

MOLECULAR STUDIES OF LIPOPROTEIN LIPASE

by

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## ABSTRACT

Molecular defects in the lipoprotein lipase (LPL) gene account for a significant proportion of the occurrence of the massive hypertriglyceridemia and other clinical manifestations of the chylomicronemia syndrome. Furthermore, some published reports suggest that the heterozygous state for LPL deficiency leads to a reduced capacity to clear triglyceride-rich lipoproteins from plasma; when this clearance is saturated through dietary or hormonal factors, moderate hypertriglyceridemia may be observed. The LPL gene of four hypertriglyceridemic subjects was examined for the presence of molecular variants. In one subject with the classical presentation of the chylomicronemia syndrome and documented deficiency of LPL activity in postheparin plasma, we have identified a mutation leading to the substitution of glutamic acid for glycine at residue 195 of the mature enzyme. The patient was homozygous for this mutation. By *in vitro* mutagenesis and transient expression in cultured mammalian cells, it was shown that the protein encoded by this mutated sequence lacked catalytic activity, and data on the homologous enzyme pancreatic lipase indicate that this mutation occurred within the catalytic domain of the enzyme. By contrast, we identified two other mutations in two of the three other hypertriglyceridemic subjects with moderate hypertriglyceridemia that did not appear of functional significance. Therefore, we have found no evidence of a role of molecular defects of LPL in these milder cases.

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## INTRODUCTION

### Lipid Transport in Plasma

Long-chain fatty acids of intestinal or hepatic origin transit in plasma after esterification with glycerol to form triacylglycerols. The transport of this hydrophobic material in an aqueous environment is achieved by associating the rather insoluble lipids with more polar ones, such as phospholipids, and combining them with cholesterol and protein to form hydrophilic lipoprotein particles. Synthesized by the intestine and the liver, lipoproteins undergo a series of catabolic steps before they are cleared by specific cellular receptors (Havel and Kane, 1989). Heterogeneity in size and density has led to the definition of discrete classes of lipoproteins, as summarized in Table 1. The characterization of their tissue of origin, lipid and protein composition, and metabolic fate has led to the model of plasma lipid transport and metabolism depicted in Figure 1.

### Disorders of Lipid Metabolism

A variety of pathological states are associated with either abnormal concentration or altered composition of some lipoproteins, referred to as hypolipidemias, hyperlipidemias and dyslipidemias. A classification of the hyperlipidemias (Table 2) has been introduced by Fredrikson and Levy (1967). Although this classification was based on an analysis of observed lipid profiles within families with several hyperlipidemic

Table 1. Physical and Chemical Features of Human Plasma Lipoprotein Classes.

Class	Density g/ml	Electrophoretic mobility	Diameter nm	Surface Components			Core Lipids	
				Ct	Pl	ApoLp	Tg	CE
Chylomicrons	0.93	Remains at Origin	75–1200	2	7	2	86	3
VLDL	0.93–1.006	Pre- $\beta$ -Lipoproteins	30–80	7	18	8	55	12
IDL	1.006–1.019	Slow-Pre- $\beta$ -Lipoproteins	25–35	9	19	19	23	29
LDL	1.019–1.063	$\beta$ -Lipoproteins	18–25	8	22	22	6	42
HDL2	1.063–1.125	$\beta$ -Lipoproteins	9–12	5	33	40	5	17
HDL3	1.125–1.210	$\beta$ -Lipoproteins	5–9	4	35	55	3	13

Legend: Surface Components and Core Lipids gives as % of Dry Mass.

Abbreviations: VLDL, Very Low Density Lipoprotein; IDL, Intermediate Density Lipoproteins; LDL, Low Density Lipoproteins; HDL, High Density Lipoproteins; CT, Total Cholesterol; PL, Phospholipids; ApoLp, Apolipoprotein; Tg, Triglycerides; Ce, Cholesterol esters.

