DETECTION OF A MUTATION IN A HUMAN LCAT GENE

by

ANN ELIZABETH HORNBY

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We accept this thesis as conforming
to the required standard

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October, 1988

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Department of Medical Genetics
The University of British Columbia
Vancouver, Canada

Date October 14, 1988
ABSTRACT

LCAT deficiency is a rare autosomal recessive disease characterized by low levels of plasma HDL and an inability of the enzyme lecithin:cholesterol acyltransferase (LCAT) to esterify cholesterol. An understanding of the structure and function of the LCAT protein will add significantly to the understanding of reverse cholesterol transport. This understanding can be gained, in part, by studying different mutations within the LCAT gene and their resultant phenotypes. Recombinant DNA technology has been used to determine the nature of a mutation in an LCAT gene of a previously described homozygote with this disorder. Southern blot analysis determined there were no major rearrangements in the genomic DNA at the LCAT locus. An attempt was made to follow segregation of the mutant alleles in three generations of a large pedigree by linkage analysis. There are known polymorphisms at the haptoglobin (Hp) locus, which is linked to LCAT on the long arm of chromosome 16, and in the adenosine phosphoribotransferase (APRT) and choesterol ester transfer protein (CETP) loci which are also on the long arm of chromosome 16, but have not been shown linked to LCAT. The information gained was uninformative in this pedigree. An extensive restriction fragment length polymorphism (RFLP) search in the immediate vicinity of the LCAT gene did not reveal any polymorphic sites. 2.4 kb of the λ phage clone SF1020, obtained from one of the homozygotes, containing exons 1-5 plus 0.5 kb of DNA 5' to the LCAT gene, but not exon 6, was subcloned into M13 and sequenced. A cytosine to thymidine (C->T) transition was discovered in exon 4. This would result in a substitution of tryptophan for arginine at amino acid 135. The amino acid arginine is positively charged and resides in one of the most highly charged segments along the amino acid chain of the LCAT protein indicating that this region is likely involved in protein folding. Tryptophan, on the other hand is the most hydrophobic of the amino acids and would, therefore, severely disrupt the interaction of charged amino acids in that region, preventing normal folding of the LCAT protein.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>apo A</td>
<td>apolipoprotein A</td>
</tr>
<tr>
<td>apo B</td>
<td>apolipoprotein B</td>
</tr>
<tr>
<td>apo C</td>
<td>apolipoprotein C</td>
</tr>
<tr>
<td>apo D</td>
<td>apolipoprotein D</td>
</tr>
<tr>
<td>apo E</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>ATP</td>
<td>riboadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CIA</td>
<td>chloroform:isoamyl alcohol 24:1 (v/v)</td>
</tr>
<tr>
<td>CE</td>
<td>cholesterol ester</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyriboadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ddATP</td>
<td>dideoxyriboadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxyribocytidine 5'-triphosphate</td>
</tr>
<tr>
<td>ddCTP</td>
<td>dideoxyribocytidine 5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyriboguanosine;osine 5'-triphosphate</td>
</tr>
<tr>
<td>ddGTP</td>
<td>dideoxyriboguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxyribothymidine 5'-triphosphate</td>
</tr>
<tr>
<td>ddTTP</td>
<td>dideoxyribothymidine 5'-triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid</td>
</tr>
<tr>
<td>Het</td>
<td>heterozygote</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
</tbody>
</table>
Horn homozygote

gm gram(s)

IPTG Isopropyl-β-D-Thiogalactopyranoside

kb kilobase pair(s)

l liter(s)

LDL low density lipoprotein

LB Luria-bertani

μg microgram(s)

μl microliter(s)

mg milligram(s)

ng nanogram(s)

N normal

NH₄OAc ammonium acetate

NaOAc sodium acetate

PEG polyethylene glycol

PL phospholipid

PSB Phage suspension buffer

RT room temperature

SDS sodium dodecyl sulphate

TCA Trichloroacetic acid

TG triglyceride

Tris tris(hydroxymethyl)aminomethane

UV ultraviolet

V volts

VLDL very low density lipoprotein

W watts

X-Gal 5-Bromo-4-Chloro-3-Indoly1-β-D-Galactopyranoside
ACKNOWLEDGEMENTS

Many people have contributed time and knowledge, without which the completion of this project would not have been possible. I would like to thank John McLean for his valuable support throughout, Jiri Frohlich for access to the Fe family, Jeff Hewitt, Rob McMaster and members of his lab, especially Anne Wallis who patiently answered any question, Hugh Brock for direction and Michael Hayden for the project itself. I also wish to express personal gratitude to Alec and Jenny McDougall for all their patience and support.
INTRODUCTION

Coronary heart disease (CHD) is the leading cause of adult death in North America (3). Although factors such as smoking, hypertension and obesity (4) contribute to its progression, the level of cholesterol in plasma is considered to be the major atherogenic factor (5). Studies have shown that the incidence of (CHD) is positively correlated with plasma levels of low density lipoprotein (LDL) cholesterol (6,7) and inversely related to the plasma concentration of high density lipoprotein (HDL) (8).

The identification of the precise genetic and biochemical defect in disorders of lipoprotein metabolism has traditionally been accomplished by investigations of the structural and functional alteration of the proteins involved. The mechanisms which regulate LDL metabolism and cellular cholesterol homeostasis have been well studied. The classical case is the elucidation of the genetic defect in familial hypercholesterolemia (FH) by Brown and Goldstein for which they jointly received the 1986 Nobel prize in Physiology or Medicine (9,10). Briefly, Brown and Goldstein showed that in normal individuals LDL is cleared from plasma by cells via a specific plasma membrane receptor, and that this receptor is defective in patients with FH. LDL accumulates in the plasma of these patients causing CHD at an early age. This work has greatly increased our knowledge of the role of the low density lipoprotein in atherogenesis.

Although the exact mechanism of the protective effects of the high density lipoprotein is not yet understood, it is generally accepted that these effects are related to the ability of HDL to remove cholesterol from peripheral tissues (11). This central role that HDL plays in reverse cholesterol transport involves the esterification of cholesterol by lecithin:cholesterol acyltransferase (LCAT), and its transfer from HDL to lower density lipoproteins by cholesterol ester transfer protein (CETP). The role of LCAT in reverse cholesterol transport will be dealt with in greater detail on page 11.
Genetic diseases which present with a familial form of HDL deficiency provide the necessary tools for studying HDL metabolism. Some of the diseases currently being investigated are (12):

1. Familial hypoalphalipoproteinemia. The mode of inheritance of this disorder is autosomal dominant. The level of HDL cholesterol in people with this disease is about 50% of normal and they are prone to premature coronary artery disease. The molecular defect causing familial hypoalphalipoproteinemia is unknown (13).

2. Fish-eye disease. Fish-eye disease is inherited as an autosomal recessive disorder in which HDL cholesterol values are less than 10% of normal. Here too the molecular defect remains unknown (14).

3. Apo A-I variant states. Several mutations in the apo A-I gene have been reported resulting in low HDL cholesterol which segregate in an autosomal codominant fashion (13).

4. Tangier disease. Tangier disease is caused by a rare autosomal recessive disorder characterized by a virtual absence of HDL cholesterol and an accumulation of cholesterol esters in many peripheral tissues (13).

5. Familial apo A-I/C-III deficiency. This is an autosomal recessive disease characterized by marked HDL cholesterol deficiency and undetectable levels of plasma apo A-I and apo C-III. The defect appears to be an inability to synthesize apo A-I and apo C-III (13).

6. Familial LCAT deficiency. Familial LCAT deficiency, the topic of this thesis, is a rare autosomal recessive disease characterized by decreased and abnormal HDL cholesterol due to an inability of the enzyme LCAT to esterify cholesterol.
BACKGROUND

(a) Role of cholesterol in CHD

Cholesterol, the most important sterol found in human tissues, is found in all mammalian cells primarily in the plasma membrane where it controls viscosity and permeability (15). The implication of cholesterol in atherogenesis comes from epidemiological studies observing the pathological deposition of cholesterol and cholesterol esters in the vascular bed (15).

Cholesterol is formed from acetyl-CoA by almost all tissues. It is the precursor of all the steroid hormones, bile salts, and vitamin D₃. It is a component of nearly all plasma membranes and some subcellular organelle membranes (16) where it intercalates among the phospholipids with its hydroxyl group at the aqueous surface. At temperatures above the transition temperature, its rigid sterol ring interacts with acyl chains of the phospholipids, limiting their movement and, therefore, decreasing membrane fluidity. At lower temperatures the interaction with the acyl chains interferes with their alignment, thus keeping the membrane more fluid (16). The fluidity of a membrane is an important regulator of its function. As membrane fluidity increases so does its permeability to water and small hydrophobic molecules. The rate of lateral movement of integral proteins also increases which can alter the transport rate. An example is the insulin receptor which binds more insulin under these conditions (16).

Virtually all tissues containing nucleated cells are capable of synthesizing cholesterol from 2 carbon (acetate) units through a complex pathway involving more than 30 enzyme catalyzed reactions (15). Approximately 50% (500mg/d) is synthesized by the body, the rest coming from dietary sources (17).

Cholesterol balance within the cell is tightly regulated. The following processes are thought to maintain this balance:

1. Uptake of lipoproteins via receptor pathways, i.e. the LDL receptor.
2. Uptake of cholesterol from cholesterol-rich lipoproteins by lipid transfer.
3. Intracellular cholesterol synthesis.

4. Efflux of cholesterol to HDL promoted by LCAT.

5. Esterification of cholesterol by ACAT (18)

6. Utilization of cholesterol for synthesis of other steroids such as hormones or bile acids in the liver (15,17).

The problem arises of how to transport lipids absorbed from the diet and synthesized by the liver between the various tissues and organs such as the adrenal glands and ovaries which require cholesterol for the synthesis of hormones and back to the liver for catabolism through the aqueous plasma environment. This is solved by the lipoprotein.

(b) Lipoproteins

Lipoproteins are complexes of both lipid and protein which exist to provide a transport system for lipids in the aqueous environment of the plasma. Composed of a nonpolar lipid core, primarily triacylglycerol and cholesteryl ester, lipoproteins are surrounded by more polar phospholipids and the apoproteins which provide both structural and functional properties.

Lipoproteins are generally classified on the basis of their flotation density which is a function of their lipid/protein ratio, although their functional properties are determined more by protein composition than lipid composition (15). The many components of lipoproteins are not in equilibrium, but exist in a non-steady state (15).
Figure 1: Lipid Transport. (Reproduced from reference 17)
Table I: Human Plasma Lipoproteins Classified by Density (Reproduced from reference 15)

<table>
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<tr>
<th>Density range (g/mL)</th>
<th>Lipoprotein fraction</th>
<th>Synonyms</th>
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<tr>
<td>d &lt; 1.006</td>
<td>Very low density lipoprotein</td>
<td>VLDL</td>
</tr>
<tr>
<td>1.006 &lt; d &lt; 1.019</td>
<td>Intermediate density lipoprotein</td>
<td></td>
</tr>
<tr>
<td>1.019 &lt; d &lt; 1.063</td>
<td>Low-density lipoprotein</td>
<td>LDL, β-lipoprotein</td>
</tr>
<tr>
<td>1.063 &lt; d &lt; 1.2</td>
<td>High density lipoprotein</td>
<td>HDL, α-lipoprotein</td>
</tr>
<tr>
<td>1.063 &lt; d &lt; 1.12</td>
<td>High density lipoprotein</td>
<td>HDL₂</td>
</tr>
<tr>
<td>1.12 &lt; d &lt; 1.21</td>
<td>High density lipoprotein</td>
<td>HDL₃</td>
</tr>
<tr>
<td>1.21 &lt; d &lt; 1.25</td>
<td>Very high density lipoprotein</td>
<td>VHDL</td>
</tr>
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Table II. Composition of the Major Human Plasma Lipoprotein Classes (reproduced from reference 23)

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Isolation method</th>
<th>Protein (w%)</th>
<th>Free cholesterol (w%)</th>
<th>Phospholipid (w%)</th>
<th>Cholesteryl ester (w%)</th>
<th>Triacylglycerol (w%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VLDL*</td>
<td>Affinity chromatography</td>
<td>10.4</td>
<td>5.8</td>
<td>15.2</td>
<td>13.9</td>
<td>53.4</td>
</tr>
<tr>
<td>IDL‡</td>
<td>Density gradient centrifugation</td>
<td>17.8</td>
<td>6.5</td>
<td>21.7</td>
<td>22.5</td>
<td>31.4</td>
</tr>
<tr>
<td>LDL*</td>
<td>Affinity chromatography</td>
<td>25.0</td>
<td>8.6</td>
<td>20.9</td>
<td>41.9</td>
<td>3.5</td>
</tr>
<tr>
<td>HDL₂‡</td>
<td>Rate zonal centrifugation</td>
<td>42.6</td>
<td>5.2</td>
<td>30.1</td>
<td>20.3</td>
<td>2.2</td>
</tr>
<tr>
<td>HDL₃‡</td>
<td>Rate zonal centrifugation</td>
<td>54.9</td>
<td>2.6</td>
<td>25.0</td>
<td>16.1</td>
<td>1.4</td>
</tr>
<tr>
<td>VHDL§</td>
<td>Preparative ultracentrifugation</td>
<td>62.4</td>
<td>0.3</td>
<td>28.0</td>
<td>3.2</td>
<td>4.6</td>
</tr>
</tbody>
</table>
(c) Apolipoproteins

Apolipoproteins are amphipathic proteins which allows them to interact with both the aqueous environment of the plasma and the lipid environment of the lipoprotein particle. Their distribution is reviewed by Morrison et al. (19) and Smith et al. (20).

