 mA. **b** From 16 unrelated patients with clinical diagnosis of definite or probable FH. SSCP seen in *lanes* 3, 5 and 10

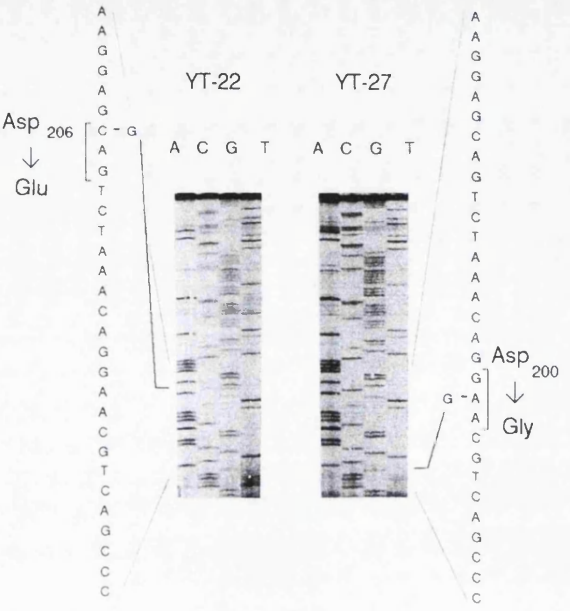

Fig. 3. DNA sequence of the amplified fragments of exon 4 from patients in whom mutations were detected. The PCR-amplified fragment of DNA was purified by electroelution from agarose gel after electrophoresis and sequenced directly using an internal oligonucleotide from base 535 to base 553 5′ AAGATGGCTCGGATGAGT 3′. *Left*, the sequence of the DNA from patient YT-22, who was heterozygous for the Asp^{206}→Glu; *right*, the sequence of the fragment from patient YT-27, heterozygous for the Asp^{200}→Gly change

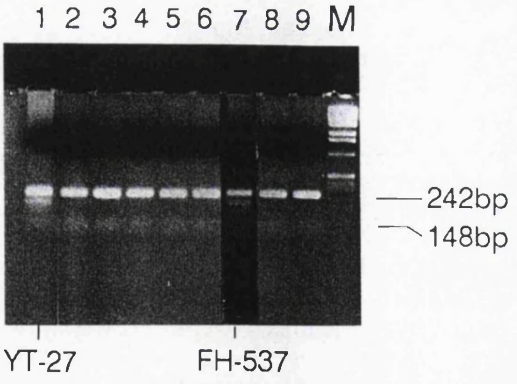

Fig. 4. Detection of the Asp200→Gly mutation in exon 4 by agarose gel electrophoresis. Fractionation of PCR fragments by agarose gel electrophoresis after digestion with *Msp*I and staining with ethidium bromide. Each lane contains *Msp*I-digested amplified DNA from patents heterozygous for FH. *Lanes 2–6, 8, 9*, the normal restriction fragment pattern; *lane 1*, the pattern from patient YT-27; *lane 7*, the digest from FH-537 marker (*M*) is kb ladder

was screened for this A→G change, and one of the patients was a carrier for this mutation.

Untreated lipid levels and clinical details for the five identified individuals are presented in Table 1. Three of the individuals, one of whom (YT-27) was obese, presented initially with elevated levels of triglycerides, which were reduced to within the normal range on diet alone. All patients had features of FH, either tendon xanthomas and evidence of CAD or evidence of a family history of hyperlipidaemia. All were white and none reported recent immigration to the United Kingdom, except YT-22, who was a recent immigrant from South Africa.

Discussion

Until recently, diagnosis of FH was for the majority of patients based on biochemical and clinical findings, in the presence of a family history of CAD and/or hyperlipidaemia. However, it has been rec-

Table 1. Clinical characteristics of the individuals with various mutations in exon 4 of the LDL receptor gene

Mutation	FH number	Sex	Age[b] (years)	TC[c]	TG[c] (mmol/l)	HDL-C[c] (mmol/l)	LDL-C[d] (mmol/l)	TX	CAD[e]
3 bp del Gly^{197}	YT-4	M	32	12.0	3.9	1.1	9.2	Yes	Mother and two brothers affected
	YT-19	F	20	11.0	1.6	1.0	9.3	No	Father CABG at 42 years
$Asp^{206}\rightarrow Glu$	YT-22	M	42	13.1	3.2	0.7	10.9	No	Xanthelasmas arcus
$Asp^{200}\rightarrow Gly$	YT-27	M	55	10.2	2.2	1.2	8.0	Yes	Angina, brother affected
	FH-537	M	41	11.6	1.2	1.2	9.9	Yes	Mother and child affected

TC, Total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TX, tendon xanthoma; CABG, coronary artery bypass graft

[a] The number is an arbitrary identification number assigned to the patient for this study

[b] Age at cholesterol measurement

[c] Untreated levels

[d] Calculated by Friedewald's formula: LDL-C = TC - HDL-C - TG/2.2 mmol/l

[e] Positive diagnosis of CAD as myocardial infarct or coronary artery bypass graft or angina pectoris diagnosed by coronary angiography or positive exercise test

ognized for many years that not all individuals with a defect in the LDL receptor fulfil the criteria for FH (e.g. [15, 20, 26]). Individuals who are carriers for a mutation in the LDL receptor may have lipid levels within the normal range because of environmental or compensatory genetic factors, or their levels may rise above the 95th percentile with increasing age [13]. Conversely, the hypercholesterolaemia may be the result of a combination of various environmental and genetic factors or due to the effect of mutation in another gene, such as for that for apolipoprotein B. A single mutation in the apolipoprotein B gene ($Arg^{3500}\rightarrow Gln$) occurs in roughly 3% of patients with a clinical diagnosis of FH in the United Kingdom and Germany [30, 35], and estimates of the frequency of this mutation in the general population are roughly 1/700.

Assays of LDL receptor function on lymphocytes have been proposed to identify patients with defects in the LDL receptor [2, 6, 29], but these are technically demanding and still show an overlap between FH patients and normals. However, once patients with FH have been identified, their hyperlipidaemia is responsive to treatment by diet and drugs [3, 36], and by extrapolation of the benefits observed in hyperlipidaemic patients in the general population [36] it is likely that such treatment will reduce subsequent CAD morbidity and mortality in patients with FH. Thus development of methods to screen for the disorder are important, firstly in individuals at high risk because of poor family history and ultimately in a wider context when population-based screening is established. For a disorder such as FH in which the pattern of inheritance is clear, it is likely that the genetic tests will become a useful part of such a screening strategy, particularly in the unequivocal detection of young relatives [16, 20].

DNA polymorphisms of the LDL receptor gene can be used to follow the inheritance of the defective gene in families [12], but this depends on the availability of affected and non-affected relatives, and that the proband is heterozygous for one or more DNA polymorphisms. In some parts of the world molecular diagnosis is easier, as particular mutations in the LDL receptor gene occur in a large proportion of patients because of "founder" effects through migration and geographical or cultural isolation [1, 10, 18, 19, 21, 33]. However, in most parts of Europe, such a founder effect in unlikely, and it is predicted that there are many different mutations in the population. Thus methods to identify mutations are required that are rapid, and that can be applied generally. One such method is the chemical cleavage of mismatch technique [7, 23], which appears to pick up all known mutations, but which is time consuming and requires the use of toxic chemicals. A second approach is the use of denaturing gradient gel electrophoresis [24], which also appears to detect all known mutations. A recent report used this method to screen the promoter region of the LDL receptor for mutations in FH patients, but none were identified [34].