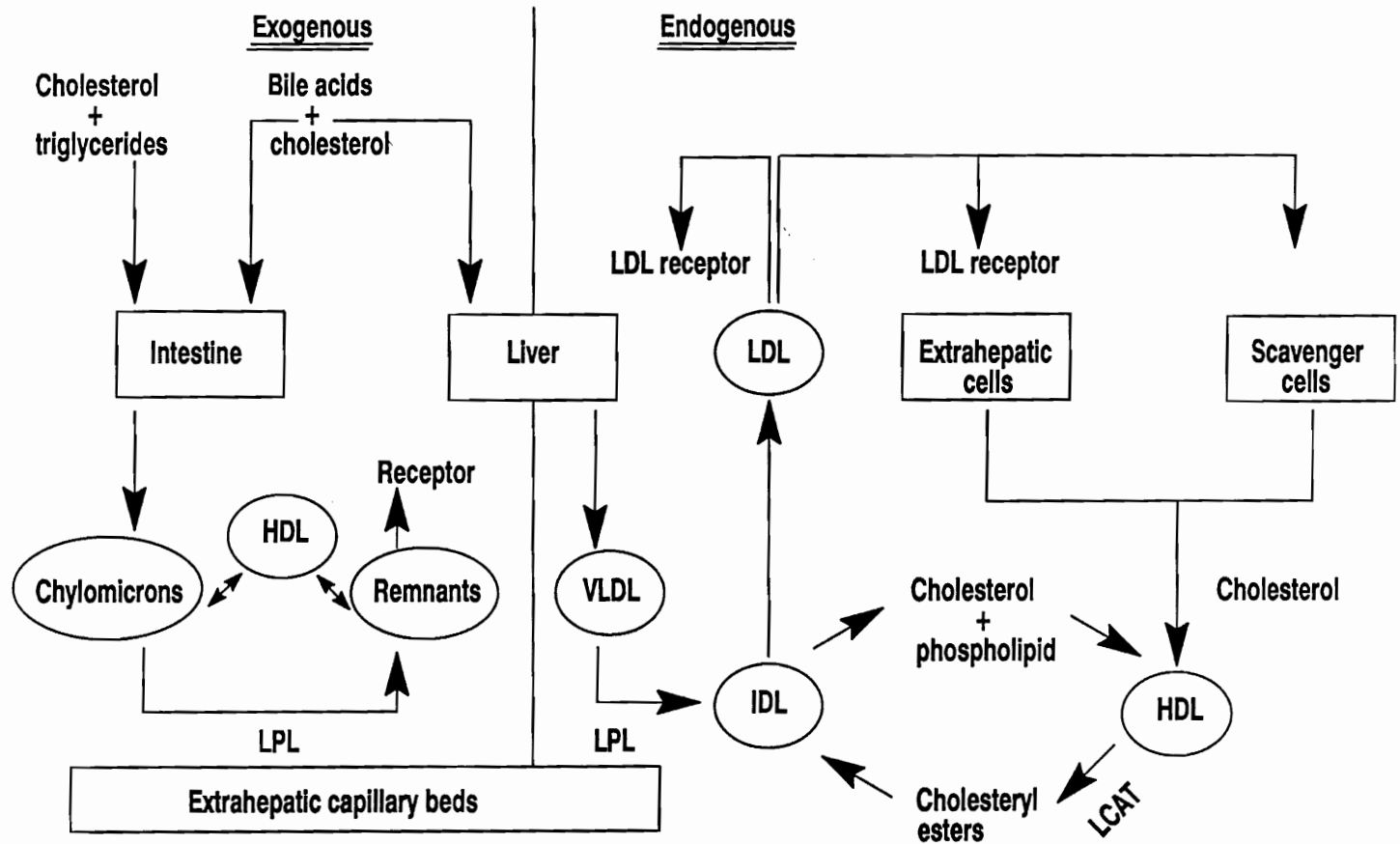


Figure 1. Lipoprotein transport.