Table III: The Major Apolipoproteins of the Human Plasma Lipoproteins

(15)

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Molecular weight</th>
<th>Lipoprotein distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo A-I</td>
<td>28,331</td>
<td>HDL</td>
</tr>
<tr>
<td>Apo A-2</td>
<td>17,380</td>
<td>HDL</td>
</tr>
<tr>
<td>Apo B-48</td>
<td>200,000</td>
<td>Chylomicrons</td>
</tr>
<tr>
<td>Apo B-100</td>
<td>350,000</td>
<td>VLDL, LDL</td>
</tr>
<tr>
<td>Apo C-1</td>
<td>7,000</td>
<td>HDL, VLDL</td>
</tr>
<tr>
<td>Apo C-2</td>
<td>8,837</td>
<td>Chylomicrons, VLDL, HDL</td>
</tr>
<tr>
<td>Apo C-3</td>
<td>8,751</td>
<td>Chylomicrons, VLDL, HDL</td>
</tr>
<tr>
<td>Apo D</td>
<td>32,500</td>
<td>HDL</td>
</tr>
<tr>
<td>Apo E</td>
<td>34,145</td>
<td>Chylomicrons, VLDL, HDL</td>
</tr>
</tbody>
</table>
The function of only a few of the apolipoproteins have been clearly identified:

1. Apolipoproteins which provide structure for the lipoprotein particle - apo B-48 in chylomicrons, apo B-100 in VLDL and Apo A-I in HDL.

2. Lipid transfer activity - apo D in HDL.

3. Ligands for interaction with cellular receptors - apo B-100 for the LDL receptor, apo E for the remnant receptor and possibly apo A-I for an HDL receptor.

4. Cofactors for enzymes - apo C-II for lipoprotein lipase, and apo A-I for LCAT.

(d) Chylomicrons and VLDL

The primary function of chylomicrons and very low density lipoproteins (VLDL) is the transport of triglycerides to extrahepatic tissues for utilization or storage. The particles known as chylomicrons are produced by the intestinal epithelium. Dietary cholesterol is adsorbed by the intestinal epithelium, reesterified by ACAT, then secreted into the intestinal lymph in a complex with apo B-48, phospholipids and small amounts of free cholesterol and enters the blood circulation via the thoracic duct (17). Secretion of chylomicrons then, is a function of the rate of fat absorption (21).

Clearance of chylomicrons is rapid - less than one hour in humans. The action of lipoprotein lipase, which is present on the walls of capillaries, results in the loss of about 90% of the triacylglycerol and of apo C. This in turn results results in chylomicron remnants which are taken up by the liver via the apo E receptor and further catabolized (17).

The liver is the major site for secretion of lipoproteins containing endogenously derived lipids. Triglyceride (TG) is secreted by the liver into the circulation as VLDL particles which contain apo B-100, apo E and apo C (22). Further apoproteins are added after secretion (15).
(e) **LDL and HDL**

The primary role of low density lipoproteins (LDL) and high density lipoproteins (HDL) is to transport cholesterol through the aqueous environment of plasma. Plasma LDL which is primarily produced by the action of lipoprotein lipase and hepatic triglyceride lipase on VLDL particles (23) resulting in the loss of TG, phospholipid (PL) and apo E (24) transports cholesterol to extrahepatic tissues, whereas HDL transports cholesterol from peripheral tissues back to the liver for catabolism, ie. 'reverse cholesterol transport'.

Plasma HDL are a heterogeneous group of particles 7-10 nm in diameter with a molecular weight of 200-400,000 daltons (25). They are composed (weight percent) of 50% protein (mainly apo A-I and apo A-II) and 50% lipid (mainly phospholipid, free and esterified cholesterol and a small amount of triacylglycerol) (26).

It was initially thought that the liver was the major source of HDL precursors (11). The "nascent" HDL structures identified in liver perfusates and intestinal lymph, were thought to be the major HDL precursors (27). However, the experiments upon which this hypothesis is based have been criticized due to the inability to identify such structures along secretory pathways. This, plus the fact that lipoprotein lipase is highly active under the conditions of the perfusion experiments and analysis of the discoidal particles themselves from liver and intestine has lead to support for the hypothesis that the major source of "nascent" HDL particles are the lipolytic products of VLDL or chylomicrons (27). Nascent HDL are discoidal phospholipid bilayers composed of polar phospholipids, unesterified cholesterol, apo A-I and the enzyme LCAT.

HDL is not catabolized as a unit, but individual constituents have differing catabolic rates. Lipid and apoprotein exchanges between HDL species and other lipoproteins make it difficult to determine the catabolic fate of HDL. Studies in humans indicate that apo A-I is catabolized faster than apo A-II (28) and animal studies have shown that cholesterol components of HDL are catabolized faster than the protein
components (29) and particles containing only apo A-I may be catabolized more rapidly than particles containing both apo A-I and apo A-II (30), although differential catabolism of HDL constituents varies tissue by tissue. The major site of terminal cholesterol-ester is the liver whereas kidney uptake of apo A-I exceeds that of cholesterol-ester (31).

**LCAT**

(a) **LCAT as Part of Cholesterol Metabolism**

Lecithin:cholesterol acyltransferase is the plasma enzyme responsible for the esterification of cholesterol. Surface phospholipid and free cholesterol are converted into cholesteryl esters and lyssolecithin in the reaction shown in figure 3.

65 - 75% of plasma cholesterol is present in esterified form (32). The esterification of cholesterol plays an important role in the transport of cholesterol by maintaining a concentration gradient. Whereas, free cholesterol is mildly soluble in aqueous environments and can, therefore, move down a concentration gradient between lipoproteins and membranes or between different lipoproteins, it requires the action of an enzyme and, therefore energy to esterify cholesterol, which in this form is insoluble in an aqueous environment, and transfer it from membranes to lipoproteins or between lipoproteins. Thus, when LCAT is not present or is defective, the whole of lipid metabolism is severely disrupted.

The nature of the LCAT reaction was determined in the 1960's by Glomset et al. (33) as seen in figure 2.
Figure 2: Reaction catalyzed by LCAT. (Reproduced from reference 33)
Maturation of HDL precursors to spherical HDL is dependent on the action of the plasma enzyme, lecithin:cholesterol acyltransferase (LCAT) (34). This progresses as a result of the net transport of cholesterol from nonhepatic cells into the circulation which is maintained by the esterification of cholesterol by the enzyme LCAT (35). LCAT is present on only a small pool of HDL, the discoidal HDL\textsubscript{3} which contains, as well, apo A-I and apo D. The nascent HDL molecule contains relatively more lecithin and free cholesterol, but less cholesterol esters than the mature spherical HDL\textsubscript{2} (34) which contain in their core a large number of cholesteryl esters.

The nonpolar cholesteryl esters move into the hydrophobic core of HDL and lysolecithin is transferred to plasma albumin. This reaction continues until a spherical HDL is formed in which a nonpolar core is covered by a surface film of polar lipids and apoprotein. The esterified cholesterol can be transferred from HDL to the lower density lipoproteins (chylomicrons, VLDL and LDL) by means of the CETP. Cholesteryl ester can then be transported to the liver via the remnants of chylomicrons and VLDL or the hepatic uptake of LDL for catabolism (17), thus maintaining the gradient of cholesterol efflux set up by the LCAT reaction.

(b) LCAT, the Enzyme

The enzyme LCAT was first discovered by Sperry in 1935 (36).

LCAT is the major, or only, factor catalyzing sterol ester synthesis in the plasma. Its action is dependent on the cofactor apo A-I and it is inhibited by apo A-II (37).

Although much less common, sterols other than cholesterol can serve as the acyl acceptor. Cholestanol, desmosterol and \(\beta\)-sitosterol are esterified in human plasma (38).

Only a small subfraction of HDL molecules are a substrate for LCAT, a population that contains LCAT, apo A-I and apo D. It has been shown that at 37\textdegree C in plasma the prebeta HDL are transformed into lipoproteins of alpha mobility within two hours (34). This is completely inhibited by 1.4 mM 5,5'-dithiobis-(2-nitrobenzoic acid)
and by 20 mM menthol, both inhibitors of LCAT. It has also been demonstrated that apo A-I is required for LCAT activity (33), bulk HDL are a poor substrate for LCAT (40) and that a complex containing apoprotein A-I, LCAT, and apo D is found in plasma in the form in which most or all of the enzyme is present (41).

Frohlich et al. have proposed a mechanism for the formation of HDL$_2$ and HDL$_3$ indicating the involvement of LCAT in both processes as is shown in figure 4.

**Figure 3:** Formation of HDL$_2$ and HDL$_3$. (Reproduced from reference 32).
LCAT has three known catalytic activities:

(1) The main function of the enzyme LCAT is the hydrolysis of the acyl group esterified at position 2 of lecithin and its transfer to the 3-hydroxyl group of cholesterol resulting in the formation of cholesteryl ester and lysolecithin (42). This acyl transferase activity is responsible for the formation of essentially all CE found in plasma (36);

(2) A phospholipase activity stimulated by apo A-I which, in the absence of an acyl acceptor, hydrolyzes the two-position fatty acid of lecithin to lysolecithin plus free fatty acid for which serum albumin is an acceptor, although this is probably not physiologically important (43,44).

(3) A lysolecithin acyltransferase (LAT) activity which is stimulated by LDL and does not require apo A-I (45).

Apo A-I, the major apoprotein in HDL is the principle physiologic activator of LCAT. Several investigators have reported in vitro activation of LCAT by apoproteins C-I, A-IV and E (46-50), although the in vivo significance of these findings remains unconfirmed. Diseases such as Tangier disease, apo A-I/C-III deficiency and Fish-Eye disease represent conditions where LCAT may be activated by proteins other than apo A-I, and may, therefore be important in determining the significance of other potential activators (32) leading to a greater understanding of the regulation of LCAT activity.

LCAT is a glycoprotein produced in the liver and secreted into the circulation (51,33). The mature protein is secreted as a single polypeptide chain containing 416 amino acids and having an apparent MW ~63,000 (63), 24% of which is carbohydrate (51,33). It has a very hydrophobic N-terminal which contains a leader sequence of 24 amino acids (52) and contains a relatively high content of the amino acids aspartic acid, glutamic acid, proline, glycine, valine and leucine (42). Biochemical studies indicate that the active site may contain a cysteine residue as activity can be blocked with reagents such as 5,5-dithio-nitrobenzioc acid (DTNB) or iodoacetic acid (IAA) (53). There are a
number of extended sequences of hydrophobic amino acids, one of which contains a hexapeptide identical to the interfacial binding site segment of the active site of porcine pancreatic lipase and is similar to the active site of rat lingual lipase (52).

(c) LCAT, the gene

The gene for LCAT has been localized to chromosome 16q21-22.1 by in situ hybridization to metaphase chromosomes, southern blot analysis of somatic cell hybrids and linkage analysis (54). McLean et al (52) have shown that the liver is the only site of mRNA synthesis, producing an mRNA product of about 1550 nucleotides. The normal LCAT gene was cloned and sequenced by McLean et al. to provide a basis for comparison of the structure of the LCAT gene with the LCAT gene of patients with LCAT deficiency (52,55). N-terminal sequence analysis was performed on LCAT protein purified from normal human plasma. Based on the amino acid sequence, oligonucleotide probes ranging from 33-60 bases were prepared and used to screen human adult liver cDNA libraries constructed in λgt 10 (56)(57). Then a human genomic library in λ Charon 30 (58) was screened with restriction fragments of cDNA clones to recover a complete genomic LCAT gene (63). The LCAT gene contains 6 exons and 5 introns covering about 4.2 kb of the genome. The 3' untranslated region is very short, consisting of only 23 bases. The signal prepeptide and first 27 amino acids are encoded in the first exon. Any functions for exons two, three and four remain unknown. The hydrophobic region homologous to the lipid interfacial binding site of porcine pancreatic lipase is encoded in exon 5 (55) and the first 59 nucleotides of exon 5 share a 66% DNA sequence identity with the 3' terminal coding region of the apo E gene (55). In fact, it is thought that exon 5 may encode a protein domain which interacts with the lipid interface containing the substrates of the LCAT reaction. The largest exon is exon 6 which contains nearly half the protein. The 3' end of the gene is
unusual in that the poly (A) signal AATAAA overlaps the COOH-terminal glutamic acid and the untranslated region is only 23 bases (55).

Familial LCAT Deficiency

(a) Genetics and Linkage

Familial LCAT deficiency is a rare autosomal recessive disease. To date there are less than 40 known cases in the world (59).

Genetic linkage was first established in 1936 by Bell and Haldane (60) when they linked the genes for colour blindness and haemophilia on the X chromosome. At present, close to 3500 genes and markers have been localized on human chromosomes (54).

Linkage analysis can be used in two ways. A gene can be localized to a particular region on a chromosome by establishing that it segregates with a marker whose position is known as was the case with Huntington's disease (61). Linkage can also be used to exclude candidate genes by showing that the disease segregates independently from a known marker. LCAT deficiency has been shown linked to the haptoglobin locus in some families (62).