We chose to use the SSCP method; in the conditions that we used small deletions were easily detected, and three single base changes ($A\rightarrow G$ in codon 200, $C\rightarrow G$ in codon 206 and $C\rightarrow A$ in codon 210) were also detectable. In addition to the SSCP

that occurred as a result of these mutations, heteroduplexes of double-stranded DNA were observed migrating more slowly than the normal DNA fragment, as has been noted by others [9, 21, 25]. These serve as a useful additional mean of identifying sequence changes. Although all the mutations available to us were detected by this technique, it is still possible that the method may not identify all base changes present in a fragment of DNA. However, it has the advantage of being technically straightforward and can be carried out without use of radiolabel by using staining of the DNA with silver [22]. In common with the other methods available, it appears to be optimal at detecting mutations in fragments of DNA 200–400 bp in length, and because of its speed and ease we believe it will be a useful first screen which should result in the rapid detection of the vast majority of defects in a group of FH patients. Other techniques could then be used in those patients in whom no SSCP is observed.

Exon 4, which is 382 bp long, appears to be a hot spot for mutations in the LDL receptor, with nine mutations reported so far ([9, 11, 17–19, 21] and this report). This region of the gene codes for repeats 4 and 5 of the receptor which are involved in interaction with apolipoprotein E and apolipoprotein B [11, 38], and screening efforts have clearly focused on this region, which may have biased the frequency estimate. However, the exon has a higher proportion of the dinucleotides CG than any other region of the receptor (1/15 bases compared to 1/22 bases on average in the coding region of the gene), and it is well known that such CG sequences are hot spots for deamination of cytosine to thymidine [5]. In the sample of 50 patients whom we examined four (8%) had a detectable mutation in the 3' half of exon 4. Two patients had the del Gly^{197} mutation, first found in a patients in the United States and recently identified as being common in Jewish patients in Israel of Lithuanian origin [21]. We have also found this to be a common mutation in London, being detected in 6 of 200 patients, all of whom reported Jewish origins [9]. Both of the new patients identified here reported Jewish ancestry. The $Asp^{206} \rightarrow Glu$ mutation was identified in an individual who had recently immigrated from South Africa. This mutation occurs in roughly 65% of patients with FH in the Afrikaner population in South Africa [19] and was also found in 3 of 200 FH patients from London [9], raising the possibility that this mutation originally occurred in an individual in England. The novel mutation detected here did not occur as a result of a change in a CG dinucleotide. Although we have not proved that the $Asp^{200} \rightarrow Gly$ change destroys the function of the LDL receptor, the neighbouring $Asp^{206} \rightarrow Glu$ change is known to affect function and to cause FH [19]. Moreover, this is not a conservative substitution, and the positively charged Asp normally at this position is likely to be important in interaction with negatively charged ligands. Studies are underway to confirm this possibility.

The lipid levels and clinical characteristics of the individuals with specific mutations detected here are typical of FH patients in general. However, it is possible that different mutations are associated with different clinical consequences or require different management, and information about this may be a major benefit for the patients. This clinically important possibility can be addressed only when sufficient numbers of patients with a specific defect have been identified. The method that we present here will form a useful basis for future development of genetic screening strategies for FH based on DNA techniques.

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Effect on Plasma Lipid Levels of Different Classes of Mutations in the Low-Density Lipoprotein Receptor Gene in Patients With Familial Hypercholesterolemia

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Abstract We used the single-strand conformational polymorphism method to screen 311 patients with familial hypercholesterolemia from London lipid clinics and Southampton and South West Hampshire health district for mutations in the 3′ part of exon 4 of the low-density lipoprotein (LDL) receptor gene. This part of the gene codes for repeat 5 of the binding domain of the LDL receptor, which is known to be critical for the receptor-mediated removal of both triglyceride-rich lipoprotein remnants and LDL. Six previously described mutations were identified in 29 apparently unrelated individuals (9.3%), with the mutations all lying within a 50-bp fragment of the gene. Three of the mutations are null alleles producing no protein, and the other three lead to production of a defective protein. The effect of the different gene mutations on lipid levels was examined, after the data were combined with information on previously reported mutations in this patient group. Mean LDL cholesterol levels were highest in those individuals with a mutation creating a null allele (9.54 mmol/L) and were similar to levels in those individuals with a mutation affecting repeat 5 that resulted in the production of a defective protein (9.37 mmol/L). In this sample, previously identified patients with a defective protein mutation outside repeat 5 had lower mean levels of LDL cholesterol (7.78 mmol/L), which were similar to levels seen in patients in whom the specific mutation had not been identified (7.31 mmol/L). Overall, these differences were highly statistically significant ($P<.001$). These data reinforce the observations of other researchers that specific mutations in the LDL receptor gene are associated with different effects on plasma lipids and indicate that the phenotype is influenced by the genotype. (*Arterioscler Thromb.* 1994;14:1717-1722.)

Key Words • single-strand conformational polymorphism • LDL receptor mutations • familial hypercholesterolemia • genotype-phenotype

Familial hypercholesterolemia (FH) is caused by mutations in the low-density lipoprotein (LDL) receptor gene.[1] To date, it has been difficult to examine whether specific LDL receptor gene mutations show a different genotype-phenotype relation except in founder populations in which large numbers of carriers for a particular mutation can be found. In such populations, recent studies have suggested that different mutations have different phenotypes such as lipid levels, expectation of clinical sequelae, and drug responsiveness.[2-5] However, because of their common origin, the patients may also share other genetic factors, and these comparisons may thus be confounded. Here, we report the first attempt to undertake such a study in a group of FH patients from a population with a complex spectrum of LDL receptor gene mutations by combining the characterized LDL receptor gene mutations into functional groups and examining the differences on baseline lipid levels among these groups.

Five classes of mutations at the LDL receptor locus have been identified on the basis of the phenotypic behavior of the mutant protein.[1] Class 1 mutations fail to produce any immunoprecipitable protein (null alleles). The other four classes all produce defective proteins and are affected at different levels of the receptor pathway because of the precise location of the missense mutation. The first 292 residues of the receptor contain 7 imperfect repeats of 40 amino acids that make up the binding domain.[6] Mutational analysis of the binding domain[7] has shown that repeat 1 is not required for binding of either apolipoprotein B (apoB) or apoE, but repeat 5 is required for both ligands. Mutations affecting this part of the protein are thus likely to have a severe effect on the binding function of the receptor. Repeats 2 and 3 as well as 6 and 7 are required for maximal binding of LDL via apoB but not for very-low-density lipoprotein (VLDL) via apoE,[7] and analysis of a naturally occurring mutation in repeat 4 has shown that the substitution of Leu for Ser at position 156 (S156L), which abolishes the binding of LDL but not that of β-VLDL, causes only a mild reduction of receptor function in vitro.[8]

The binding domain of the LDL receptor is coded by exons 2 through 6 of the gene, with the 3′ end of exon 4 coding for the apoE/apoB binding repeat 5 (amino acids 172 to 210), and both we[9,10] and others[11] have observed that mutations occur frequently in this part of the gene. It has been suggested that the high mutation rate observed in this part of the gene is caused by a selection bias, because individuals with such mutations are more severely affected than individuals with mutations in

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other parts of the gene.[11] To examine for possible differences of effect of mutations on lipid phenotype, we have compared patients with any mutation causing a null allele with those in whom a missense mutation occurs in repeat 5 and those with a missense mutation elsewhere in the gene. Comparisons were made by combining data from the current screening of the 3′ part of exon 4 of the gene with results from previous studies on this group of patients in whom gross deletions[12] or missense mutations in exon 3[13] or exon 14[14] had been detected.