Both the exogenous and endogenous cycles begin with the secretion of triglyceride-rich particles (chylomicrons and VLDL) that are converted to cholesteryl ester-rich particles (remnants, IDL, and LDL) through interaction with LPL. Abbreviations: LPL, lipoprotein lipase; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin cholesterol acyltransferase.

Table 2. Classification of Hyperlipoproteinemias.

Type	<u>Lipoproteins</u>			<u>Lipids</u>	
	Chy	VLDL	LDL	CH	TG
1	+++	N or ↑	N	↑	↑↑↑
2a	0	N	↑↑	↑↑	N
2b	0	↑	↑	↑	↑
3	0	↑↑ N or ↓ (abnormal)		↑↑	↑↑
4	0	↑↑	N	N	↑↑
5	++	↑↑	N	↑	↑↑↑

Note: Chy, chylomicrons; VLDL, very low density lipoproteins; LDL, low density lipoproteins; CH, cholesterol; TG, triglycerides; N, normal

subjects, it does not correspond to well defined genetic entities. Furthermore, clustering of different types of hyperlipidemias within families is rather common. This is particularly the case for the hyperlipidemias characterized by increased plasma concentration of triglycerides, where Type I, Type V, and Type IV profiles are often seen among close relatives.

High plasma concentrations of triglycerides may result from increased synthesis, impaired plasma catabolism, or decreased clearance of triglyceride-rich lipoproteins. By hydrolyzing triglycerides from chylomicrons and very low density lipoproteins (VLDL), the enzyme lipoprotein lipase plays a central role in their remodeling and eventual clearance. This graduate research work has consisted of the molecular analysis of the lipoprotein lipase gene in four hypertriglyceridemic subjects. Pertinent information regarding the biochemistry and gene structure of LPL, LPL deficiency states, and outline the rationale of the study will be summarized.

#### Lipoprotein Lipase : General Properties

Lipoprotein lipase (LPL; triacylglyceroprotein acylhydrolase, EC 3.1.1.34) is an extracellular enzyme synthesized by a variety of parenchymal cells including adipocytes, skeletal and cardiac muscle cells, the mammary gland and activated macrophages. After secretion from such producing cells, the enzyme diffuses through the vascular tree and becomes anchored to the surface of the capillary endothelium of extrahepatic tissues by an ionic interaction with the glycosaminoglycan heparan sulfate. The enzyme, which requires the specific cofactor apolipoprotein C-II for efficient catalytic activity, hydrolyses triacylglycerols by binding to the surface of chylomicrons and very low

density lipoproteins (VLDL), thereby releasing free fatty acids for uptake into the tissues where they can either be used immediately as fuel or reesterified for storage. General properties of the enzyme are presented in Figure 2. Thus the enzyme plays a key role in the metabolism of large triglyceride-rich lipoproteins to smaller particles, and determines the distribution of fatty acid energy among various tissues. General reviews summarize a vast literature on the biochemistry of this enzyme, such as Smith and Pownall (1984), Garfinkel and Schotz (1987), or Olivecrona and Bengtsson-Olivecrona, (1987).

Hormonal regulation of LPL has mainly been studied in adipose tissue or isolated adipocytes. Stimulation of adipose tissue enzyme activity has been demonstrated by insulin, glucocorticoids, adenosine analogues, gastrin and pancreozymin. Inhibitory effects on LPL activity in adipose tissue have been observed with catecholamines, dibutyryl cAMP and estrogens. Aspects of hormonal regulation of LPL activity are reviewed in Weinberg (1987).