There are two types of polymorphisms which can be detected with radioactively labelled fragments of DNA. One is the presence or absence of restriction enzyme sites giving rise to DNA fragments of different lengths called restriction fragment length polymorphisms (RFLP's). The other type of polymorphism does not depend on the presence or absence of a cleavage site, but on a difference in length of the same fragment of DNA. These segments consist of a series of short sequences which can vary in number of repeats from one individual to another. These are known as VNTR's or variable number of tandem repeats.
The establishment of linkage localizes genes and markers on a genetic map with 1% recombination equal to one map unit or centimorgan. A centimorgan corresponds roughly to one million base pairs (54).

(b) LCAT deficient patients

The importance of the LCAT reaction in lipoprotein metabolism and its involvement in the maintenance of the equilibrium between tissue and plasma cholesterol, is demonstrated by the fact that virtually all lipoproteins are abnormal in people with LCAT deficiency (63,64,85)

Much of the knowledge gained so far on the mechanism of sterol efflux has come from the study of LCAT deficient patients (43) and in vitro studies where the activity of LCAT is inhibited (65). The in vitro studies have shown cholesterol efflux is unaffected when LCAT in plasma is inhibited, however, there is no net transport of sterol; ie., efflux is balanced by an equivalent rate of influx (65). In vitro replacement of LCAT in plasma samples from LCAT deficient patients has resulted in normalization of some parameters (66,67). A proposed mechanism for the formation of HDL\textsubscript{2} and HDL\textsubscript{3}, figure 2, demonstrates the important role LCAT plays in the 'reverse' transport of cholesterol from peripheral tissues to the liver.

In LCAT deficient patients small HDL particles resembling nascent HDL accumulate in the plasma along with abnormally large VLDL and LDL particles containing little or no CE (43). Eighty to 100% of the plasma cholesterol is unesterified and plasma phosphatidylcholine (PC) is increased. The small amount of cholesteryl esters present in the plasma are believed to be of intestinal acyl:cholesterol acyltransferase (ACAT) origin (32). The handling of TG's in LCAT deficient individuals is severely impaired and LDL levels are very low, probably because of the lack of esterifying activity which allows the exchange of TG for CE in the particle core (67). HDL does not migrate in the usual electrophoretic position so that the alpha band
is practically absent (32). Instead, when seen with the electron microscope, the HDL particles are discoidal and vesicular structures which look like HDL precursors (11,68,69), and there is an absence of disc to sphere transformation which can be induced with the addition of LCAT enzyme to the patients plasma (71-75).

The changes in lipoprotein structure and composition seen in LCAT deficiency states are consistent with the concepts presented in the model seen in figure 2 (63,76). The virtual absence of CE in the plasma of LCAT deficient patients leads to abnormally large VLDL and LDL particles and to TG enrichment in all classes of lipoproteins (63).

The HDL particles that are present in electron microscopy are small spherical species that resemble the "stacked discs" of nascent HDL (32). In vitro incubation with purified LCAT (77) results in an increase in the size of the HDL particles. The authors of these studies have suggested that LCAT induces particle rearrangement to provide sufficient surface components for the expanding core, and that the alterations of lipoproteins induced by the action of LCAT may be necessary to maintain reverse cholesterol transport from peripheral cells (33,51).

The genetic background of the patients diagnosed to date is diverse and the wide range of enzyme mass and activity values found in the different families indicate that several different mutations probably have occurred which cause LCAT deficiency. 46% (22) are of Scandinavian, Irish or English origin, 28% (15) are Asian, 15% (9) have at least one ancestor of Italian or French origin and three families have at least one ancestor from either Dutch or Swiss Mennonite background indicating that the gene for LCAT deficiency may be more frequent in that population (59).

Alber's has classified families with LCAT deficiency into three types(78):

(1) apparent absence of both LCAT activity and mass (the family in this study fits into this category) (79,92);

(2) functionally defective LCAT in which there is some protein present in the plasma, but little or no activity (73);
(3) a small amount of apparently normal LCAT is present in the plasma (80).

Table IV: Range of LCAT Mass and Activity Values Reported in the Homozygotes and Heterozygotes for Familial LCAT Deficiency and in Normal Controls (59).

<table>
<thead>
<tr>
<th></th>
<th>LCAT MASS (mg/l)</th>
<th>ASA (mol/min/l)</th>
<th>SN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygotes</td>
<td>0 - 2.65</td>
<td>0 - 9.1</td>
<td>0 - 12</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>2.6 - 4.8</td>
<td>42 - 64</td>
<td>34 - 67</td>
</tr>
<tr>
<td>Controls</td>
<td>4.3 - 7.8</td>
<td>80 - 163</td>
<td>40 - 93</td>
</tr>
</tbody>
</table>

ASA: artificial substrate assays
SN: Stokke-Norum assay

Patients with LCAT deficiency present with a number of clinical abnormalities (81,82):

(1) Corneal opacities are the earliest sign of LCAT deficiency and are present in all patients from early childhood (83);

(2) hemolytic anemia is present in the majority of patients. The erythrocytes show abnormalities in appearance and lipid composition (84) and 'target cells' are found in dry smears of both peripheral blood and bone marrow. Measurements of erythrocyte membrane preparations of some patients show increased amounts of unesterified cholesterol and phosphatidylcholine (83). This causes the membranes to be more rigid and fragile making the red blood cells (RBC's) less able to deform when passing through small capillaries as normal red blood cells do. The
RBC membranes therefore burst, releasing amongst other things, the oxygen carrying heme (32).

(3) Kidney abnormalities are also found in the majority of patients. Almost all patients with familial LCAT deficiency have proteinuria detectable at an early age. This usually does not increase until the fourth or fifth decade of life as renal function deteriorates. With the patient SF in this study, renal function became severely impaired during the second decade of life necessitating kidney dialysis, whereas the sibling DH still has minimal proteinuria and normal kidney function (93). Dietary differences between the affected siblings may be the cause of the increased rate of deterioration of renal function. SF, who is now in kidney failure consumes a high protein, high cholesterol diet, whereas DH, who has mild proteinuria consumes a low protein, low fat diet (93).

Foam cells have been found in the glomerular tufts of all renal biopsies examined with the light microscope so far. Lipid analysis of isolated glomeruli have shown higher than usual amounts of unesterified cholesterol and phospholipid (83). Electron microscopic studies have shown capillary lumens that are partly filled with a meshwork of membranes and particles with an amorphous mottled structure. The capillary walls appear to be abnormal - endothelial cells are frequently absent, the basal lamina is of irregular thickness, and endothelial foot processes are fused (86).

The presence of large low density lipoproteins (LDL) in the plasma of LCAT deficient patients has been proposed as a possible cause of renal injury, however it remains unknown whether kidney lipid deposits are causally related to renal failure since an Irish patient without proteinuria was found to have glomerular lipid deposits (87).

(4) Atherosclerosis: Although no early atherosclerosis has been reported in the Fe family, many patients with familial LCAT deficiency have developed early
atherosclerosis. Post mortem examinations have revealed atherosclerosis of the aorta and large arteries even before the age of 40 (83).

(c) Assessment of LCAT activity and mass

All functional assays of LCAT activity measure the increase in CE or the decrease in unesterified cholesterol (88). Early methods used plasma as the enzyme source. The problem with these assays is that they measure and LCAT activity which is dependent on the composition and physical state of the plasma (32). Originally, Glomset and Wright (89) utilized pooled human plasma as the substrate for LCAT. This required long incubation times to measure esterification and the assay was not reproducible over extended periods. Stokke and Norum (53) developed a method which uses the patient's own plasma lipoproteins as substrates for the enzyme, i.e. an endogenous substrate. This method then, measures activity that is a function of both enzyme quantity and substrate availability, but it is also time consuming.

Recently, methods using artificial substrates containing apo A-I and lipids have become the preferred approach for assessing LCAT activity (38,90). These assays are more sensitive, rapid, and reproducible, and measure LCAT activity independent of the subject's lipoprotein status. Activity measured by these methods has been shown to correlate well with LCAT mass measurements (58). LCAT mass can be detected by a sensitive immunoassay using purified antibody against LCAT (91,92). The normal range of LCAT activity is 50-100 nmol/hr/ml of plasma and LCAT mass is 4.5-8.0 mg/L of plasma (32).

Although it is known that there is a mutation in the LCAT gene of some LCAT deficient patients (J. McLean, personal communication) resulting in a lack of CE, in others it is not known whether these changes in lipoprotein composition result from the lack of CE for transfer or from and abnormality of LPL or its substrate.
(d) Fe Family

A Canadian family of Dutch Mennonite–Italian background (figure 6) has been extensively biochemically studied by Frohlich et al. (92–94). It has been demonstrated that both of the homozygotes in this family, III-6 (SF) and III-7 (DH) in figure 6 have a virtual absence of LCAT protein mass or enzyme activity. The heterozygotes in this family have approximately one half normal levels of LCAT mass and activity and those that are unaffected fall within the normal range (92,94).
Figure 4: Family Tree (Fe). (Courtesy of J. Frohlich)

Figure 4 shows the pedigree of the family Fe. Solid symbols are homozygotes, half-solid symbols are heterozygotes and open symbols are unaffected or untested family members.
It is possible to determine the status - homozygous, heterozygous, or unaffected - in the Fe family by either of two methods. Figure 5 (92) shows the mass of LCAT protein in the plasma of several members of the Fe family by reaction with LCAT antibody. The status of each individual is determined by the height of the 'rocket'. Figure 6 presents the differences detected in LCAT activity (94).
Figure 5: Electroimmunodiffusion of LCAT mass of several members of the Fe family.

(Reproduced from reference 92)

Figure 5 demonstrates the detectable LCAT protein mass in homozygotes, heterozygotes and unaffected individuals in the Fe family.
Table V: Status of Fe family members based on LCAT activity.

(Reproduced from reference 94)

<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>LCAT Activity (nmol/h/mL)</th>
<th>Cholesterol Esterrification Rate (nmol/h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unaffected</td>
<td>II-3</td>
<td>28.0</td>
<td>66.0</td>
</tr>
<tr>
<td></td>
<td>II-8</td>
<td>28.4</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>III-9</td>
<td>30.2</td>
<td>95.2</td>
</tr>
<tr>
<td></td>
<td>III-14</td>
<td>28.2</td>
<td>57.7</td>
</tr>
<tr>
<td></td>
<td>III-18</td>
<td>21.4</td>
<td>65.0</td>
</tr>
<tr>
<td></td>
<td>III-19</td>
<td>26.6</td>
<td>77.5</td>
</tr>
<tr>
<td></td>
<td>III-21</td>
<td>28.3</td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>III-25</td>
<td>32.1</td>
<td>54.7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>27.9 ± 3.1</td>
<td>62.6 ± 17.9</td>
</tr>
<tr>
<td>Heterozygotes</td>
<td>I-2</td>
<td>11.4</td>
<td>65.0</td>
</tr>
<tr>
<td></td>
<td>II-4</td>
<td>16.0</td>
<td>73.2</td>
</tr>
<tr>
<td></td>
<td>II-10</td>
<td>12.5</td>
<td>56.3</td>
</tr>
<tr>
<td></td>
<td>III-2</td>
<td>15.9</td>
<td>82.0</td>
</tr>
<tr>
<td></td>
<td>III-4</td>
<td>16.8</td>
<td>55.9</td>
</tr>
<tr>
<td></td>
<td>II-13</td>
<td>14.7</td>
<td>72.2</td>
</tr>
<tr>
<td></td>
<td>III-15</td>
<td>15.9</td>
<td>83.4</td>
</tr>
<tr>
<td></td>
<td>III-17</td>
<td>15.5</td>
<td>67.7</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>14.8 ± 1.9*</td>
<td>69.5 ± 10.4</td>
</tr>
<tr>
<td>Homozygotes</td>
<td>III-6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>III-7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>31.7 ± 4.5</td>
<td>67.4 ± 12.2</td>
</tr>
</tbody>
</table>

*Significantly different (P < .05) from unaffected group.
†Data from 14 normolipidemic subjects.

In Table V (94) individuals from the Fe family have been classified homozygous, heterozygous, or unaffected on the basis of LCAT activity. The assays, which were carried out in J. Frohlich's lab, used single bilayer vesicles to which was added $^3$H-cholesterol and apo A-1 plus patient's plasma (94). These results concur with the LCAT immunoassay results measuring mass in figure 5.
There are several possible causes of familial LCAT deficiency:

1. A defect within the LCAT gene itself leading to no protein or a non-functional protein produced;
2. A defect in the regulatory region of the LCAT gene interfering with the promotion of transcription. No mRNA and therefore no protein would be produced in this case;
3. A defect within the major activator of LCAT - apo A-I. In this case the LCAT enzyme would be produced, but not activated and therefore no cholesteryl esters would be produced;
4. A defect in a suppressor protein such as apo C-2 such that the suppressor protein was always present and active. Here again, although the LCAT enzyme would be present, it could not act on its substrate, therefore no cholesteryl esters would be formed.

The fact that the addition of exogenous LCAT enzyme to plasma samples of LCAT deficient patients normalizes some of the parameters of their lipoproteins (67) is strongly suggestive that the defect causing LCAT deficiency is in their LCAT gene itself or its regulatory region rather than a regulatory protein such as apo A-I or apo C-II.

The question asked in this thesis, therefore, is 'where is the mutation causing LCAT deficiency in this kindred?'.