Methods

Selection of Patients

A total of 311 apparently unrelated patients with heterozygous FH were studied. They were from four Lipid Clinics in London (Hammersmith Hospital, Charing Cross Hospital, St. Mary's Hospital [n=189] and the Department of Medicine, University College of London Medical School [n=72]). The patients from the first three clinics have been described in earlier studies.[9,10,12] In addition, 50 patients were recruited from Southampton and South West Hampshire health district (I.N.M.D., unpublished data). Standard diagnostic criteria for FH were used,[1,12] including a serum total cholesterol of more than 7.5 mmol/L and plasma level of LDL cholesterol higher than 4.9 mmol/L, with tendon xanthomas in the patient, and a first- or second-degree relative less than 55 years of age with tendon xanthomas and/or definite myocardial infarction. Cholesterol and triglyceride analyses were by laboratories participating in national quality-control schemes. LDL cholesterol was calculated according to Friedewald et al.[15] Patients who were found to carry the mutation for familial defective apoB were excluded.[16] The 30 patients showing no single-strand conformational polymorphism (SSCP) in the 3′ part of exon 4, who were selected for sequencing, all had tendon xanthoma as well as the full standard diagnostic criteria.

Amplification of Genomic DNA

Parts of exon 4 were amplified by polymerase chain reaction (PCR)[17] using two sets of oligonucleotide primer pairs. For the 3′ end, primer 1 from bp 528 to bp 547 in exon 4 and primer 2 in intron 4 (5′-GGGACCCAGGGACAGGTGATAGGAC-3′) were used giving a 236-bp fragment (designated fragment 1). The whole of exon 4 (designated fragment 2) was amplified using primer 3 (5′-AAAGTCGACGGTCTCGGCCATC-CATCCCTG-3′) from intron 3 (including *Sal* I cutting site, as that primer was found to give the most consistent results) and 5′ biotinylated primer 2. Oligonucleotides were obtained from Severn Biotech Ltd. The amplifications were performed in an automated thermal cycler (Cambio) with *Taq* DNA polymerase (GIBCO BRL) in the buffer recommended by the manufacturer and a total volume of 25 μL for fragment 1 and 50 μL for fragment 2. The conditions for both fragments were 95°C for 5 minutes and subsequently 68°C for 6 minutes once and 95°C for 1 minute and 68°C for 6 minutes for 30 cycles, with 15 minutes at 68°C after the last cycle.

Single-Strand Conformational Polymorphism

Fragment 1 was labeled by PCR amplification using the conditions above with the addition of 0.1 μL per reaction mixture of [α-^{32}P]5′-deoxycytidine triphosphate (800 Ci/mmol, 10 μCi/μL; Amersham). A quantity of 5 μL of the PCR mixture was diluted with 25 μL of 0.1% sodium dodecyl sulfate and 10 mmol/L EDTA. Five microliters of this dilution was mixed with 5 μL formamide dye (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The PCR DNA was denatured by boiling for 3 minutes and then put immediately on ice. Samples (4 μL) were loaded onto a 4.5% polyacrylamide nondenaturing gel (ratio of acrylamide to bisacrylamide, 49:1) in 0.089 mol/L Tris-borate, 0.002 mol/L EDTA buffer, with or without 10% glycerol. Gels were 40 cm×30 cm×0.4 mm. Electrophoresis was at 20 mA for 16 hours at room temperature in the case of the 4.5% polyacrylamide gels with 10% glycerol and at 45 mA for 3 hours at +4°C in the case of the 4.5% gel without glycerol. The gels were then transferred onto Whatman 3MM chromatographic paper and dried and exposed to hyperfilm β max (Amersham) for 12 to 24 hours at −70°C before developing.

Direct Sequencing

For sequencing, biotinylated PCR fragment 2 was captured onto Dynal-beads (Dynal UK Ltd) for purification. The single strands were separated by denaturing in NaOH according to the manufacturer's protocol, and the DNA was then sequenced using the Sequenase kit (version 2.0, United States Biochemicals) following the manufacturer's protocol. The oligonucleotides used for sequencing were primer 4 from bp 606 to 588, primer 5 from nucleotides 534 to 552, primer 6 from the intron 4–exon 4 boundary (antisense), and primer 7 from nucleotide 685 to 704.

ApoE Genotyping

ApoE genotype was determined by PCR and an *Hha* I enzyme digest as previously described.[18]

Statistical Analysis

Analysis of the pretreated plasma lipid levels and age at the time of diagnosis was carried out using the SPSS/PC+ computer programs (Northwestern University) and STATGRAPHICS II (Statistical Graphics Corp). Plasma levels of triglycerides and lipoprotein(a) were log 10 transformed before statistical analysis. Means of plasma lipid levels among groups were compared by ANOVA or Student's t test, both with and without adjustment for age and gender by multiple linear regression. Statistical significance was taken at a value of $P<.05$. For many patients, data on weight at the time of measurement of pretreatment lipid levels were unavailable, so adjustment for body mass index was not possible. In general, FH patients are not obese, and in samples from the general population adjustment for body mass index, with the normal range, has only moderate effects on cholesterol levels. In addition, no adjustment was made for possible differences in lipid measurements from the different participating clinics. Such differences are likely to be small (<5%), and because each clinic contributed roughly equally to the patients identified with each class of mutations, this is unlikely to have introduced a significant bias.

Results

Identification of Mutations by SSCP

Samples from all 311 FH patients in this study were analyzed by SSCP at the 3′ region of exon 4 of the LDL receptor gene. Several combinations of conditions were used, including either 4°C or room temperature with a 4.5% polyacrylamide gel (with and without glycerol). The clearest results were obtained when samples were run in a 4.5% polyacrylamide gel and 10% glycerol at room temperature for 18 hours; Fig 1 shows the SSCP pattern obtained for the six mutations detected. Complete sequencing (not shown) demonstrated that all of these mutations had previously been identified in this region of the gene, either in a subset of the patients analyzed here[9,10] or in the Dallas collection (E207X).[11] The mutations are easily detected by changes in the upper single-strand migration pattern. The smallest mobility shift (0.5% or 1 mm on a 220-mm-long run) was seen for the Cys to stop at codon 210 (designated

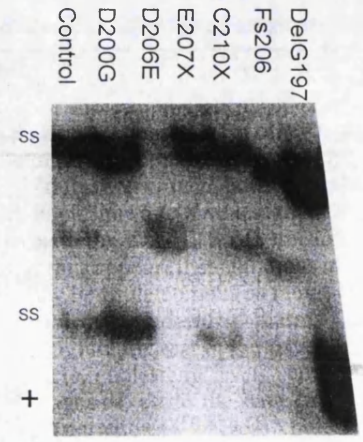

FIG 1. Autoradiograph shows single-strand conformational polymorphism of the 3′ end of exon 4 as described in "Methods." + denotes the anode; SS denotes the single strands. The slowest migrating band is representative of one single strand, with the lowest band representing the other single strand (faint). The middle bands are "shadows" that are possible polymerase chain reaction artifacts.[19] The D206E single strands are particularly faint in this picture, which was not usually seen and is probably related to lower polymerase chain reaction efficiency in the experiment.

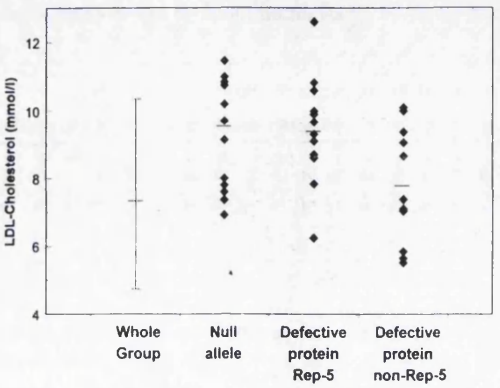

FIG 2. Scatterplot shows pretreatment individual unadjusted low-density lipoprotein (LDL) cholesterol levels in patients in the various groups analyzed. Transverse lines represent the mean LDL cholesterol levels in each group. For the whole familial hypercholesterolemia group, 95% confidence intervals are shown; for the other groups, individual levels are plotted. Rep-5 indicates repeat 5.