#### Lipoprotein Lipase: Functional Domains

Similar to other lipases, it is assumed that LPL requires intact catalytic as well as interfacial lipid binding sites in order to hydrolyze lipid emulsions and lipoprotein particles. Although LPL readily binds to lipid-water interfaces, the enzyme also requires a protein cofactor, apolipoprotein C-II, to become fully active. The cofactor requirement of LPL disappears when soluble substrates are hydrolyzed. The striking homology that has been demonstrated between LPL, hepatic triglyceride lipase, and pancreatic lipase,

Serine Esterase (Ser-Asp-His Catalytic triad)

Active Enzyme: homodimer

Monomer: native 475 aa, mature 448 aa, 55 kDa, 8% carbohydrates  
glycosylated at Asn 43 and Asn 359

Substrates: triacylglycerols of lipoproteins, DAG, phospholipids, p-nitrophenylesters

pH Optimum: 8-9

Activated by Apo C-II

Figure 2. Physical properties of lipoprotein lipase.



may actually hint at the structure of some of the functional domains of LPL. In these enzymes there is a large region of homology, from residue 105 to residue 209 in human LPL. This region includes a putative sequence for interfacial lipid binding (human residues 126-135). References can be found in the general reviews quoted in the previous section.

Some work on bovine LPL and canine pancreatic lipase suggested that the active serine of human LPL may be located at residue 132. Recently, the three-dimensional structure of the human pancreatic lipase gene has been solved (Winkler et al., 1990), confirming that the catalytic site of pancreatic lipase (PL) consists of the Ser-Asp-His catalytic triad commonly found in serine proteases. The specificity of the enzyme for lipid substrates appears to be related to the buried localization of the active serine in a pocket with a high density of hydrophobic residues. The homology of human LPL and PL becomes manifest when both sequences are optimally aligned (Figure 3).

Other functional domains of LPL, such as the cofactor binding site and the heparin-binding site, remain to be identified. Furthermore, although the noncovalent homodimer structure of the active enzyme has been demonstrated, the sequence elements involved in this interaction are unknown. Consequently, the identification of molecular variants of LPL may provide an opportunity to identify such functional domains.

#### Lipoprotein Lipase: Gene Structure

The human LPL complementary DNA (cDNA) has been cloned from adipose tissue (Wion et al, 1987). It encodes a protein of 475 amino acids including an apparent