OBJECT OF RESEARCH

The object of this thesis is to define, at the molecular level, a genetic defect causing lecithin:cholesterol acyl transferase (LCAT) deficiency in a large kindred of Dutch Mennonite and Italian ancestry.
MATERIALS AND METHODS

I. MATERIALS

Media:

2 X YT
- 1.6% Bacto-tryptone
- 1.0% Yeast Extract
- 0.5% NaCl
- pH 7.5

1 X YT
- 0.8% Bacto-tryptone
- 0.5% Yeast Extract
- 0.5% NaCl
- pH 7.2 - 7.4

LB broth - 1L.
- 10g Bactotryptone
- 5g Yeast Extract
- 10g NaCl
- pH 7.5

NZYCM - 1L.
- 10g NZ-amine Type A
- 2g MgCl₂·6H₂O
- 5g Yeast Extract
- 1g Casamino Acids
- 5g NaCl

TB - 1L.
- 10g Bactotryptone
- 5g NaCl

TB* - 1L.
- 12g Bacto-tryptone
- 24g Yeast Extract
- 4ml Glycerol
dH₂O to 900ml - autoclave
add 100ml 0.17M KH₂PO₄/0.72M K₂HPO₄ - filter sterilized

M9 - 1L:
- 880 mls dH₂O - autoclaved
add: 100 mls 10 X M9 salts - autoclaved
- 2 mls 1M MgSO₄ - autoclaved
- 1 ml CaCl₂, 0.1M - autoclaved
- 10 mls 20% Glucose - filter sterilized
- 10 mls 100 X vitamin mix - filter sterilized
10 X M9 salts: 60 g \( \text{Na}_2\text{HPO}_4 \)  
30g \( \text{KH}_2\text{PO}_4 \)  
5g \( \text{NaCl} \)  
1ml 0.1M \( \text{FeCl}_3 \) pH 7.4  
\( \text{dH}_2\text{O} \) to 1 L.  

100 X Vitamin mix - 40 mls: 240ng Uridine  
4.8mg Thiamine  
24mg Thymidine  
120mg Tryptophan  

Buffers:  

**TE**  
10mM Tris-HCl  
1mM EDTA  

**10 X TBE**  
0.9M Tris Base  
0.9M Boric Acid  
25mM EDTA  
ph 8.3  

**20 X SSC -1L**  
175.3g NaCl  
88.2g Sodium Citrate  
ph to 7.0  

**20 X SSPE - 1L:**  
174.0g NaCl  
27.6g \( \text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O} \)  
7.4g EDTA  
ph to 7.4  

**10 X MG**  
0.5M Tris Base  
0.5M Boric Acid  
10mM EDTA  
ph8.3  

Depurinating Solution: 0.25M HCl  

Denaturing Solution: 0.5M NaOH  
1.0M NaCl  

Neutralizing Solution: 1.0M Tris-HCl pH7.5  
1.5M NaCl  

Denhardt's Solution - 50 X:  
5g Ficoll  
5g polyvinylpyrrolidone  
5g BSA (Pentax Fraction V)  
\( \text{dH}_2\text{O} \) to 500 mls  

Prehybridization Solution:  
5 X SSPE  
10 X Denhardt's  
0.3% SDS  
100mg/ml Salmon Sperm DNA
Hybridization Solution:  
5 X SSPE  
5 X Denhardt's  
0.1% SDS  
100mg/ml Salmon Sperm DNA

PSB (phage suspension buffer)-100mls:  
0.58g NaCl  
0.20g MgSO$_4$$^\cdot$7H$_2$O  
5.0mls 1M Tris-HCl pH7.5  
0.5mls 2% Gelatin

Oligo Stop:  
20mM NaCl  
20mM Tris-HCl pH7.5  
2mM EDTA  
0.25% SDS  
1 $\mu$M dCTP

Sequencing Reagents:

Promega does not provide information on the make-up of its sequencing reagents.

Sequenase:

Sequenase Buffer (5 X concentrate):

- 200 mM Tris-HCl pH7.5  
- 50 mM MgCl$_2$  
- 250 mM NaCl

Labelling Mix (dITP) 5 X concentrate:

- 15 $\mu$M dITP  
- 7.5 $\mu$M dCTP  
- 7.5 $\mu$M dTTP

ddG Termination Mix (for dITP):

- $160$ $\mu$M dITP, $80$ $\mu$M dATP, $80$ $\mu$M dCTP, $80$ $\mu$M dTTP, $1.6$ $\mu$M ddGTP

ddA Termination Mix (for dITP):

- $160$ $\mu$M dITP, $80$ $\mu$M dATP, $80$ $\mu$M dCTP, $80$ $\mu$M dTTP, $8$ $\mu$M ddATP

ddT Termination Mix (for dITP):

- $160$ $\mu$M dITP, $80$ $\mu$M dATP, $80$ $\mu$M dCTP, $80$ $\mu$M dTTP, $8$ $\mu$M ddTTP
ddC Termination Mix (for dITP):

160 $\mu$M dITP, 80 $\mu$M dATP, 80 $\mu$M dCTP, 80 $\mu$M dTTP, 8 $\mu$M ddCTP

Stop Solution: 95% Formamide
20 mM EDTA
0.05% Bromophenol Blue
0.05% Xylene Cyanol FF
II. SUBJECTS

(a) Control Subjects

DNA was extracted from the blood of healthy individuals with normal lipid profiles. Blood samples from members of the Fe family including unaffected, heterozygotes and those homozygous for LCAT deficiency was obtained by J. Frohlich.

(b) Case History

SF, a 29 year old male, presented at age 16 with asymptomatic proteinuria and microscopic hematuria. On physical examination, the only abnormal finding was bilateral corneal opacity with peripheral arcus and slight anemia with occasional target cells. On further examination an electron micrograph of a kidney biopsy showed numerous vacuoles with lipid deposits in the thickened glomerular basement membrane and fusion of the foot processes. Lipoprotein electrophoresis showed abnormalities in all lipid classes and no LCAT enzyme could be detected immunologically (106). The paternal branch of his family originated in Italy and the maternal branch in Sweden.

III. DNA PURIFICATION

(a) Isolation and Lysis of White Blood Cells. Blood samples from EDTA tubes were centrifuged for 10 min. at 2500 rpm. and the serum removed. Ten mls of cells were added to 10 mls of 37°C NH₄Cl:Tris solution in 50 ml Falcon tubes, then filled to 50 mls with NH₄Cl:Tris, vortexed briefly, and incubated 15 min. at 37°C to lyse the red blood cells. The mixture was then centrifuged for 10 min. at 2500 rpm. The supernatant was aspirated and the pellet resuspended in 40 mls .85% saline, vortexed, and centrifuged for 10 min. at 2000 rpm. The supernatant was again aspirated and the pellet resuspended in 10 mls 10 X TE. The white blood cells were lysed by injecting 0.5
volume lysis mix (0.2M Tris-HCl pH8.0, 0.2M EDTA, 0.2% SDS) using a 10 ml syringe. 0.01 volume of predigested proteinase K (10mg/ml) was added and the mixture incubated overnight at 37°C to aid in dissociation of protein from the DNA.

(b) **DNA Extraction.** The DNA was extracted twice with phenol TE saturated, then two times with CIA. It was precipitated by the addition of 0.1 X volume 4M NH₄OAc plus 2.5 volumes of 95% ethanol. The tube was rocked gently until the DNA precipitated. The DNA was then rinsed with 70% ethanol, dried in a vacuum desicator, resuspended in TE and stored at 4°C.

**IV. SOUTHERN BLOTTING OF GENOMIC DNA.**

(a) **Digestion and Electrophoresis.** Samples containing 8µg of genomic DNA were digested with restriction enzymes using the buffer and incubation temperature recommended by the supplier. A 1µg sample of each digest was separated electrophoretically in a 0.8% agarose gel and checked for complete digestion. The remainder of each sample was separated on a 0.6% to 1.2% agarose gel depending on the size of the fragments to be separated.

(b) **DNA Transfer.** The DNA was depurinated 10 min. in 0.25M HCl (when the average fragment size was greater than 9kb), denatured 45 min. in 0.5M NaOH, 1.0M NaCl, then neutralized 45 min. in 1.0M Tris-HCl pH7.5, 1.5M NaCl. The DNA was then transferred onto a nylon membrane (NYTRAN) overnight (1) using 10 X SSC at room temperature. The membrane was then rinsed in 10 X SSC and baked 1.5 hours at 80°C to fix the DNA to the membrane.
(c) Hybridization and Washing Conditions. Prehybridization was carried out at 65°C for 4 hours or overnight in 5 X SSC, 10 X Denhardt’s, 0.3% SDS, 100mg/ml Salmon sperm DNA. The filters were then hybridized at 65°C overnight in 5 X SSC, 5 X Denhardt’s, 0.1% SDS, 100mg/ml Salmon sperm DNA plus 25ng of radioactively labeled probe. The filters were washed 2 X at room temperature in 0.5 X SSPE, 0.1% SDS to remove excess probe. The next 2 or 3 washes were done at 65°C using 0.1% SDS with the concentration of SSPE ranging from 0.5 X to 0.1 X depending on the specific activity of the probe and its efficiency in binding to its target sequence. The filters were then dried, wrapped in saran and exposed to XAR-5 film at -70°C for one to four days with one or two intensifying screens before being developed.

V. OLIGO LABELING OF DNA. Purified DNA fragments were oligo labelled by the method of Feinberg and Volgelstein (2).

Solution A. 1000 μl Solution O (1.25M Tris-HCl pH8.0, 0.125M MgCl₂)
18 μl β-mercaptoethanol
5 μl 0.1M dATP in TE (3mM Tris-HCl pH 7.0, 0.2mM EDTA)
5 μl 0.1M dTTP in TE
5 μl 0.1M dGTP in TE

Solution B. 2M HEPES (titrated to pH 6.6 with 4 N NaOH)

Solution C. Hexadeoxynucleotides (final concentration of 90 O.D. U/ml in TE).


The reaction was carried out in a 1.5ml Eppendorf tube containing 25 ng of DNA plus dH₂O to 7.5 μl, boiled for 5 min., cooled on ice for 5 min., then centrifuged 2 secs. Ten microliters of OLB, 2 μl BSA (10 mg/ml), 5 μl α²³²P-dCTP, and 0.5 μl Klenow (6.3 U/μl, Pharmacia Biochemicals) were added. The mixture was incubated 4-6 hours at RT, then stopped with 200 μl stop buffer (20mM NaCl, 20mM Tris-HCl pH 7.5, 2mM EDTA, 0.25% SDS, 1μM dCTP). Specific activity, measured on a scintillation counter after TCA precipitation, was 1-5 X 10⁹ cpm.
VI. ISOLATION OF PLASMID DNA. Plasmid DNA was isolated using a variation on the alkaline lysis method for rapid, small-scale isolation of plasmid DNA. A single bacterial colony containing the desired plasmid was used to inoculate 5 ml of LB or TB* containing antibiotic selection (tet. - 12.5 mg/ml or amp. - 50.0 mg/ml) and grown overnight at 37°C to stationary phase. 1.5 ml of the overnight culture was transferred to a 1.5 ml Eppendorf tube, centrifuged 1 min. to pellet the cells and the supernatant was removed. The pellet was resuspended in 100 μl glucose solution (50 mM glucose, 100 mM Tris-HCl pH8.0, 20 mM EDTA, fresh lysozyme to 2 mg/ml). This was stored at RT for 5 min., then 200 μl of lysis solution (1% SDS, 0.2M NaOH) was added. The contents were mixed gently by inversion, then allowed to sit at RT 5 min. 150 μl of 3M NaOAc pH5.2 was added, mixed gently, then placed on ice for 5 min. The mixture was then centrifuged 5 min. at 4°C to precipitate the lysed cell debris. 400 μl of the supernatant was transferred to a clean Eppendorf tube and extracted once with an equal vol. of phenol (TE saturated), once with 0.5 X vol. phenol plus 0.5 X CIA, and once with an equal vol. of CIA. The DNA was precipitated by the addition of 0.1 X vol. of 3M NaOAc pH5.2 plus 2.5 X vol. 95% ethanol, incubated at 23°C or 4°C for 30 min. or overnight, centrifuged 30 min. at 4°C, washed with 70% ethanol to remove the salt, dried and resuspended in TE plus 20 μg/ml RNase A. The quantity and concentration of DNA obtained was determined by spectrophotometric analysis.

VII. ISOLATION OF LAMBDA (λ) DNA. 300 μl of a fresh overnight culture of LE392 cells (grown in NZYCM plus 0.2% maltose), 300 μl 10 mM CaCl₂/MgCl₂ plus 100 μl phage λ (1 plaque reuspened in 1 ml PSB plus 50 μl chloroform) were incubated for 20 min. at 37°C to allow the phage to adsorb to the E. coli phage receptors, then added to 50 ml NZYC medium and incubated overnight at 37°C. The overnight culture was then transferred to 50 ml corex tubes and centrifuged for 10 min. at 10,000 rpm to pellet the
cell debris. The supernatant was transferred to a clean tube, 100 μl DNase I (1.0 mg/ml) plus 100 μl RNase I (10 mg/ml) were added and incubated for 30 min. at 37°C. 10 mls of 20% PEG/2.5M NaCl was added to a clean tube, topped with the supernatant from above, placed on ice (0°C) for 60 min., then centrifuged for 10 min. at 10,000 rpm. The supernatant was discarded and the tube drained well. The pellet was resuspended in 1 ml PK buffer (10mM Tris-HCl pH7.8, 10mM NaCl, 10 mM EDTA, 0.5% SDS) plus 2.5 μl PK (proteinase K, 10 mg/ml) and incubated for 30 min. at 37°C. This was extracted with an equal vol. of CIA 3 or 4 times or until there was no PEG at the interface, twice with phenol (TE saturated), to remove proteins, then once with CIA to remove any residual phenol. The λ DNA was precipitated by adding 0.1 vol. 3M NaOAc plus 2.5 X vol. 95% ethanol at RT, rocked gently to precipitate, washed with 70% ethanol, dried and resuspended in 25 μl TE. 1-2 μl were used to test digestibility.