C210X). Two of the mutations show very similar changes in mobility and cannot easily be distinguished from each other by the SSCP pattern alone. These are the D206E mutation and the mutation not previously reported in the United Kingdom (E207X). This mutation was shown to be different from the D206E by direct sequencing of PCR-amplified fragment from DNA from the individuals showing these SSCP patterns (not shown). All of the 3′ exon 4 mutations detected in this study reside within 50 bases of the gene.

To evaluate whether the SSCP method had detected all the mutant alleles present in this part of the gene, we chose PCR-amplified DNA from 30 of the patients with definite FH (with tendon xanthomas) but no SSCP in exon 4 and reamplified and directly sequenced the DNA. No other mutations were detected (data not shown).

Mutations and Lipid Levels

Only patients in whom pretreatment lipid values were available were included in the analysis. For the analysis the data obtained from individuals with an identified mutation in repeat 5 were combined with data from previous studies on this patient group.[9,12-14] Three of the mutations (occurring in 12 patients) are predicted to cause "null alleles," FS206, E207X, and C210X, and lipid data from these patients were compared with data from the 17 patients with mutations predicted to cause a defective protein in repeat 5, del G197, D200G, and D206E (designated defective protein repeat 5), and with data from a group consisting of 4 patients with the missense mutation E80K,[13] 3 with the P664L mutation,[14] 2 with the S156L mutation,[9] and 2 with gross deletions causing a defective protein[12] (designated defective protein excluding repeat 5). Fig 2 shows a scatterplot of the LDL cholesterol levels in the different patient groups, and the Table shows the plasma lipid levels and characteristics of the patients. The group of patients with any detected mutation had unadjusted total cholesterol and LDL cholesterol levels that were 15% and 22% higher, respectively ($P<.0001$), and triglyceride levels 18% lower ($P<.05$) compared with the rest of the FH patients with no detected mutation. This difference was also highly significant ($P<.0001$ and $P<.05$, respectively) when the analysis was performed on sex- and age-adjusted values (data not shown). Among the patients, the group with a mutation causing a null allele had the highest mean total and LDL cholesterol levels, with very similar levels being seen in patients with a defective protein caused by a mutation affecting repeat 5. By contrast, patients with a defective protein mutation outside repeat 5 had mean total and LDL cholesterol levels that were significantly lower and were similar to levels seen in the group of patients with an unknown mutation.

To examine the possibility that the common apoE variation might explain the differences in plasma lipids, we determined apoE genotype. The frequency of the apoE alleles did not differ between those patients with a mutation in repeat 5 compared to the rest of the FH patients (E2, 0.054 versus 0.047; E3, 0.786 versus 0.774; and E4, 0.160 versus 0.179, respectively) nor between those patients in the subgroups of null alleles and defective proteins (data not shown).

Fig 3 shows the distribution of serum cholesterol with age (untreated lipid levels at age of diagnosis) in all heterozygous FH patients in whom the mutation had been identified, including those previously described from this sample, with patients grouped according to the predicted class of mutation of either any defective protein or any null allele. It can be seen that patients with a defective protein (Fig 3a) display a statistically significant correlation of plasma cholesterol with age, whereas there is no statistically detectable correlation in the group with null alleles (Fig 3b). The slope of the regression line for the defective allele group is 0.8 mmol/L per decade.

Discussion

In our hands, SSCP is an effective and rapid method for detecting sequence changes in PCR-amplified DNA

Comparison of Unadjusted Lipid Levels Between Various Groups of Probands With Familial Hypercholesterolemia

	Whole Group	Any Mutation	Null Mutations	Defective Protein Repeat 5	Defective Protein Excluding Repeat 5
n	118*	44†	12	17	11‡
Male/female	75/43	25/19	5/7	11/6	7/4
Age, y	44.9 (14.5)	41.1 (12.8)	40.1 (11.2)	41.4 (11.1)	42.0 (16.7)
TC, mmol/L	9.37 (1.77)	10.74 (1.90)§	11.31 (2.11)‖	11.23 (1.65)¶	9.64 (1.77)**
LDL, mmol/L	7.31 (1.86)	8.93 (1.82)§	9.54 (2.05)‖	9.37 (1.52)¶	7.78 (1.73)**
TG, mmol/L	1.65 (0.65-3.36)	1.38 (0.67-2.55)§	1.03 (0.4-2.55)‖	1.63 (0.73-3.90)#	1.47 (0.67-2.09)
HDL, mmol/L	1.30 (0.42)	1.19 (0.28)	1.29 (0.31)	1.13 (0.22)	1.20 (0.36)
Lp(a), mg/dL	39.1 (3-105)	30.5 (5-93)	22.2 (4-60)	31.0 (5-93)	64.7 (20-102)
	[n=70]	[n=25]	[n=8]	[n=10]	[n=5]

TC indicates total cholesterol; LDL, low-density lipoprotein cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; and Lp(a), lipoprotein(a). All values are mean (±SD) except for TG and Lp(a) showing mean (95% confidence interval). LDL was calculated by Friedewald's formula: LDL=TC−HDL−TG/2.2 mmol/L. For Lp(a), the number of individuals measured is shown in brackets.

*Untreated plasma lipid values were not available for all patients with familial hypercholesterolemia.

†Four individuals with gross deletions[12] but unknown functions excluded from the subgroups of mutations.

‡Includes 4 individuals with the E80K mutation,[13] 2 with the S156L mutation,[9] 3 with the P664L mutation,[14] and 2 with gross deletions FH28 and FH218.[12]

§$P<.0001$, ‖$P<.05$, ¶$P<.01$, compared with the whole group.

#$P<.05$ between defective protein in repeat 5 and null.

**$P<.05$ between defective protein in repeat 5 and defective protein excluding repeat 5.

in the 3′ end of exon 4 of the LDL receptor gene. This region codes for repeat 5 of the binding domain of the LDL receptor, which we and others have shown is a frequent mutation site in FH patients.[9-11] SSCP detected mutations in 29 of the 311 patients studied (9.3%). SSCP is known to be sensitive to the fragment size as well as the electrophoresis conditions,[20,21] and we observed that a relatively small change in ambient temperature resulted in a variation in the ability to detect an SSCP. It was therefore important to control the experimental conditions for the SSCP run to achieve the most consistent outcome. Complete sequencing of this same DNA fragment from 30 FH patients who showed no SSCP changes did not reveal any additional mutations. We thus conclude that for this 236 bp fragment, single-condition SSCP has detected most of the mutations present in the sample.

In this group of FH patients, 9.3% had a mutation in the 3′ end of exon 4, and this high frequency has also been reported from the Dallas collection of fibroblasts from homozygous FH patients,[11] which represents patients from a wide geographic spectrum. The mutations are concentrated in a region of fewer than 50 bases, and to date, 13 mutations have been identified in this region,[9-11] suggesting that a DNA sequence–specific mechanism may underlie the high frequency of mutations in this region of the gene (discussed in Reference 9). Another possibility for the high frequency is that patients with mutations in this region of the gene are more severely affected[11] and are thus more frequently