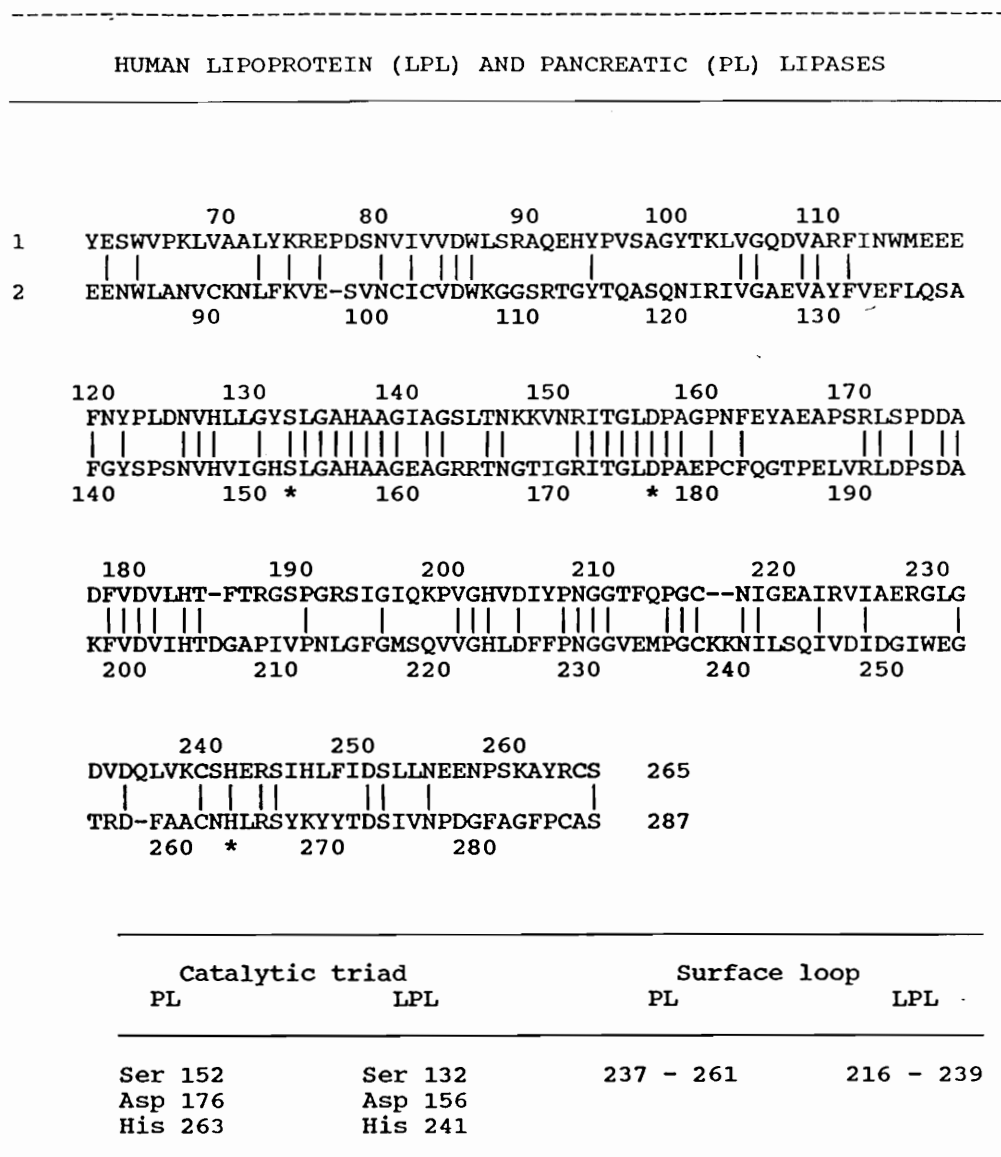


Figure 3. The homology of human lipoprotein lipase and pancreatic lipase: central region of homology.

signal peptide of 27 amino acids and three potential asparagine-linked glycosylation sites. LPL mRNA was detected in adipose tissue, adrenals, kidney and intestine, but not in liver, the HepG2 hepatoma cell line or white blood cells. Two hybridizing species of 3350 and 3750 nucleotides seem to represent LPL message terminating with roughly equal frequency at polyadenylation sites separated by 395 nucleotides. LPL shows extreme conservation across species, with 7% and 6% amino acid differences between human and murine or bovine proteins respectively (Datta et al., 1988). Furthermore, it displays strong evolutionary relationships with other lipolytic enzymes such as hepatic lipase and pancreatic lipase. The LPL gene maps to human chromosome 8, and its genomic structure has been determined (Deeb and Peng, 1989). It spans 30 kilobases, and includes 10 exons. Exons 1 to 9 range in size from 106 to 276 bp; exon 10 encodes the entire 3' noncoding sequence of the gene and is 1948 bp in length (Figure 4).

#### Lipoprotein Lipase Deficiency

Familial lipoprotein lipase deficiency is a rare autosomal recessive condition characterized by massive chylomicronemia, with triglyceride (TG) concentrations typically ranging from 1500 to 4500 mg/dl, normal or moderately elevated VLDL concentrations, and markedly depressed concentrations of both LDL and HDL (Nikkila, 1983; Brunzell, 1989). It is usually detected in infancy or childhood on the basis of repeated episodes of abdominal pain, recurrent acute pancreatitis, and eruptive xanthomas, but it can also remain asymptomatic and be detected through routine blood tests. Absent or very low LPL activity in postheparin plasma or adipose tissue