VIII. ELECTROELUTION OF DNA FRAGMENTS. Dialysis tubing (Spectapor membrane tubing, Spectrum Medical Industries, Inc. - 15.9 mm or 6.4 mm) was cut into pieces of 10 - 20 cm, boiled for 10 min. in a large vol. of 2% sodium bicarbonate, 1mM EDTA, rinsed with distilled water, boiled 10 min. in 1mM EDTA and stored at 4°C. The tubing was washed inside and out with distilled water before using and clamped at one end. The pieces of agarose and a small amount of TE were put inside, the air bubbles removed and the other end clamped. It was then electrophoresed in 1 X TBE at 60V for 1-2 hours. The solution was removed from the tubing, ethanol precipitated, and resuspended in TE. The concentration of DNA was determined by comparison of 1 μl of sample to known DNA concentrations on a 1% agarose plus ethidium bromide plate.
Ω. λ-FIX LIBRARY.

(a) Preparation of SF DNA. 100 µg of SF DNA were phenol extracted with 1 X vol. phenol, 0.5 X vol. phenol + 0.5 X CIA, then 1 X CIA, ethanol precipitated with 0.1 X vol. 3M NaOAc pH5.2 + 2.5 X vol. 95% ethanol at RT, rocked gently to precipitate, washed with 70% ethanol and dried. The DNA was resuspended in 200 µl TE and the concentration determined to be 0.4 µg/µl by measuring O.D.260 with a spectrophotometer.

(b) MboI Partial Digest.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 µl</td>
<td>SF DNA (0.4 µg/µl)</td>
<td></td>
</tr>
<tr>
<td>100 µl</td>
<td>10 X High Salt Buffer</td>
<td>(1M NaCl, 100mM Tris-HCl pH7.5, 100mM MgCl2)</td>
</tr>
<tr>
<td>660 µl</td>
<td>dH2O</td>
<td>(1.5 µl of 8.0 U/µl MboI (Pharmacia) was diluted with 47 µl dH2O immediately prior to use).</td>
</tr>
<tr>
<td>40 µl</td>
<td>MboI (0.25 U/µl)</td>
<td></td>
</tr>
</tbody>
</table>

This sample was incubated at 37° C. At 5 min. intervals (5, 10, 15, 20 & 25 min.), 200 µl aliquots were removed, EDTA was added to a final concentration of 10 mM the sample heated to 68° C for 10 min., and placed on ice. 1 µg of each aliquot was electrophoresed through a 0.2% agarose gel to determine which aliquot(s) would be best for ligating into λ-Fix. Each aliquot was ethanol precipitated (0.1 X vol. 3M NaOAc pH5.2 + 2.5 X vol. 95% ethanol; 0° C, 10 min.; centrifuged 15 min. at 4° C; 70% ethanol washed; dried) and resuspended in 30 µl T̄E/10 (10 mM Tris-HCl pH7.5, 0.1 mM EDTA). Concentrations were determined on a spectrophotometer. The 5 and 10 min. aliquots were combined.
(c) **Fill-in Reaction.** Two base pairs of the Mbo I sticky end were filled in with dGTP and dATP:

\[
\begin{align*}
\text{GATC} & \quad \text{GA} \\
\text{AG} & \quad \text{CTAG}
\end{align*}
\]

GATC---------GA
AG----------CTAG

to prevent the formation of concatamers and to make them compatible with the \(\lambda\)-Fix vector which had been cut with XhoI and 2 base pairs filled in:

\[
\begin{align*}
\text{CTC} & \quad \text{TGCAG} \\
\text{GAGCT} & \quad \text{CTC}
\end{align*}
\]

This will prevent self-ligation and, therefore reduce the background of false positives which are religated vector with no insert.

The fill-in reaction:

\[
\begin{align*}
30 \mu l & \quad \text{SF DNA (12.5 \mu g)} \\
15 \mu l & \quad \text{10 X Ligase Buffer (500 mM Tris-HCl pH8.0, 70 mM MgCl}_2 \quad \text{10 mM DTT)} \\
5 \mu l & \quad \text{10 mM dGTP} \\
5 \mu l & \quad \text{10 mM dATP} \\
91.5 \mu l & \quad \text{dH}_2\text{O} \\
3.5 \mu l & \quad \text{Klenow (7.2 u/\mu l - Pharmacia)}
\end{align*}
\]

was incubated 30 min. at 37\(^\circ\)C, EDTA to a final concentration of 10 mM added and the reaction mixture stored at -20\(^\circ\)C overnight. The fill-in reaction was then phenol extracted (0.5 vol. phenol + 0.5 vol. CIA), ethanol precipitated (0.1 vol. 3 M NaOAc + 2.5 vol. 95% ethanol; 0\(^\circ\)C, 15 min.; centrifuged 30 min. at 4\(^\circ\)C; washed with 70% ethanol; dried) and resuspended in 10 \(\mu l\) TE. The concentration of DNA was determined with a spectrophotometer and diluted to 0.5 \(\mu g/\mu l\) with TE.

(d) **Ligation of SF DNA with \(\lambda\)-Fix DNA.** 0.5 \(\mu g\) of SF DNA was ligated to 1.0 \(\mu g\) of \(\lambda\)-Fix DNA according to the conditions recommended by Stratagene except that the ligation was carried out at 14\(^\circ\)C overnight.
(e) **Packaging Reaction.** λ-Fix will accommodate inserts of 9-22 kb. Gigapack Plus (batch no. 128) was used to package 0.75 μg of the SF + λ-Fix ligation. Most efficient packaging is 0.5 - 1.0 μg of concatamerized DNA according to the protocol recommended by Stratagene.

(f) **Titre of packaged λ phage.** 300 μl of a fresh overnight culture (NZYCM + 0.2% maltose) of LE392 or P2393 cells was mixed with 300 μl of 10 mM CaMg plus 5 μl of a 10⁻² or 10⁻³ dilution of packaged λ phage. This was incubated 15 min. at 37°C to allow the phage to adsorb to the cell receptors, then 10 mls of NZY top agarose at 50°C was added. This was poured onto NZYC plates and incubated at 37°C overnight and the plaques counted. The titre was low at 1.5 X 10⁵ phage per μg of DNA. The expected was 1 X 10⁶ phage per μg of DNA.

(g) **Screening.** A mixture containing:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>500 μl</td>
<td>CES201 cells (fresh overnight culture grown in TB + 0.2% maltose)</td>
</tr>
<tr>
<td>500 μl</td>
<td>10 mM CaMg</td>
</tr>
<tr>
<td>167 μl</td>
<td>SF + λ phage.</td>
</tr>
</tbody>
</table>

was incubated 15 min. at 37°C, then 10 mls of NZYCM top agarose (50°C) was added, the mixture poured onto NZYCM plates and incubated overnight at 37°C.

(h) **Plaque Lift and Hybridization.** The plates were chilled at 4°C, then Hybond-N (Amersham) filters were placed on the plates and marked to orient. The first filter was left on for 3 min. and the second for 5 min. Each was denatured 3 min. (0.5 M NaOH, 1.0 M NaCl), neutralized 5 min. (1.0 M Tris-HCl pH7.5, 1.5 M NaCl), soaked 5 min. in 2 X SSC, then dried and exposed to UV radiation to crosslink the DNA to the nylon membrane. The membranes were prehybridized for 4 hours at 65°C in 50 mls of prehybridization solution (5 X SSPE, 10 X Denhardt’s, 0.3% SDS, 100 mg/ml Salmon Sperm DNA), then hybridized overnight at 65°C in 25 mls of hybridization solution (5
X SSPE, 5 X Denhardt's, 0.1% SDS, 100 mg/ml Salmon Sperm DNA) plus 25 ng denatured, radioactively labelled LCAT cDNA as probe. The membranes were washed 2 X at RT in 0.5 X SSPE, 0.1 % SDS for 30 min. at 65°C in 0.5 X SSPE, 0.1% SDS, 30 min at 65°C in 0.2 X SSPE, 0.1% SDS and 30 min. at 65°C in 0.1 X SSPE, 0.1% SDS, air dried, wrapped in saran and exposed to XAR-5 film at -70°C for 1.5 hours.

(i) Probe Preparation. The 5' probe was 25 ng of the 0.44 EcoRI- KpnI LCAT cDNA insert containing exons one, two and three from the pUC19 plasmid obtained from J.W. McLean (Genentech). The 3' probe was a 0.2 kb PstI fragment subcloned into pUC19 from LCAT-14B, an LCAT cDNA pKT218 plasmid from S.E. Humphries (116). The 3' probe contained the mid portion of exon 6. The probes were prepared according to section 3, oligo labelling. The specific activities were $1.4 \times 10^9$ cpm for (0.44)EcoRI-KpnI 5' probe and $1.5 \times 10^9$ cpm for (0.2)Pst I 3' probe.

(j) Plaque Purification. There was one positive plaque detected with the 5' probe which was picked, placed in phage suspension buffer with chloroform and allowed to diffuse. These phage were subject to 2 rounds of purification before DNA was extracted to be used for subcloning.

X. COMPETENT CELLS AND TRANSFORMATIONS.

(a) JM83. 20 mls of LB + 1 JM83 colony were grown at 37°C until they reached a density of $O.D_{600} = 0.2$ (~5 X $10^7$ cells /ml), placed on ice 10 min., centrifuged 5 min. at 4,000 rpm and 4°C. The supernatant was discarded and the pellet resuspended in 0.5 vol. 50 mM CaCl$_2$, 10 mM Tris-HCl pH8.0, placed on ice 10 min, centrifuged 5 min. at 4,000 rpm and 4°C. The supernatant was again discarded and the cells resuspended in
0.067 vol. 50 mM CaCl$_2$, 10 mM Tris-HCl pH8.0. The cells not used for transformations were quick frozen (dry ice plus ethanol) and stored at -70°C in 15% glycerol. For each transformation, 200 µl of competent JM83 cells were mixed with 40 - 100 ng of plasmid DNA, placed on ice 30 min., then heat shocked 2 min. at 42°C. 1.0 ml of LB was added and the cells incubated 1 hour at 37°C. Varying amounts of cells, 10-20 µl, were plated on LB + amp plates with 50 µl 2% X-Gal plus 10 µl 2% IPTG and incubated overnight at 37°C.

(b) JM101. 5 mls of M9 medium plus 1 JM101 colony from an M9 plate were grown at 37°C overnight. 750 µl of the overnight culture was placed into 60 mls of YT medium and grown at 37°C until the O.D$_{600}$ = 0.5 (~4 X 10$^8$ cells/ml), placed on ice 10 min., centrifuged 10 min. at 4,000 rpm and 4°C. The supernatant was discarded and the cells resuspended in 0.5 X vol. 50 mM CaCl$_2$, placed on ice 20 min., then centrifuged 5 min. at 4,000 rpm and 4°C. The supernatant was again discarded and the cells resuspended in 0.1 vol. 50 mM CaCl$_2$. 300 µl of competent JM101 cells were mixed with approximately 10 ng of DNA and placed on ice 40 min. During this time 4 mls of 6% YT top agarose, 45°C, was added to a 16 ml Falcon tube for each transformation with 50 µl 2% X-Gal, 2% IPTG and 200 µl of exponential JM101 cells. At 40 min. the competent cells plus DNA were heat shocked at 42°C for 2 min., then added to the top agarose, poured onto YT plates and incubated 8-12 hours at 37°C. The unused competent JM101 cells were discarded to ensure the maintenance of the F' pili.

XI. PLASMID LIGATIONS. Vector plus insert DNA were incubated 4 hours at RT or overnight at 14°C using a molar ratio of vector:insert of 1:1 or 3:1 in the following reaction mixture: 10 X Ligase buffer (0.5 M Tris-HCl pH8.0, 0.1 M MgCl$_2$, 0.1 DTT, 10 mM Spermidine, 1 mg/ml BSA), 10mM ATP, 3U T4 DNA Ligase (Pharmacia) and
dH$_2$O to 15 µl. Following incubation, 10 ng of the reaction mixture was diluted 5 fold with TE before adding to competent cells for transformation.