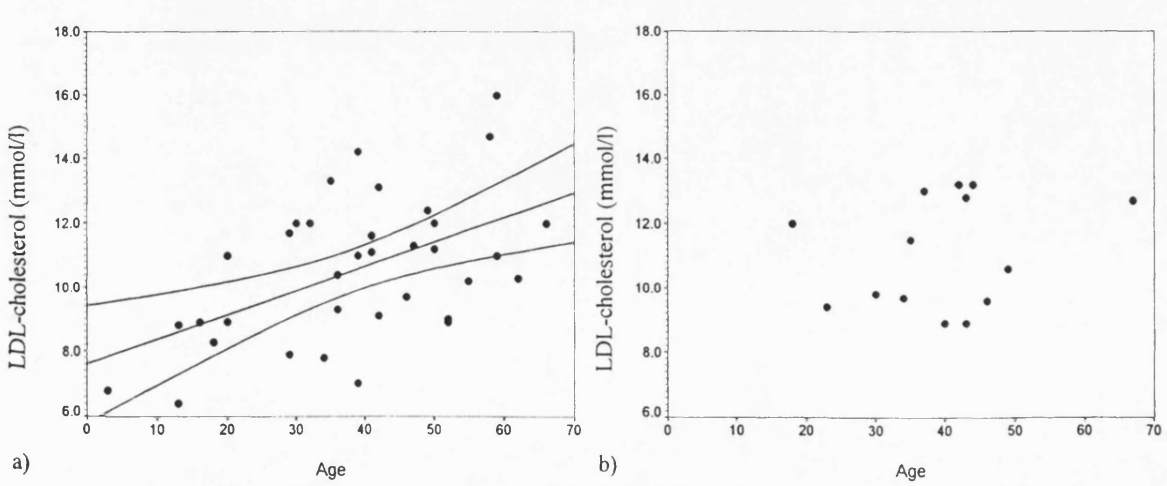

FIG 3. Scatterplots show serum low-density lipoprotein (LDL) cholesterol levels against age in patients with a defective protein (a) ($r=.53$, $P=.001$) and null alleles (b) ($r=.24$, $P=.4$). The 95% confidence interval lines for the regression line are shown in panel a. Interactive outlier analysis showed that the offspring included in panel a do not alter the outcome on either plot.

found in lipid clinics. The data from our study support this idea and show that these patients have significantly higher pretreatment total and LDL cholesterol levels than the rest of the group, particularly compared with patients with missense mutations in other regions of the gene. Of the mutations detected in this study that result in a repeat 5–defective protein, both the del G197 and D200G have been shown to have less than 2% receptor activity[8,11,22] and the D206E a 5% to 15% receptor activity.[23-27] It thus appears that mutations in repeat 5 of the protein may be as deleterious as mutations causing null alleles, at least in regard to plasma lipid levels. However, it is of interest that in the patient group with a defective allele, cholesterol levels show an increase with age of diagnosis, as do the data from the entire patient group (not shown), whereas cholesterol levels are high at all ages in patients with a null allele. The explanation for this observation is unclear but is in agreement with results from other researchers.[4] The increase in plasma cholesterol levels is approximately 0.8 mmol/L per decade in the group with the defective protein compared with approximately 0.5 mmol/L per decade in the general British population.[28,29]

In the general population a clear relation exists between the magnitude of hypercholesterolemia and the prevalence and incidence of coronary artery disease (CAD)[30,31] as well as the onset of symptoms, with an increase of 1.3 mmol/L in plasma cholesterol associated with a CAD onset 10 years earlier. Thus, the 1.88 mmol/L higher total cholesterol in those individuals with missense mutations in repeat 5 and the 1.43 mmol/L higher levels in those with a null allele raise the possibility that these individuals would be more severely affected with CAD compared with the other FH patients. With the clinical information currently available for these patients, a definitive analysis of this could not be carried out at this time. In homozygous FH there is clear evidence that patients with lower levels of receptor activity have higher LDL cholesterol[32] and a more rapid progress of atherosclerosis.[1] Although a consistent correlation has not been demonstrated between serum cholesterol levels and the age of CAD onset in heterozygous FH individuals, there is some evidence to support the relation.[33-36] In a large study of FH patients,[33] men with CAD had significantly higher LDL cholesterol than those free of disease (7.13 versus 6.51 mmol/L), and in a recent study from South Africa,[4] FH patients with CAD had 8% higher cholesterol than those without CAD. A significant positive correlation between intima-media thickening in the femoral artery and total serum cholesterol levels in FH patients has been reported.[37] In a group of French Canadian heterozygous FH patients, individuals with the T66W mutation in exon 3 of the LDL receptor gene had lower plasma cholesterol levels (7.2 mmol/L) than individuals who were heterozygous for the French Canadian 10-kb deletion (8.0 mmol/L).[3] Although there was no difference in the frequency of CAD between the individuals in the families, those who carried the deletion had CAD at an earlier age, supporting the view that the plasma level of cholesterol has an influence on CAD onset.

In the light of the observations in this report, as well as those by other researchers,[2-5] it is now clear that different mutations are associated with differences in lipid levels, and it is likely that this will be associated with clinically different effects. It is also apparent that the phenotypic effect of the mutation is modulated by other genetic or environmental factors.[4,38] Studies that identify the specific mutations in large groups of unrelated FH individuals of heterogeneous background such as have been described here will be important in defining the genotypic background on which the genetic and environmental effects on phenotype can be analyzed.

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Effect of the StuI polymorphism in the LDL receptor gene (Ala 370 to Thr) on lipid levels in healthy individuals

Gudnason V, Patel D, Sun X-M, Humphries S, Soutar AK, Knight BL. Effect of the StuI polymorphism in the LDL receptor gene (Ala 370 to Thr) on lipid levels in healthy individuals.
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We have examined the effect on plasma lipid levels of a single base change in exon 8 of the LDL receptor gene that causes an amino acid change Ala 370 to Thr in a sample of 318 Icelandic individuals selected at random from the general population. The change destroys a *StuI* restriction site and was detected by digestion of pooled samples in groups of 5. The frequency of the loss of the cutting site was 0.05 (95%CI=0.014–0.054). In men, those with the Thr allele (n=18) had 8.3% higher total cholesterol, 11.8% higher LDL cholesterol and 10.3% higher apolipoprotein B than those with the common Ala allele, whereas in women those with the Thr allele (n=12) had levels lower by 7.4%, 13.3% and 10.1% respectively. These differences reached statistical significance only in the men ($p<0.05$). Functional analysis of CHO cells transfected with constructs of the LDL receptor cDNA carrying the Ala370 and Thr370 alleles showed that within the limits of the assays there was no difference in function of the LDL receptor protein as measured by uptake and degradation of LDL. The data raise the possibility that amino acid substitutions that could affect LDL receptor function below the limits of detection by conventional assays, may have an effect on plasma lipid levels in the general population.

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Key words: LDL cholesterol levels – LDL receptor – PCR – RFLP

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High levels of plasma cholesterol are associated with increased risk of coronary artery disease (CAD), particularly when carried in low density lipoprotein (LDL) (Goldstein et al. 1973). Most of the cholesterol in the blood is carried in LDL particles of which the major protein is apolipoprotein B (apoB) (Kane & Havel 1989). Levels of LDL in the blood are determined by the rate of production of LDL, as well as its rate of removal (Sparks & Sparks 1985), and LDL is removed both by non-receptor mediated pathways, and by receptor-mediated endocytosis through the LDL-receptor (Goldstein & Brown 1989). Major defects in this receptor occur in approximately one in 500 individuals in the general population, and lead to familial hypercholesterolaemia (FH), which is associated with grossly elevated levels of plasma cholesterol and premature atherosclerosis. The gene for the LDL-receptor has been fully characterised (Südhof et al. 1985), and over 150 mutations in the LDL-receptor gene have been described to date (reviewed by Hobbs et al. 1992). Different mutations in the LDL receptor gene affect the function of the protein in different ways, and a number of studies have found a correlation between a particular mutation and variation in plasma lipid levels (Leitersdorf et al. 1993, Moorjani et al. 1993, Kotze et al. 1993, Koivisto et al. 1993).