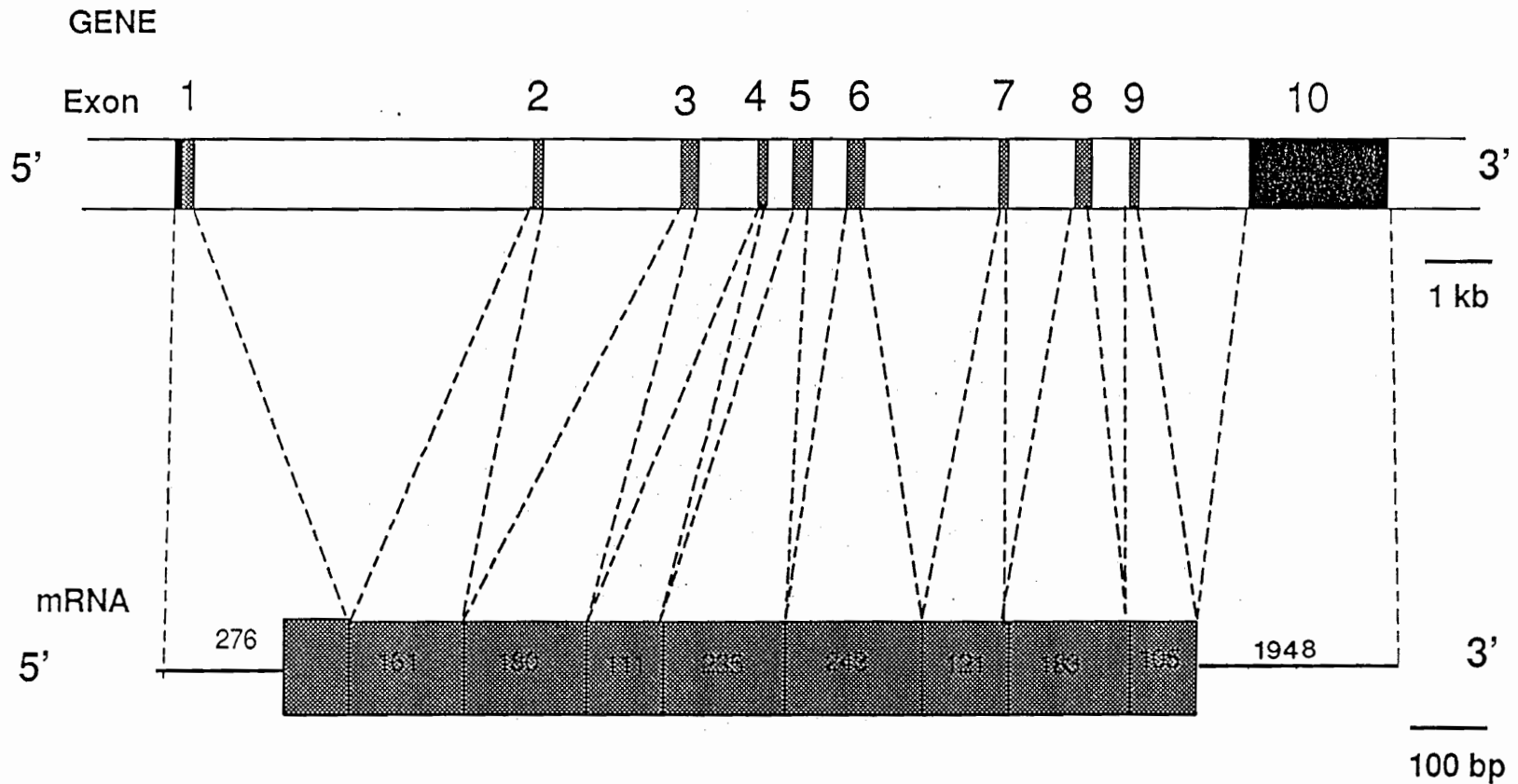


Figure 4. Structure of the human LPL gene.