XII. ISOLATION OF M13 SINGLE-STRANDED TEMPLATE DNA. A single JM101 colony was picked from an M9 agar plate and grown in M9 medium overnight at 37°C with rapid agitation (250 rpm). 1 ml of the overnight culture was used to inoculate 100 mls (100 X dilution) of 2 X YT medium and incubated at 37°C and 250 rpm until the O.D.$_{600}$ = 0.4. A single M13 phage plaque plus 2 mls of JM101 culture were incubated overnight at 37°C and 250 rpm. 1.5 mls of each overnight culture was transferred to a 1.5 ml Eppendorf tube and the cells pelleted with a 5 min. spin at RT. 1 ml of the supernatant was transferred to a fresh Eppendorf tube to be used for preparing template DNA. 100 µl of the supernatant was stored at 4°C as a phage stock. The cell pellet was used to recover double stranded M13 DNA by the alkaline lysis method described in 4. 250 µl of sterile 20% PEG/2.5M NaCl was added to 1 ml of supernatant and incubated 10 min. at RT. The phage were pelleted by centrifugation for 10 min. at RT. The supernatant was aspirated using a drawn out Pasteur pipette. To remove the last of the PEG, the tube was centrifuged again for 1 min. and the remainder of the supernatant aspirated. The phage pellet was resuspended in 150 µl of TE and extracted with 0.5 vol. phenol (TE saturated) plus 0.5 vol. CIA 2 or 3 times (until there was no PEG at the interface). The DNA was precipitated with 0.1 vol. of 3M NaOAc (pH5.2) plus 2.5 X vol. 95% ethanol, incubated 30 min. at RT, then centrifuged 30 min. at RT. The pellet was rinsed with 70% ethanol, dried under vacuum and resuspended in 20 µl of TE to 0.2 µg/µl and stored at -20°C.
XIII. DIDEOXY SEQUENCING

The SF (2.4)Bam-H1/SalI LCAT clone was sequenced by the dideoxy method. Except for regions of GC secondary structure, the sequencing was done using reagents ordered from Promega (K/RT™ Universal Sequencing System). The regions containing GC secondary structure were sequenced with Sequenase (USB) using dITP instead of dGTP to reduce the formation of secondary structure.

**Reactions:** Promega

**Annealing reaction:** 5 µl (1 µg) of (2.4 BamH1/SalI) SF template DNA, 1 µl of 10 X Klenow buffer (100mM Tris-Cl pH7.5, 500 mM NaCl), 1 µl (1 pmole) of primer (except for M13 forward primer from Promega, the primers were a gift from J. McLean, Genentech) plus 3 µl dH2O were mixed together, incubated in a water bath at 55°C for 6 minutes, then cooled slowly to 37°C. 100 µl of 100mM DTT, 5U Klenow and 5 µl (α35S)dATP (500Ci/mole - NEN) were added to each annealing reaction.

**Termination reaction:** 3 µl of the appropriate nucleotide mix was dispensed to each GATC tube, then 3 µl of the template/primer/label reaction was added to each GATC tube and incubated 20 minutes at 37°C after which, 1 µl of chase solution was added to each tube and incubated another 15 minutes at 37°C. Each reaction was stopped by adding 5 µl of stop solution. The reactions were heated to 90°C for 3 minutes, then placed on ice prior to loading 2.5 µl onto a 6% or 8% polyacrylamide sequencing gel. The gels were electophoresed in MG Buffer (0.5M Tris base, 0.5M Boric acid, 10mM EDTA, pH8.3) at 35 W for one to four hours. After electrophoresis, the gels were fixed for 15 minutes in 5% acetic acid/5% methanol, transferred to 3MM Watmann paper and dried down under vacuum using a gel drier at 80°C for 20 minutes. The gels were exposed to Kodak XRP-1 film overnight at room temperature.

Several short strings of G's could not be resolved by this procedure or by sequencing the antisense strand, therefore Sequenase (USB) which uses dITP in place of
dGTP to prevent secondary structure formation was used to sequence through these regions.

**Reactions Sequenase**

**Annealing reaction:** 5 µl (1 µg) of SF (2.4 BamH1/Sal1) template DNA, 2 µl of 5 X Sequencing Buffer (200mM Tris-Cl, pH7.5, 50mM MgCl₂, 250mM NaCl), 1 µl of primer - same as for Promega reactions, and 2 µl of dH₂O were mixed, incubated at 55°C for 6 minutes, then cooled slowly to 35°C. 1 µl of 100mM DTT, 2 µl of labelling mix (15 µM dITP, 7.5 µM dCTP, 7.5 µM dTTP diluted 1:5 with dH₂O), 2 µl (α³⁵SdATP 500 Ci/mole - Amersham) and 2 µl of Sequenase diluted 1:8 with TE were added to the annealing reaction, and incubated at room temperature 5 minutes.

**Termination reaction:** 2.5 µl of the appropriate ddNTP termination mix was aliquoted to each GATC tube then the tubes were capped and and warmed at 37°C for one minute. 3.5 µl of the template/primer/label mix was then added to each GATC tube and the reaction incubated 5 minutes at 37°C. The reactions were stopped by the addition of 4 µl of stop solution. The DNA was denatured at 80°C for 2 minutes, then placed on ice prior to loading 2.5 µl of each reaction onto a 6% or 8% polyacrylamide gel. The remainder of the protocol was the same as for the Promega reactions.
RESULTS

I. INTRODUCTION

The search for a mutation in the region of the LCAT gene was organized in the following manner. DNA extracted from blood samples of the homozygotes was analyzed with several restriction endonucleases and compared with normal controls to determine if there were any major rearrangements. Next the family was analyzed to determine if there was linkage between LCAT deficiency and any of the known polymorphic sites on nearby regions of chromosome 16. A search for new restriction fragment length polymorphisms (RFLPs) was then undertaken in the region of the LCAT gene which not only had the possibility of providing a marker for this family, but would be the first polymorphic site detected in the immediate vicinity of chromosome 16 and therefore, useful to researchers studying other diseases in this region of the chromosome. Finally, a gene from one of the homozygotes was cloned and sequenced in order to determine the precise nature of a mutation causing LCAT deficiency in this kindred.

II. Restriction Enzyme and Southern Blotting Analysis

In this section the results obtained from analyzing total genomic DNA with restriction endonucleases and Southern blotting are presented and analyzed.

(a) Major Rearrangements

Total genomic DNA of a normal control, a heterozygote (II-4, fig. 6) and a homozygote (III-6, fig. 6) was digested with the restriction endonucleases BamHI, SstI, PstI and AccI and analyzed by Southern blot hybridization to compare the LCAT gene from an LCAT deficient individual with that of a normal control.

Although DNA from the heterozygote did not digest to completion with the restriction enzymes PstI and AccI, the hybridizing fragments of the DNA from the homozygote are the same as those of the control DNA and both are consistent in size
with predictions (Table V) based on sequencing of the normal LCAT gene (55). In each case a single, faint, anomalous hybridizing fragment can be seen which might indicate that homology exists elsewhere in the genome to a limited part of the cDNA sequence. These enzymes would have allowed differences of about 0.1 kb to be detected, therefore there were no major rearrangements in the LCAT gene of the LCAT deficient patients in this family using these criteria.
Figure 6 shows the results of digestion of human DNA with several different restriction enzymes probed with isolated insert fragment of the human LCAT cDNA clone pL12. This probe was chosen because it contains nearly all of the coding region, but no poly (A) tail.
Figure 6: Southern blot analysis of human genomic DNA cleaved with various restriction enzymes and probed with a $^{32}$P-labelled cDNA insert of clone pL12 (52).
Table V: Predicted sizes of genomic DNA at the LCAT loci digested with several restriction enzymes and probed with LCAT cDNA. (courtesy of J.McLean).

<table>
<thead>
<tr>
<th>Restriction Endonuclease</th>
<th>Size kb</th>
<th>Gene Location</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SstI</td>
<td>1018</td>
<td>5' - exon 1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2866</td>
<td>exon 2 - part of exon 6</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>2055</td>
<td>part of exon 6 - 3'</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extra band at about 4.0 kb</td>
<td>faint</td>
</tr>
<tr>
<td>BamHI</td>
<td></td>
<td>only sites are in the 5' flanking region</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>and 18 kb downstream</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extra band at about 4.9 kb</td>
<td>faint</td>
</tr>
<tr>
<td>PstI</td>
<td>975</td>
<td>exon 1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>694</td>
<td>exons 2 - 4</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>2321</td>
<td>exon 5 - part of exon 6</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>1539</td>
<td>part of exon 6 - 3'</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extra band at about 3.6 kb</td>
<td>faint</td>
</tr>
<tr>
<td>AccI</td>
<td>1339</td>
<td>exon 1 - part of exon 2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>109</td>
<td>part of exon 2</td>
<td>off gel</td>
</tr>
<tr>
<td></td>
<td>484</td>
<td>exon 3 - part of exon 5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2264</td>
<td>part of exon 5 - part of exon 6</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>3.6 kb</td>
<td>part of exon 6 - 3'</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>extra band at about 2.6 kb</td>
<td>faint</td>
</tr>
</tbody>
</table>

Table V shows the predicted size of each hybridizing fragment seen in figure 7. All the predicted fragments are present in the DNA from both the control and homozygote. The location of each fragment within the LCAT gene is shown. The intensity of each band corresponds to the length of $^{32}$P-labelled cDNA which can hybridize to each genomic fragment.
(b) **Linkage Analysis**

(i) **Haptoglobin (Hp):**

Teisberg and Gjone, in 1974 (62), described non-random distribution between LCAT deficiency and serum haptoglobin types reporting a recombination fraction of 0.05.

There are two types of polymorphisms in the Hp gene. One is due to a partial gene duplication caused by an unequal crossover event. The other is due to a mutation resulting in an amino acid substitution which leads to an altered charge on the protein. There are three main Hp alleles: Hp1S (slow) and Hp1F (fast) which differ at only one amino acid and Hp2 which is the result of a partial gene duplication between Hp1F and Hp1S and is 1700 base-pairs larger than either Hp1 allele (95).

DNA from members of the Fe family was digested with the enzymes EcoRI, HindIII, XbaI, Accl, PstI, BclII. Southern analysis using the α2 PstI/BamHI fragment of the Hp6-18 haptoglobin cDNA clone as a probe showed that all members of the kindred had the haplotype 2-2. The known RFLP's at the haptoglobin locus were therefore, uninformative in this family.
Table VII: Hp haplotypes (96)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Allele</th>
<th>Size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HindIII</td>
<td>$H_p^R$</td>
<td>3.4 (constant)</td>
</tr>
<tr>
<td></td>
<td>$H_p^1$</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>$H_p^2$</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Table VII shows the possible alleles detected using the enzyme HindIII.

Three main haplotypes exist which vary in frequency between different populations. The average frequencies found in a study by Hill et al. (96) were:

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hp2-2</td>
<td>8%</td>
</tr>
<tr>
<td>Hp2-1</td>
<td>44%</td>
</tr>
<tr>
<td>Hp1-1</td>
<td>48%</td>
</tr>
</tbody>
</table>
Figure 7: Hybridizing fragments of DNA from Fe family members digested with the restriction enzyme HindIII and probed with the α2 haptoglobin cDNA fragment.

Figure 7 is an example of the 2-2 haplotype present in the Fe family. In this case each DNA was digested with the restriction enzyme HindIII, then probed with the 2 haptoglobin cDNA fragment. It shows that the HpR 3.4 kb constant band is present as well as the larger Hp2 4.9 kb band in every case.
(ii) APRT (adenosine phosphoribotransferase)

The APRT gene was first mapped to chromosome 16q12-22 (97). In situ hybridization, recombinant DNA technology and somatic cell hybrids have since placed the APRT gene at 16q24. No linkage has been established between APRT and the LCAT locus, although APRT and Hp, which is linked to LCAT, have been shown to be unlinked. An APRT (2.2 kb BamHI) genomic fragment which detects a TaqI polymorphism (98) was used to look for linkage between the APRT locus and the LCAT deficient gene.

For several reasons linkage between APRT and the LCAT deficient gene could not be established. Phase of the parents of the homozygotes could not be determined (Figure 9). Only those offspring that are homozygous for LCAT deficiency or unaffected are informative for both LCAT and APRT. There are four offspring which are heterozygous for the APRT polymorphism and either homozygous LCAT deficient, heterozygous LCAT deficient, or homozygous unaffected. This means that there is at least one recombinant in this sibship. Therefore, a calculation of the lod score would be based on too little information to make the result significant.

Table VIII: Size and frequency of the APRT polymorphism (98).

<table>
<thead>
<tr>
<th>kb</th>
<th>Frequency</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>constant</td>
<td></td>
</tr>
<tr>
<td>0.6/2.1</td>
<td>0.64</td>
<td>B</td>
</tr>
<tr>
<td>2.7</td>
<td>0.36</td>
<td>A</td>
</tr>
</tbody>
</table>
Figure 3: Hybridizing fragments of DNA from the Fe family digested with the restriction enzyme TaqI and probed with a cDNA fragment of the APRT gene.

Figure 3 shows those members of the Fe family analyzed to determine whether or not linkage exists between the APRT locus and the LCAT deficient gene. The 0.5 kb constant band and the lower 0.6 kb band of the B allele (not shown) were present in each individual. Those individuals who have only the 2.1 kb band have the haplotype BB, whereas those with both the 2.1 and 2.7 kb bands have the haplotype AB.
In figure 9 the haplotype of each individual is presented on the family tree. The numbers at the top of figure 9 correspond to the numbers at the top of figure 8 and each lane in figure 9 lines up with the appropriate individual in figure 8. There has been a crossover event between the APRT and LCAT loci in this family (figure 10, number 6) which is indicated by an 'X' at the most probable meiosis, although this cannot be determined with any certainty. There is not enough information in this pedigree to obtain relevant lod scores on linkage between APRT and LCAT. The phase of the parents (6 and 6) cannot be determined with the information available. Without knowing the phase of the parents, the offspring (III) are uninformative in this case.
CETP: Cholesterol Ester Transfer Protein

The gene for CETP has been localized to chromosome 16q21 also by in situ hybridizations on metaphase chromosomes, recombinant DNA methodology and somatic cell hybrids. No linkage has been established between CETP and the LCAT locus.