It is quite possible that, in addition to the mutations that cause FH, there are sequence changes in the gene that have only a small effect on the function of the receptor and as a consequence, on LDL cholesterol concentration. If such sequence changes were common, they might make an important contribution in determining lipid levels within the normal population. In support of this hypothesis there is evidence for variation in LDL-receptor activity measured in fibroblasts from different normal individuals (Maartmann-Moe et al. 1981a, Maartmann-Moe et al. 1981b, Magnus et

al. 1981). In addition, associations between plasma cholesterol concentration and a PvuII restriction fragment length polymorphism (RFLP) in intron 15 in the LDL-receptor gene have been shown in a number of studies (Pedersen & Berg 1988, Schuster et al. 1990, Humphries et al. 1991). The PvuII polymorphism has also been shown to mediate some of the effects attributable to the apolipoprotein E (apoE) polymorphism (Pedersen & Berg 1989, Pedersen & Berg 1990). A common RFLP in the LDL-receptor gene of the baboon has also been found to be associated with differences in plasma cholesterol concentration (Hixson et al. 1989). These sequence changes do not alter amino acids and are likely to be markers for another change causing the effect rather than being the cause themselves. To date, only one amino acid substitution in the LDL-receptor has been described that is not associated with FH, an alanine to threonine change in codon 370, caused by substitution of an A by a G in exon 8 (Kotze et al. 1989). This nucleotide change causes a loss of a restriction site for the *Stu*I endonuclease (Kotze et al. 1986), and the frequency of the loss of this restriction site ranges from 0.04–0.08 in the populations studied so far (Taylor et al. 1988, Kotze et al. 1989, Leitersdorf et al. 1989). We have investigated the effect of this polymorphism on plasma lipid levels in healthy Icelandic individuals and have examined the effect of the amino acid substitution on LDL receptor function *in vitro*.

Materials and methods

Subjects

The subjects, all healthy, 152 men and 166 women age 15–78 years, were participants in The Icelandic National Diet Survey 1990, for which they had been randomly selected from the general population. All subjects came from the south-west part of Iceland, and the greatest proportion from the capital, Reykjavik. Those with diabetes, on lipid-lowering drugs or thyroxin were excluded and no biochemical measures were done to further identify the subclinical state of thyroid dysfunction. The subjects were unrelated; in particular, no first-degree relatives are included.

Blood sampling and lipid analyses

Blood samples were collected at The Icelandic Heart Association Research Centre after an overnight fast. Total serum cholesterol (TC) and serum triglyceride were measured by automated enzymatic colorimetry (Cobas Mira, Roche). HDL-cholesterol was measured enzymatically after phosphotungstic/magnesium precipitation of apoB-containing lipoproteins on the day of blood sampling (Lopes-Virella et al. 1977). Both internal and external laboratory controls were used. Apolipoprotein B was measured with an automated turbidimetric method (Roche MA 30: Cobas Mira), and apo(a) by radioimmunoassay (Pharmacia Diagnostics AB, Sweden). All the measurements were carried out at The Icelandic Heart Association Research Centre. LDL cholesterol concentration was calculated as by Friedewald et al. (1972).

DNA analyses

DNA was extracted from 10 ml of EDTA blood (Miller et al. 1988). A 196 bp fragment of exon 8 of the LDL receptor gene was amplified by PCR (Saiki et al. 1988), using 250 ng of each of the two primers P1:

5'AATGTCGACCAAGCCTCTTTCTCTCT-CTTC3'

and P2:

5'AAGTCGACCCACCCGCCGCCTTCCCGT-GC3'

with 50 ng of genomic DNA and 0.02 U of *Taq* polymerase (Bethesda Research Laboratories) in a total volume of 10 µl of buffer recommended by the manufacturer. The reactions were performed on a Hybaid Omnigene Intelligent Heating Block at 95°C for 5 min, 57°C for 3 min and 72°C for 5 min for one cycle, and subsequently 30 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 1 min. After analysis of the PCR product (2 µl of the reaction mix) by agarose gel electrophoresis, samples (1 µl of the reaction mix) were pooled in groups of 5 and digested overnight with 5U of the restriction enzyme *Stu*I (Boehringer Mannheim) in a total volume of 10 µl of buffer recommended by the supplier. The DNA fragments were separated by non-denaturing polyacrylamide gel (7.5%) electrophoresis and stained with silver (Merrill 1990). Where the presence of larger bands suggested the presence of an uncut sample in a pool, 1.5 µl of the individual PCR products were digested with 2U of *Stu*I as described above. ApoE genotyping for these samples has been described elsewhere (Gudnason et al. 1993).

Site-directed mutagenesis of the LDL receptor cDNA and expression in Chinese Hamster Ovary (CHO) cells

The mutation was introduced into pLDLR4, kindly provided by Dr. D. Russell (Dallas, TX), as described by Sun et al. (1994). A 3.2 kb EcoRI-SacI fragment comprising the 3' part of the coding region was cloned into p-SELECT (Promega Ltd., Madison USA). Mutations were introduced into the single strand DNA using Altered sites *in vitro*

mutagenesis kit® (Promega Ltd.) following the supplier's protocol. Mutant plasmids were identified by restriction enzyme analysis of PCR amplified fragments of plasmid DNA from colonies (Newman & VanToai 1990). The mutant inserts were cloned into pLDLR4 as described by Webb et al. (1992). To confirm the loss of the *Stu*I cutting site in an otherwise normal sequence, 200 bases flanking the *Stu*I site were determined.

The mutant and normal LDL receptor cDNAs were cotransfected with pSV2neo into LDL receptor deficient CHO A7 cells (Sege et al. 1986), kindly provided by Dr. M. Krieger (Cambridge, Mass). Stable transfectants were isolated as Geneticin-resistant colonies of transfected cells and LDL receptor expression was analysed as described by Sun et al. (1994). The LDL receptor protein was identified by immunoblotting as described before (Webb et al. 1992). Uptake and degradation of I^{125} labelled LDL by transfected cells was carried out in duplicate as previously described (Knight & Soutar 1982).

Statistical analyses

Statistical analyses were performed using the software package SPSS/PC+(Northwestern University, Chicago, USA). Lipid and lipoprotein concentrations were adjusted by linear regression for age and body mass index in men and women separately. Plasma triglyceride and lipoprotein(a) (Lp(a)) levels were log10 transformed for analyses. Student's *t*-test, analysis of variance (ANOVA), and Kruskall-Wallis one-way ANOVA were used to compare differences between means. Two-way interaction between genotype and sex in the determination of plasma total and LDL cholesterol, and apoB levels was estimated by ANOVA. Allele frequencies were estimated by the gene counting method. χ^2-analysis was used to compare the allele frequencies of the *Stu*I polymorphism and to test for Hardy-Weinberg equilibrium.

Table 1. Unadjusted lipid concentrations and characteristics of the sample of 318 individuals from the general population of Iceland. Mean (±SEM)

	Men	Women	All
n	152	166	318
Age (years)	41.1 (1.2)	42.6 (1.2)	41.7 (1.2)
BMI (kg/m^2)	25.1 (0.3)	24.1 (0.3)	24.6 (0.3)
TC (mmol/l)	5.58 (0.09)	5.51 (0.09)	5.54 (0.09)
TG (mmol/l)	1.15 (0.05)	0.98 (0.04)*	1.06 (0.05)
HDL-C (mmol/l)	1.16 (0.02)	1.44 (0.03)**	1.31 (0.03)
LDL-C (mmol/l)	3.89 (0.08)	3.63 (0.09)*	3.76 (0.09)
ApoB (mg/dl)	120.10 (2.08)	111.82 (2.22)	115.78 (2.15)
Lp(a) (mg/dl)	24.6 (0–92.4)†	26.3 (0–100.6)†	25.5 (0–93.7)†

* $p<0.05$, ** $p<0.01$, † 95% Confidence interval.
BMI=body mass index; TC=total cholesterol; TG=triglycerides; HDL-C=high density lipoprotein cholesterol; LDL-C=low density lipoprotein cholesterol; ApoB=apolipoprotein B; Lp(a)=lipoprotein (a).