The length of LPL gene is approximately 30 kb (the top portion of the figure). The 10 exons are represented by the filled boxes. Solid portions of exons represent non-coding sequences. The cDNA is interrupted by 9 introns giving rise to 10 exons. Exons 1-9 are of average size (105-276 bp) whereas exon 10, which codes for all of the 3' untranslated region of the mRNA, is 1948 bp in length (lower portion of the figure).

establishes the diagnosis. A recent study of LPL-deficient, unrelated probands revealed a major genomic rearrangement in 4 of 19 alleles (Langlois et al., 1989) resulting from an internal duplication disrupting the normal structure of the gene (Devlin et al., 1990). Other mutations of the gene, including several amino acid substitutions, have been reported (Emi et al., 1990; Hata et al., 1990; Beg et al., 1990; Dichek et al., 1991). Less often, a similar syndrome can be ascribed to functional deficiency or absence of the LPL cofactor, apo C-II, which can be diagnosed by assaying cofactor activity of the patient's plasma (Nikkila, 1983; Brunzell, 1989).

### Hypertriglyceridemia

The heterozygous state for LPL deficiency ( $LPL_h$ ) remains poorly characterized. Obligate heterozygotes are either normal or exhibit mild hyperlipidemia, and multiple lipoprotein phenotypes have been observed in some pedigrees. Measuring both mass and activity of LPL, Babirak et al. (1989) suggested that Hypertriglyceridemia in relatives of LPL deficient probands, possibly of the type seen in familial combined hyperlipidemia (HLP), segregated with  $LPL_h$ ; only about half of the subjects thus classified as heterozygotes exhibited HTG. Identifying carriers at the molecular level in a large pedigree, Wilson et al. were able to characterize the phenotypic expression of  $LPL_h$  (Wilson et al, 1990), with delayed expression of HTG, high VLDL cholesterol, subnormal LDL cholesterol and reduced HDL cholesterol concentrations.

Familial forms of HTG may result from  $LPL_h$ , at least in some families. Four familial lipid disorders featuring HTG have been defined (Havel and Kane, 1989): (1) in familial hypertriglyceridemia (FHTG), affected relatives have elevated plasma VLDL

but normal or low LDL concentrations (type IV); (2) in primary type V HLP, chylomicronemia and/or elevated VLDL are observed in family members (types V and IV); (3) in familial combined HLP (FCHL), multiple lipoprotein phenotypes are observed among relatives, including isolated elevation of VLDL (type IV), isolated elevation of LDL cholesterol (type IIa), or elevation of both (type IIb); (4) in type III HLP, a rare condition associated with premature atherosclerosis and usually homozygosity for the E2 electrophoretic variant of apolipoprotein E, high cholesterol and triglyceride concentrations result from the accumulation of abnormal  $\beta$ -VLDL particles. Although this purely phenotypic classification is unlikely to be of much etiological significance, it has served as a basis for clinical investigations over the past two decades.

The mechanisms underlying increased plasma VLDL remain poorly understood. Extrinsic factors such as obesity, diabetes, excessive alcohol intake, or medication with exogenous steroids contribute to the etiology of HTG. Some metabolic investigations in various HTG subjects (type IV or type IIb) would support a predominant role for VLDL overproduction, while others suggest that defective VLDL removal is of greater significance (Grundy, 1984; Grundy and Vega, 1988). Compositional differences of VLDL particles have been reported between type IV and type IIb probands. In type V subjects, turnover studies best support a combination of both overproduction and impaired clearance of VLDL. Assessment of the published evidence is further complicated by differences in patient selection and experimental procedures. At present, it is reasonable to conclude that both factors are at play to varying degrees in HTG patients. Whether overproduction or decreased clearance account for familial aggregation can also be debated. In the only large series where kinetic parameters were investigated

among type IV relatives of FHTG or FCHL probands (Sane and Nikkila, 1987), it was found that reduced clearance, but not overproduction, followed a familial pattern, and that no particular feature of VLDL triglyceride kinetics could distinguish type IV subjects in FHTG from type IV subjects in FCHL.