The restriction enzyme TaqI detects two separate polymorphisms at the CETP locus and was therefore used to try to determine linkage with the LCAT-deficient gene in the Fe family. Table IX shows the size and frequency of each allele of the two TaqI polymorphisms.

Table IX: TaqI polymorphisms at the CETP locus (54)

<table>
<thead>
<tr>
<th>(1)</th>
<th>Size kb</th>
<th>Frequency</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.0</td>
<td>12%</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>88%</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>(2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.3</td>
<td>48%</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>52%</td>
<td>D</td>
<td></td>
</tr>
</tbody>
</table>
Figure 10 shows the hybridizing fragments of DNA from members of the Fe family digested with the restriction enzyme TaqI and probed with a CETP gene fragment. The haplotype is shown at the bottom of each lane.
Figure 10: Southern blot analysis of DNA from members of the Fe family.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| CD | CC | CD | CC | CD | CD | CD | CD | CD | CD | CD | CD | CD | CD | CD | CD | CD | CD | CD | CD |

kb
-9.0/7.5
-5.3
-4.4
Figure 11: Fe family tree showing CETP haplotypes

Figure 11 shows the haplotype of each individual analyzed in the Fe family. The numbers above figure 11 correspond with the numbers in figure 1. The same problems that prevented a relevant linkage analysis between APRT and LCAT apply here. Given the small number of informative meioses and the large amount of information that would be based on statistical probabilities, the outcome of the lod score would not be relevant for any practical use.
(c) **Search for new Restriction Fragment Length Polymorphisms in the region of the LCAT gene**

None of the polymorphic sites on chromosome 16 used to analyze the Fe family were informative in a linkage analysis. There are no known markers in the immediate vicinity of the LCAT gene, therefore an RFLP in this region would be an important marker, useful in the study of LCAT-deficient families as well as other defects in this region of chromosome 16.

The minimum number of unrelated individuals required to detect a polymorphism in the population at a frequency of at least 20%, is five. As the value of a polymorphism is not just as an isolated event but something to be used in further research, a polymorphism occurring at less than 20% would not be useful.
Figure 12: Diagram of the LCAT region of chromosome 16. (Adapted from J. M. Lean)
Several genomic fragments flanking the LCAT gene were used as probes in the analysis of DNA from normal controls in order to look for RFLP's in this region.

Table X: DNA fragments flanking the LCAT gene used as probes

<table>
<thead>
<tr>
<th>Probe</th>
<th>Fragment Size</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>(0.485)SstI</td>
<td>5'</td>
</tr>
<tr>
<td>(2)</td>
<td>(2.8)BamHI</td>
<td>5'</td>
</tr>
<tr>
<td>(3)</td>
<td>(4.0)SstI</td>
<td>5'</td>
</tr>
<tr>
<td>(4)</td>
<td>(3.26)SstI</td>
<td>3'</td>
</tr>
<tr>
<td>(5)</td>
<td>(4.2)EcoRI/BamHI</td>
<td>3'</td>
</tr>
<tr>
<td>(6)</td>
<td>(0.44)EcoRI/KpnI</td>
<td>exons 1-3, cDNA</td>
</tr>
<tr>
<td>(7)</td>
<td>(0.2)PstI</td>
<td>middle section of exon 6, cDNA</td>
</tr>
</tbody>
</table>

Table X lists the probes used in searching for RFLP's in the region immediately flanking the LCAT gene and in cloning an LCAT gene. Probes 1-3 are 5' flanking DNA fragments, whereas probes 4 and 5 are DNA fragments from the 3' flanking region of the LCAT gene (figure 13). Probes 6 and 7 are cDNA fragments used to aid in the cloning of an LCAT gene from an LCAT deficient patient.

DNA from 5 normal individuals was digested with 21 different restriction endonucleases (table XI) using the conditions recommended by the supplier of each enzyme. Restriction enzymes recognize short sequences of DNA usually composed of four, five or six base pairs. Since the four bases are not distributed evenly throughout the genome, the recognition sites have an average distribution frequency which differs from one enzyme to the other. Formulas have been derived which allow one to calculate the average frequency of an enzyme restriction site based on the number of base pairs and the specific nucleotides present in the recognition site. (99). In general, the smaller
the recognition site, the more frequently it is present in the genome and, therefore, the smaller the fragments that are generated. For the purpose of this RFLP search two basic criteria were used in the selection of enzymes. First, the frequency of the restriction enzyme site should be such that the average size of the fragments generated be between 0.5 and 10.0 kb. Second, that the cost of the enzyme be reasonable. This was for the practical reason that it would not be useful as a marker for any future screening should an enzyme detecting a polymorphic site be too expensive.

Table XI: List of Restriction Endonucleases

<table>
<thead>
<tr>
<th>EcoRI</th>
<th>BglII</th>
<th>BcII</th>
</tr>
</thead>
<tbody>
<tr>
<td>PstI</td>
<td>XbaI</td>
<td>PvuII</td>
</tr>
<tr>
<td>HindIII</td>
<td>MspI</td>
<td>TaqI</td>
</tr>
<tr>
<td>SstI</td>
<td>HpaI</td>
<td>KpnI</td>
</tr>
<tr>
<td>EcoRV</td>
<td>DraI</td>
<td>StyI</td>
</tr>
<tr>
<td>XmnI</td>
<td>NaeI</td>
<td>SphI</td>
</tr>
<tr>
<td>NcoI</td>
<td>AccI</td>
<td>HaeI</td>
</tr>
</tbody>
</table>

Table XI lists the restriction enzymes used to digest the 5 control DNA's. Southern blot analysis was carried out using nitrocellulose membranes. The membranes were analyzed with the 5' probe number 2 (table 9). This probe was then removed and the membranes rehybridized with probe 5, a 3' probe. The detected fragments were the same in all individuals for each restriction enzyme used to analyze the DNA. No RFLP's were found in the region of probes 2 and 5.
New filters were prepared using a nylon membrane, NYTRAN, which is much more durable than nitrocellulose. This time the DNA from 10 normal individuals was digested with 30 restriction endonucleases, then transferred to the NYTRAN membranes to be analyzed. As five unrelated individuals is the minimum number of individuals required to detect a polymorphism occurring in the population at a frequency of at least 20% and no RFLP's were detected, the number of unrelated individuals was doubled for the subsequent analysis. Table XI lists the restriction enzymes used to search for an RFLP in the regions flanking the LCAT gene.

Table XII: Restriction Enzymes used to analyze DNA from 10 normal individuals.

<table>
<thead>
<tr>
<th>EcoRI</th>
<th>SstI</th>
<th>PstI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccI</td>
<td>HincII</td>
<td>BclII</td>
</tr>
<tr>
<td>XbaI</td>
<td>HindIII</td>
<td>KpnI</td>
</tr>
<tr>
<td>MspI</td>
<td>BglII</td>
<td>PvuII</td>
</tr>
<tr>
<td>EcoRV</td>
<td>TaqI</td>
<td>HaeIII</td>
</tr>
<tr>
<td>XmnI</td>
<td>RsaI</td>
<td>AluI</td>
</tr>
<tr>
<td>Hinfl</td>
<td>BamHI</td>
<td>SphI</td>
</tr>
<tr>
<td>StuI</td>
<td>MboI</td>
<td>BanI</td>
</tr>
<tr>
<td>DraI</td>
<td>AvaII</td>
<td>ApaI</td>
</tr>
<tr>
<td>NcoI</td>
<td>NsiI</td>
<td>BglII</td>
</tr>
</tbody>
</table>

These membranes were probed with two 5' flanking DNA fragments (probes 1 and 3, table 9) and one 3' flanking DNA fragment (probe 4, table 9). No differences in numbers of fragments or size of fragments were detected amongst the 10 controls for any of the enzymes analyzed. McLean (personal communication) and Rogne et al. (100) have also extensively analyzed the region of the LCAT gene and been unable to detect any RFLP's.
III. Cloning and Sequencing Strategy

There was no direct genetic evidence that the defect causing LCAT deficiency in the Fe family was in fact within the LCAT gene or regulatory region. Therefore, based on biochemical evidence alone (discussed in the introduction) an LCAT gene from one of the homozygotes in the Fe family (SF,III-6, figure 6) was cloned and sequenced in order to analyze one of the LCAT genes in detail.

(a) Cloning The LCAT Gene

(i) EMBL-3 Library

SF and DH DNA were digested to completion with the restriction endonuclease BamHI. The restriction enzyme BamHI was chosen for a number of reasons:

1. the entire LCAT gene is contained within an 18 kb BamHI fragment;
2. the λ phage vector, EMBL-3 contains a BamHI cloning site; and
3. the size insert accepted by EMBL-3 which allows for replication and packaging of viral plus insert DNA is 9-21 kb.

There was one positive plaque in each library. These were picked, put through two rounds of purification, and five positive clones from the final purification plus the original positive plaque were kept for analyzing. DNA was prepared from the recombinant λ phage clones and the clones were mapped by Southern blot analysis.
Table XIII: Restriction endonucleases used to analyze the recombinant EMBL-3 clones.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>There was no 18 kb band as was expected from the genomic digests which show an 18 kb band containing the LCAT gene. Instead, there was a 4.0 kb band.</td>
</tr>
<tr>
<td>BamHI/NsiI</td>
<td>The normal sequence predicts a 5.2 kb band containing the LCAT gene. Both SF and DH clones have a 4.0 kb band the same as with the BamHI digest alone, although their genomic DNA contains the 5.2 kb band.</td>
</tr>
<tr>
<td>HindIII</td>
<td>Two bands were expected with this enzyme at 4.7 and &gt;4.0. Although this site is present in their genomic DNA, HindIII does not cut the cloned fragments.</td>
</tr>
<tr>
<td>BamHI/HindIII</td>
<td>The expected fragment sizes are 0.9 and 17 kb. A single 4.0 kb band was present in both clones, again the same as with a BamHI digest alone.</td>
</tr>
</tbody>
</table>

The loss of the NsiI site in the clones could be explained by a recombination event between Alu sequences that exist both within intron 5 and 3' to the LCAT gene. With the loss of the HindIII site, though, the clone had been rearranged too extensively to be useful for determining what a defect causing LCAT deficiency in SF or DH is, therefore no further work was done with these clones.
(ii) \(\lambda\)-Fix Library

A different method was needed for obtaining an intact LCAT gene from SF or DH, one that would eliminate the possibility of recombination. That meant finding an enzyme or enzymes which would include the LCAT gene, exclude the Alu-type repetitive sequences 3' to the gene to eliminate the possibility of Alu-Alu recombination and be an appropriate size for cloning. Further, the clones needed to be propagated in a cell type that was recombination deficient to reduce the possibility of any other rearrangements.

\(\lambda\)-Fix was chosen to make the library for two reasons. One, partial MboI digests could be used to generate fragments which would potentially exclude the Alu-type repetitive sequences 3' to the LCAT gene. Two, construction of the vector DNA and the method of preparation of the insert DNA reduces the possibility of vector religation or concatamers of insert being formed, thereby increasing the yield of recombinant phage in the library.

SF genomic DNA was dialyzed in TE pH7.5 overnight to remove ions and other small molecules as this made the subsequent restriction endonuclease digests much more controllable. 80 \(\mu\)g of SF DNA was partially digested with the restriction enzyme MboI. Aliquots were removed at 5 minute intervals in order to obtain the bulk of the DNA within a different size range in each aliquot with the ideal size being 9-20 kb. These were run on a 0.2% agarose gel to determine the fragment sizes of each aliquot. The 5 and 10 minute digest aliquots were combined, both having the majority of DNA fragments in the 9-20 kb range and used to prepare a \(\lambda\)-Fix library. The library contained 2.2X10^5 independent phage.

CES201, rec\(^{-}\), cells were infected with the recombinant phage and screened with probes 6 and 7, table 9. Probe 6, an LCAT cDNA clone containing exons 1-3 was used to determine whether or not the 5' end of the LCAT gene was present in any of the recombinant phage. Probe 7 is a cDNA clone containing the middle portion of exon 6
and was used to determine whether or not the 3' end of the LCAT gene was present in any of the recombinant phage. Ideally, a positive plaque with one of the probes would also be detected with the other, indicating that the entire LCAT gene was present.

Only probe 6 hybridized to a recombinant plaque indicating that the 5' end of the LCAT gene was present. This clone was called SF1020, but it was not known how much of the LCAT gene it contained. It was mapped using the restriction enzymes BamHI, SstI and SalI.
Figure 13: Restriction Map of the Genomic Insert Contained in the -Fix Clone SF1020.

Figure 13 is a map of the -Fix clone SF1020. The entire insert is about 14 kb in length. It contains exons 1-5 plus about 11.5 kb of DNA 5' to the LCAT gene.
(b) **Sequencing Strategy**

Biochemically, SF has no detectable LCAT mass or activity (93), therefore it was much more likely that there was a mutation in the regulatory region or early in the gene that would cause this phenotype, than in the last exon. There are examples of mutations occurring near the 3' end of the gene causing phenotypes as severe as that seen in the homozygotes in this family such as those reported in the Factor VIII gene (117). It was reasonable, though to sequence this clone to search for a mutation.