Results

Lipid and lipoprotein levels of 318 individuals from the general Icelandic population are shown in Table 1. All of these individuals were genotyped for the *Stu*I polymorphism by restriction enzyme analysis of an amplified fragment of the LDL receptor (Fig. 1). The frequency of the Thr370 allele (Stu-) in the sample was 0.06 (95%CI=0.022–0.097) for men and 0.04 (95%CI=0.010–0.070) for women and was not significantly different between the two groups (Table 2). There were no subjects homozygous for the Thr370 allele in these groups.

Figure 2 shows a scattergram of the LDL cholesterol values according to age for both men and women. Generally, LDL cholesterol concentrations in subjects carrying the rarer Thr370 allele

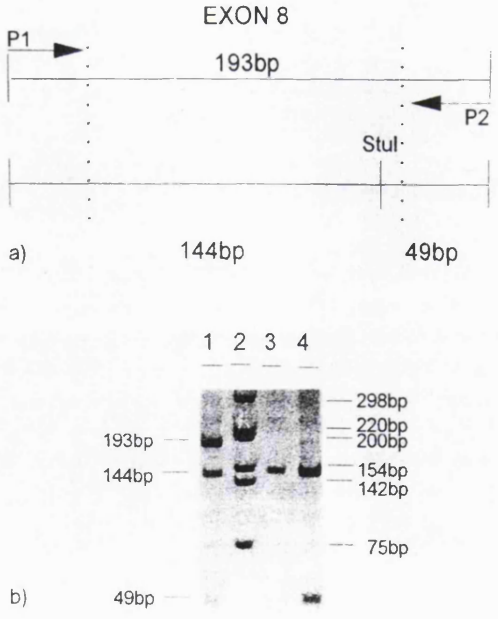

Fig. 1. a) Schematic drawing of the fragment sizes of the cut and uncut PCR of exon 8 of the LDL receptor gene. Primers are denoted by arrows. The intron-exon junction is showed by dotted line. *b)* Silver-stained polyacrylamide gel, showing the pattern of the restriction fragments after digestion with *Stu*I. Lane 1 shows a digest pattern from an individual heterozygous for the *Stu*I cutting site, lane 2 shows the 1 kb ladder markers, and lanes 3 and 4 are from individuals homozygous for the cutting site. The sizes of the digested and undigested fragments are on the left and the sizes of the marker bands are on the right.

Table 2. Adjusted lipid and lipoprotein characteristics of men and women according to their genotype

Genotype		Ala/Ala	Ala/Thr
Men	n	134§	18
	Age (years)	42.4 (15.6)**	32.8 (12.9)
	TC (mmol/l)	5.52 (0.96)*	6.02 (0.83)
	LDL-C (mmol/l)	3.83 (0.91)*	4.34 (0.85)
	ApoB (mg/dl)	118.50 (21.92)*	132.02 (19.41)
	TG (mmol/l)	1.18 (0.54–2.34)	1.20 (0.26–3.16)
	HDL (mmol/l)	1.17 (0.29)	1.13 (0.26)
	Lp(a) (mg/dl)	23.6 (0–93.8)	32.2 (5.6–91.8)¶
Women	n	154	12
	Age (years)	42.1 (14.8)	42.1 (16.8)†
	TC (mmol/l)	5.54 (0.97)	5.13 (0.52)†
	LDL-C (mmol/l)	3.69 (0.94)	3.20 (0.74)†
	ApoB (mg/dl)	112.64 (22.63)	101.26 (19.81)†
	TG (mmol/l)	0.95 (0.46–1.71)†	0.90 (0.5–1.58)
	HDL (mmol/l)	1.43 (0.32)†	1.54 (0.41)†
	Lp(a) (mg/dl)	25.9 (0–99)	30.7 (0–111.8)

All values are mean (±SD) except for Lp(a) which is mean (95% CI).
§ X^2 for frequency of genotypes NS.
* $p<0.05$ between genotypes.
** $p<0.01$ between genotypes.
† $p<0.05$ between men and women.
† $p<0.01$ between men and women.
¶ $p<0.05$ between genotypes by Kruskall-Wallis 1-way ANOVA.
Abbreviations as in Table 1.

were above the regression line in the male population but below the line in the female population (Fig. 2). Scattergrams of total cholesterol and apoB concentrations showed similar effects (data not shown). Although there was a significant correlation between cholesterol and BMI ($r=0.31$ in males and $r=0.25$ in females, $p<0.05$), there was no difference of the effect in the two genotype groups. The plasma lipid and lipoproteins were studied in greater detail after values had been corrected for age and body mass as, shown in Table 2. Men with the Thr370 allele had 8.3% higher mean total cholesterol, 11.8% higher LDL cholesterol, and 10.3% higher apoB concentrations compared with those with the Ala370 allele ($p<0.05$). This difference was still observed when the two individuals with the highest LDL levels were excluded from the analysis ($p<0.05$). In contrast, women carrying the Thr370 allele had lower mean total (7.4%) and LDL cholesterol (13.3%), and 10.1% lower apoB levels than those with the Ala370 allele, although these differences did not achieve statistical significance. Men who carried the Thr370 allele were on average 9.6 years younger, but the reason for this is unclear; no such difference was seen in the women.

All the individuals were also genotyped for the apoE polymorphism. When the analysis was carried out on men with the apoE 3/3 genotype only, those with the genotype Ala/Thr ($n=13$) still had significantly higher levels of TC, LDL-C and apoB compared with those with only the allele for Ala370 ($n=74$), 4.34 mmol/l and 3.83 mmol/l for LDL cholesterol respectively ($p<0.05$). In the women there was no effect on the Ala/Thr of the apo E genotype. In the whole group there was a statistically significant difference between men and women in both unadjusted (Table 1) and adjusted (Table 2) LDL cholesterol values. These sex differences were maintained when men and women carrying the allele for the Thr370 were compared, but were not observed when men and women with the common genotype Ala/Ala were compared (Table 2). A two-way ANOVA between sex and genotype for plasma total- and LDL cholesterol levels and apoB levels shows a highly significant interaction, ($p=0.004$, 0.001, and 0.002 respectively). In both men and women Lp(a) levels were consistently higher in those carrying the allele for Thr370 (Table 2), though this was only statistically significant for men.

To examine whether these differences in plasma lipid levels could be attributed to an effect of the amino acid change on LDL receptor function, a cDNA coding for the Thr370 variant was constructed by site-directed mutagenesis and both the normal and the mutant constructs were expressed

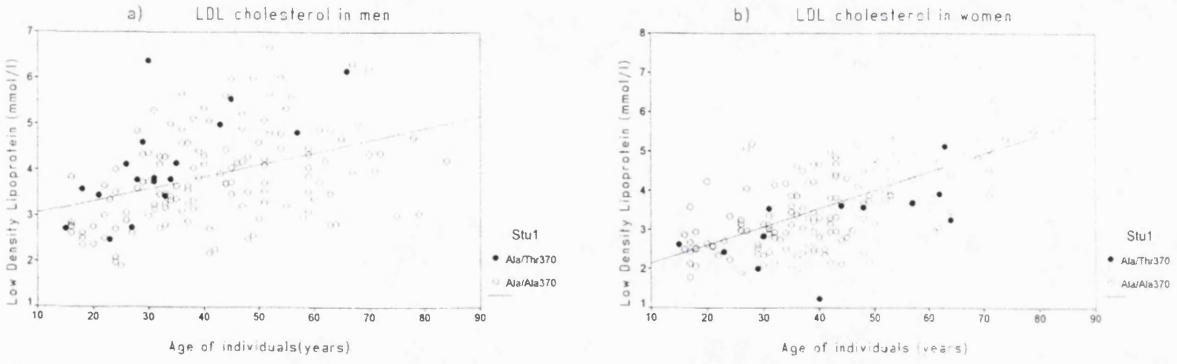

Fig. 2. Scatterplots of the unadjusted LDL cholesterol levels in (*a*) men, and (*b*) women.

as stable transfectants in LDL receptor-deficient CHO cells. Immunoblotting of cell extracts confirmed that the transfected cells expressed LDL receptor proteins and provided no evidence for any atypical accumulation of precursor proteins in the cells carrying the Thr370 allele (data not shown). Uptake and degradation of LDL were measured for two separate cell lines carrying the Thr370 allele and two carrying the Ala370 allele. There were no detectable differences in the affinity for uptake or degradation of ^{125}I labelled LDL between the cell lines carrying the different alleles (Fig. 3). Similar results were obtained from two other separate experiments.

Discussion

The *Stu*I polymorphism is the only reported common polymorphism of the LDL receptor gene that causes an amino acid change, and to date it is also the only described naturally occurring amino acid substitution that does not cause FH. It changes an alanine residue to threonine at position 370 in the receptor protein, which is the amino acid residue next to the last cysteine in repeat B of the EGF precursor homology domain (Südhof et al. 1985). A comparison between species shows that the residue is either an alanine (human and rabbit) or a valine (mouse, hamster and rat) in the species investigated (Südhof et al. 1985, Yamamoto et al. 1986, Hoffer et al. 1993, Bishop 1992, Lee et al. 1989). These amino acids are structurally similar, suggesting that a change to the polar threonine could produce some effect on the function of the protein.

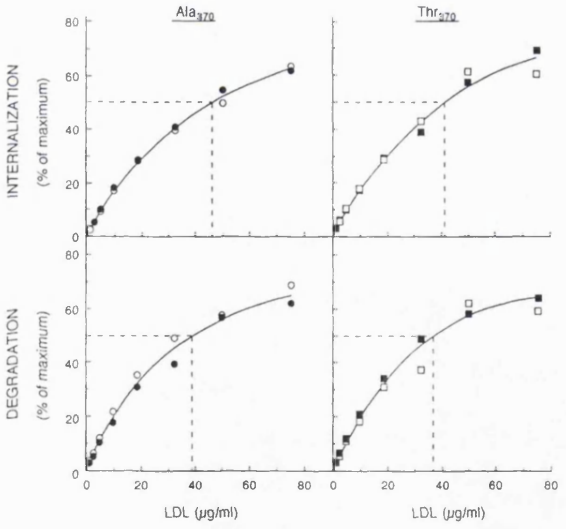

Fig. 3. Internalization and degradation of LDL by CHO cells transfected with human LDL receptor cDNA. Cells were incubated with the indicated concentrations of labelled LDL for 4 h at 37°C and the amount of LDL internalized or degraded was determined. Values were corrected for non-saturable internalization or degradation observed in the presence of an excess of unlabelled LDL (1 mg/ml) and were expressed as percentage of the maximum, calculated with the assumption that the points followed a rectangular hyperbola. Points are the averages of duplicate incubations for two separate cell lines carrying the Ala370 allele (○, ●) and two carrying the Thr370 allele (□, ■). Individual values for maximum internalization and degradations (ng/mg protein) and for concentration giving one-half maximum (μg/ml) were; ○, 767 and 1640 ng/mg protein, 47 and 35 μg/ml; ●, 129 and 265 ng/mg protein, 45 and 44 μg/ml; □, 96 and 208 ng/mg protein, 41 and 42 μg/ml; ■, 101 and 226 ng/mg protein, 42 and 37 μg/ml.

We carried out a study of the impact of the A370T change on lipid traits in a sample of healthy men and women from the general Icelandic population, which is genetically relatively homogeneous. This sample has been used previously to examine the effect of variation at the apoAI and apoE gene loci, and has proved useful in detecting small effects on lipid and lipoprotein levels (Sigurdsson et al. 1992, Gudnason et al. 1993). In this sample the allele for Thr370 was associated with raised levels of total plasma cholesterol, as well as LDL-cholesterol and apoB, although the effect was only seen in men. This holds, even when the analysis is carried out on those individuals with an apoE 3/3 genotype only, indicating that the effect is independent of the apoE genotype. The apoE 2 and apoE 4 genotypes are known to have a small but significant affect on the levels of cholesterol in the general population (reviewed by Davignon et al. 1988). The average male with an allele for Thr370 is in the 75th percentile of the cholesterol distribution according to his age. The impact of this genotype on risk of CAD can be inferred from prospective studies, which indicate that a 1% rise in cholesterol levels above 5.2 mmol/l is associated with a 2% increase in risk of CAD (reviewed by Grundy 1986). Thus compared with those carrying the Ala allele only, men with a Thr allele would have approximately 20% greater risk of CAD.

Several studies have reported that variation detected with the PvuII polymorphism in the LDL receptor gene is associated with differences in plasma lipid levels, with those carrying the less common allele (P+) having about 0.5 mmol/l lower LDL cholesterol concentration (Pedersen & Berg 1988, Schuster et al. 1990, Humphries et al. 1991). This difference is similar in extent to that observed here in men. In a sample from Germany (Schuster et al. 1990), the Thr370 allele occurs most frequently on the P+ allele. However, the effect associated with the A370T change clearly cannot explain the effect associated with the PvuII polymorphism, as they produce changes in different directions. In addition, the P+ allele is much more frequent than the Thr370 allele (0.27 vs 0.05),

respectively. To date, the functional mutation that is responsible for the PvuII effect on plasma lipid levels has not been identified.

None of the studies that have estimated the effect of the PvuII polymorphism on lipid levels have analysed the data from men and women separately, but there are a number of examples known where the effect of genetic polymorphisms on lipid traits differs between men and women. For example, an increase in plasma cholesterol concentration associated with the apoE4 allele was only observed in men (Reilly et al. 1991). Similarly in this sample of Icelandic individuals an increase in HDL cholesterol concentrations associated with an apoAI gene promoter polymorphism (G-75A), was also only seen in men (Sigurdsson et al. 1992). Since in the Icelandic women the Thr allele is not associated with raised levels of cholesterol, it is not inconceivable that the effect of the A370T change of the LDL receptor protein is being influenced by sex-specific factors. One such mechanism could be hormonal differences, as it is known, for example, that oestrogen affects the expression or activity of the LDL receptor in the liver of rats (Windler et al. 1980).

The effect of the *Stu*I polymorphism may be specific to the Icelandic population and needs to be confirmed in a second sample. It could be that this polymorphism is not functional, but is in linkage disequilibrium with another functional variation somewhere else in the gene. To see if the observed difference in plasma lipid and lipoprotein levels was reflected in receptor activity, we constructed expression vectors, carrying the Thr370 or the Ala370 sequence, which were transfected into CHO cells and the cells were tested for the presence of a human receptor and for uptake and degradation of LDL. No difference was seen between the transfected CHO cell lines in the production of the receptor or in its activity. The cell experiments thus do not support the contention that the differences observed in men are related to a large difference in LDL-receptor activity. However, this does not exclude the possibility that there is a functional difference *in vivo*, as some mutations that are known to cause FH result in only a minimal loss of activity when studied *in vitro*. For example, Koivisto et al. (1993) have described a deletion of exon 15 of the LDL receptor gene in patients with FH in Finland, in whom functional activity of the LDL receptor is within the normal range in lymphoblastoid cells. Also, Kigawa et al. (1993), have described a deletion of exons 2 and 3 of the LDL receptor gene in FH patients from Japan, for which there was no detectable abnormality in receptor activity in heterozygous fibroblasts. Thus, studies such as those performed here do not exclude small differences in receptor activity, and it is possible that more accurate assays or assays that measure different aspects of LDL-receptor function *in vitro* will be needed to analyze the effect of some mild mutations in the LDL receptor gene.

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