The 2.38 kb BamHI/Sall fragment containing exons 1 - 5 was subcloned into M13 in both orientations using M13mp18 and M13mp19. Single stranded template was prepared using JM101 cells and purified by the method described in Materials and Methods. Both strands were sequenced by the dideoxy method using the protocols described in Materials and Methods.

All the exons plus the 5' regulatory region of the coding strand were sequenced at least 2 times and most of the complementary strand was sequenced at least once. The sequencing reactions were initiated by LCAT-specific 17 base pair oligonucleotide primers prepared at Genetech.
Figure 14: Sequencing Strategy

Figure 14 diagrams the sequencing strategy used to analyze the -Fix clone SF1020. The 5' and 3' ends of the insert were sequenced using a 17-mer universal primer (Promega). In the remainder of the insert sequencing was initiated with LCAT-specific primers (courtesy of J. M'Lean).
There were several regions along the DNA in which there were strings of guanines or cytosines that initially did not give clear results due to compression on the sequencing gel. These compressions were the result of interactions between the guanines and cytosines leading to secondary structure formation within the template DNA which prevented the movement of the DNA polymerase along the template DNA. For these regions, reactions containing dITP, a base analogue of guanine, was substituted for dGTP. This prevented interaction with the cytosines and therefore, secondary structure formation, allowing the DNA polymerase to move along the template DNA.

A single base pair change in exon 4 at amino acid 135, CGG—>TGG, was the only difference from the published normal sequence (55) that was detected.
Figure 15: Sequence data at the location of the mutation in the clone SF1020.

CODING STRAND

<table>
<thead>
<tr>
<th>G</th>
<th>A</th>
<th>T</th>
<th>C</th>
</tr>
</thead>
</table>

COMPLEMENTARY STRAND

<table>
<thead>
<tr>
<th>G</th>
<th>A</th>
<th>T</th>
<th>C</th>
</tr>
</thead>
</table>

Figure 15 shows clearly that a thymine (T) has been substituted for the normal cytosine (C) on the coding strand. This was confirmed on the complementary strand where there is an adenine (A) in place of the normal guanine (G).
This single base pair change occurred at the first nucleotide of an arginine codon changing it to a tryptophan codon.
DISCUSSION

The region of chromosome 16 containing the LCAT gene appears to be replicated with a higher degree of fidelity than usual. Several observations lead one to this conclusion, although none explain why it should be so. The complete lack of the LCAT gene product is not lethal at any time during development, therefore individuals live to a reproductive age (and well beyond) passing the mutant gene causing LCAT deficiency from one generation to the next. In spite of this there are very few reported cases of LCAT deficiency in the world. Less than 40 cases have been reported over a wide geographical range including Japan, Europe and North America. The biochemical phenotype of these individuals varies considerably (table IV)(78) indicating that several different mutational events have occurred which cause LCAT deficiency. Of those individuals with LCAT deficiency whose LCAT genes have been cloned and sequenced to date, only single base pair changes have been found and each family has had a different mutation (J.McLean, personal communication).

The mutation detected in an LCAT gene of SF is a C-->T transition resulting in an amino acid substitution, arginine to tryptophan (CGG-->TGG). A paper has recently been published by Cooper and Youssoufian (101) discussing the CpG dinucleotide and human genetic disease. They collated the reports of single base-pair mutations within gene coding regions causing human genetic disease and found that 35% of these occurred within CpG dinucleotides. Over 90% of these mutations were C-->T or G-->A transitions which is 42-fold higher then that predicted by random mutation. Each mutation changed a codon specifying an amino acid, and the subsequent altered protein has given rise to the disease state.

The CpG dinucleotide occurs in vertebrate genomes at ~ 25% of the expected frequency (102) which is thought to be due to the susceptibility of cytosine, methylated at the 5' position, to undergo deamination to form thymidine (103)(106).
Five other mutations in the LCAT gene have been detected (J. McLean, personal communication). They are all single base pair changes, although none are C->T or G->A transitions. Two of the mutations cause a frameshift which leads to premature termination. The other three mutations each cause an amino acid substitution. A leucine to proline substitution in exon 2 causes a bend in the main chain of amino acids, thereby disrupting normal folding of the LCAT protein. LCAT enzyme activity in this individual is reportedly zero and there is no information on mass. In exon 6 two independent mutations have occurred resulting in amino acid substitutions. In one case arginine has been replaced by glycine resulting in an enzyme activity of 12% and protein mass of 24%. In the other case isoleucine has been substituted for methionone resulting in an enzyme activity of 8% and protein mass of 41%.

It is not surprising that a frameshift mutation leading to a premature stop codon would cause LCAT deficiency because if any protein were produced at all, it would only be part of the protein and unable to assume a normal conformation. With the mutations resulting in amino acid substitutions the most likely explanation is that proper folding of the LCAT protein was inhibited, resulting in severely decreased activity and mass. The decreased mass could be the result of either a failure to be secreted, or rapid degradation due to abnormal conformation.

There are examples of mutations in a gene that alter the protein product such that it cannot move from the rough endoplasmic reticulum to the Golgi complex in the LDL receptor (107). In the animal model, Watanabe heritable hyperlipidemic (WHHL) rabbits, a small in-frame deletion of 12 base pairs removing four amino acids from a cysteine-rich sequence of the LDL receptor disrupts its movement from the endoplasmic reticulum to the Golgi complex. Although the mutation does not remove any of the cysteine residues, it is thought to be likely that the folding pattern is disrupted preventing formation of proper disulfide bonds. Other precedents for mutant proteins that are blocked in transport from the endoplasmic reticulum to the Golgi
complex have been obtained through the study of induced mutations inexpressible cDNA's introduced into cultured cells by transfection (108,109).

Although the mechanism by which abnormal proteins are blocked in their movement is not yet clear, it has been suggested that these proteins are so denatured that they aggregate nonspecifically and therefore fail to be transported. (107) "It seems reasonable to propose that mutant proteins are trapped by detection mechanisms that are designed specifically to prevent the movement of abnormally folded proteins to the cell surface. Such fail-safe mechanisms may be crucial in biology because the surface appearance or secretion of even a small number of denatured molecules might lead to a harmful immune response." (107)

It has been noted in other regions of the genome, such as the gene for the LDL receptor (108), that Alu type sequences can be hot spots for recombination. There are three Alu-type repetitive elements, all in the same orientation in intron 5 within the LCAT gene and four Alu-type repetitive elements downstream of the 3' end of the LCAT gene, all of which are in the opposite orientation to those in intron 5 (55). Alu-Alu recombination resulting in the deletion of exon 6 has been a recurrent problem in the cloning of LCAT deficient genes. As discussed in 'Results' this was one of the cloning artifacts observed in the first positive LCAT clones obtained from the two individuals in the Fe family homozygous for LCAT deficiency. Some of the positive clones obtained by J. McLean also had exon 6 deleted due to Alu - Alu recombination (personal communication).

It has been suggested that recombination between Alu sequences may be a frequent cause of deletions in the human genome. Alu sequences consist of two tandem repeats referred to as the left arm (~132 nucleotides in length) and the right arm (~166 nucleotides in length), the latter being a duplication of the left arm with a 34-nucleotide insertion.
Alu sequences have been involved in 4 deletions characterized in the LDL receptor and four deletions reported in the γ, δ, β-globin gene complex (110). Deletions caused by recombination between two Alu sequences that are oriented in the same direction are most likely caused by homologous recombination in which unequal crossing over follows mispairing of homologous Alu sequences on different chromatids during meiosis (110). It has been proposed that recombination between Alu sequences oriented in opposite directions result from the formation of double stem-loop structures involving the same chromosome in which the complementary sequences at the ends of the deletion joint are brought into proximity (112)(113).

Alu recombination has not been observed in the LCAT gene of patients with LCAT deficiency. The alu-type sequences in intron 5 of the LCAT gene and 3' to the LCAT gene are oriented in opposite directions, therefore the second mechanism of recombination proposed above is likely the cause of the deletion of exon 6 during cloning of the LCAT gene.

When looking at the sequence of the DNA in the LCAT gene itself, the DNA appears to have been used very economically. The 3' region of the LCAT gene is unusually short. Only 23 nucleotides separate the final glutamine codon from the poly(A) tail. The poly(A) signal AATAAA is partially contained in the codon for the C-terminal glutamate (GAA) and the stop codon TAA (55). To my knowledge, such economy of DNA sequence has been reported in only two other eukaryotic nuclear genes (104,105).

Another unusual feature of the region of the LCAT gene is the lack of RFLP's. RFLP's are caused by single base pair change resulting in the creation or disappearance of a restriction enzyme cut site. They rarely are the cause of a mutation in a gene as a vast amount of the genome does not appear to code for a gene product. These changes are, therefore silent, nondeleterious changes in an individuals DNA which are passed through the generations. RFLP's can be used as markers to follow a particular
chromosome from generation to generation. This thesis presents the results of an extensive RFLP search in the 5' and 3' flanking regions of the LCAT gene with the result that not a single base pair change was detected. K. Wion (personal communication) has analyzed the LCAT gene itself for polymorphisms and been unable to detect any as has Rogne et al. The only other region of DNA detected so far that is replicated with such fidelity in the human genome that I am aware of is the X-linked granulomatous disease locus in which Orkin (111) reported that after extensive analysis of the locus, no RFLP's were found.

Given that the LCAT region of chromosome 16 is replicated with such a high degree of fidelity and that the LCAT gene itself is not essential for viability, it is possible that other genes or regulatory sequences adjacent to LCAT are essential for viability or that LCAT may reside within the intron of an essential gene coded for on the opposite strand, such as was found within the GART locus in Drosophila (114).

Significance of the mutation

A single base pair change (C->T) was the only difference detected from the published sequence of the normal LCAT gene (55). This single base pair change occurred in the first position of the codon for arginine, CGG-->TGG, causing an amino acid substitution to typtophan which is a very severe change. Arginine is a positively charged amino acid which in this case resides next to two more charged amino acids suggesting that this region may have a role in the folding of the LCAT protein. Tryptophan, on the other hand is a bulky, neutral amino acid, but more importantly, is the most hydrophobic of all the amino acids and therefore, would likely completely disrupt the function of this region.
Research tools

It is only with the advent of cloning and sequencing that a detailed investigation of the human, or any other genome, has been possible, but these are not the only methods of gaining insight into development and function of living systems. It is now possible to map the human genome using restriction endonucleases, so that, potentially there would be a nearby marker to aid in the study of any disease locus. More ambitious, but technically possible now, is to sequence the entire genome. This would create an enormous bank of information of loci coding for specific gene products and their regulatory regions, as well as providing information on the structure of the genome itself, possibly giving to the vast amount of DNA that appears to be present for no reason.

Site specific mutagenesis to gain understanding of the function of genes we are able to locate and the introduction of specific genes into mice to determine their effect on development are becoming powerful tools.

Ultimately, given enough understanding of the human system, gene therapy may be possible for individuals who lack specific gene product or produce an abnormal one.
FUTURE RESEARCH

More research is needed to understand how a mutation in exon 4 of the LCAT gene causes the phenotype seen in SF and DH, that of no immunologically detectable LCAT mass and no detectable LCAT activity (92,94):

1. LCAT mRNA extracted from a liver biopsy of one of the homozygotes could be analyzed to determine if a normal message was produced.

2. Liver tissue obtained from SF or DH and stained with antibody to LCAT would determine whether LCAT protein was present in the cells. If produced, the protein could be degraded within the cells or rapidly after being secreted from the cells. It is possible one would see an antibody reaction to LCAT protein within the hepatocytes indicating that the LCAT protein was produced, but degraded within the cell. Alternatively, a reaction could be detected both within the hepatocyte and in the region immediately surrounding the cell indicating that the protein was degraded immediately after secretion.

LCAT deficiency is an autosomal recessive disease, therefore there must be two mutant alleles present in an individual to cause the phenotypic expression of the disorder. It is possible that each allele contains a distinct mutation. Whether or not the homozygotes in this family are compound homozygotes could be determined by melting point hybridization experiments. By using an allele specific oligonucleotide containing the base pair substitution found in the LCAT gene of SF as a probe to analyze the heterozygotes from both sides of the family it could be determined whether the same, or a different mutation existed on each allele. This experiment would also confirm that this is actually a real mutation and not just a cloning artifact which is a possibility.

To address the question of why the LCAT gene and flanking DNA are replicated with a higher than usual degree of fidelity it would be interesting to probe RNA from
humans, and a number of other species representing various degrees of complexity in evolution, with the 5' and 3' flanking probes used to search for RFLP's. The information sought from this experiment would be whether or not the DNA in this region codes for a gene product and if it does, ask, whether or not the product is evolutionarily conserved. The argument for looking for conserved sequences is that they must be important for basic cellular function to remain essentially unchanged throughout evolution. With the DNA available for sequence analysis one could determine if the gene coded for a known product and thus place it accurately on the human gene map.
CONCLUSIONS

1. A cytosine to thymidine transition in exon 4 of an LCAT gene resulting in the amino acid substitution, arginine to tryptophan, is likely a cause of LCAT deficiency in a homozygote.

2. The LCAT region of chromosome 16 is replicated with a higher than usual degree of fidelity, although the significance of this is not yet known.
REFERENCES

16. Harpers Biochemistry, Murray et al. (eds), 21st ed., Appleton and Lange, Norwalk, Connecticut, USA.


59. unpublished results.