A common, saturable removal system for which both endogenous and exogenous triglycerides depend and compete was revealed by dietary manipulations in HTG subjects and appears related to LPL (Brunzell et al., 1973). An inverse relationship exists between fasting plasma TG concentration and postheparin plasma or adipose tissue LPL activities, the latter being negatively correlated with obesity. The relationship between HTG and LPL activity is blurred by variations in assay procedures. Furthermore, measurement of total LPL activity does not distinguish between differences in specific activity and differences in total pool of the enzyme; the latter is subject to a host of hormonal influences (Weinberg, 1987). Consequently, LPL activity in plasma collected after an heparin injection cannot serve as a predictor of the heterozygous state for LPL deficiency.

A combination of both overproduction and decreased clearance appears to be involved in most HTG subjects, and the phenotypic definition of familial syndromes must admit substantial etiological heterogeneity. Observations in such families, as well as on LPL<sub>h</sub>, support a role for an inherited defect of LPL in an as yet undefined subset of familial HTG. Interactions with other factors, genetic or environmental, account for variation in phenotypic expression. This is clearly the picture emerging from studies of familial type V HLP (Grundy and Vega, 1988). Overproduction may result from obesity, alcohol, estrogen use, or diabetes, while decreased clearance may result either from down

regulation or from inherited, partial deficiency in LPL.

### Rationale and Significance of the Present Study

Genetics, nutrition, and metabolic or hormonal disturbances influence plasma TG concentrations. Mutations leading to quasi-complete deficiency lead to type I HLP in the homozygous state, with a prevalence in the order of  $10^{-6}$ . Even such patients, however, express type IV HTG when adhering to a stringent low fat diet (Nikkila, 1983; Brunzell, 1989).  $LPL_h$ , a much more prevalent genotype, represents a latent lipolytic defect leading to delayed expression of HTG (type IV or type V) when triggered by nutritional, metabolic or hormonal factors.

Consequently, current study has examined the hypothesis that molecular variants of the LPL gene may account for HTG in 4 subjects referred to us by Dr Gerald Luc, from the Hospital Hotel Dieu in Paris, France. In subject K2042, the quasi-absence of LPL activity in post-heparin plasma suggested LPL deficiency in the homozygous state. Other subjects examined presented plasma triglycerides in excess of 1000 mg/dl, with either normal or elevated VLDL cholesterol (Type IV or Type V hyperlipidemia). The hypothesis was that these subjects could carry mutations of the LPL gene in the heterozygous state.



## MATERIALS AND METHODS

### Subjects

Four individuals with hypertriglyceridemia and one normal individual as a control (samples 1 to 5) were screened for the presence of molecular variants of LPL. (Table 3). One subject had classical symptoms of the chylomicronemia syndrome and documented deficiency of LPL activity in postheparin plasma. Other exhibited triglyceride levels in excess of 1,000 mg/dl, but no underlying metabolic disorder which could account for hypertriglyceridemia. Blood samples collected on ACD tubes were forwarded to this laboratory by special courier.

### Southern Blot Analysis

Total genomic DNA was isolated from peripheral blood by a standard method. For each subject, 3.6 µg of total genomic DNA was digested with the restriction enzymes PstI and StuI, respectively. Following 37°C overnight incubation, DNA was loaded on 0.5% and 0.7% agarose gels, electrophoresed at 22 to 25 Volts for 16 hours, and transferred to nylon membranes (Bio Trace™ RP, Gelman Sciences, Ann Arbor, MI). Hybridization was performed with the <sup>32</sup>P labeled cDNA clone pLPL35 as a probe for 10 hours. The membranes were washed in 0.1 X SSC/0.1% SDS washing solution

Table 3. Subjects Used in the Present Study.

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Subject Source	Sample #	Pedigree ID	Clinical Dianosis
France	1	K 2039	Hypertriglyceridemia
	2	K 2040	Hypertriglyceridemia
	3	K 2041	Hypertriglyceridemia
	4	K 2042*	LPL Deficiency
	5	Control	

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\* The offspring of a consanguineous marriage.

(1x SSC= 0.15M NaCl/15 mM Na citrate, ph 7.0) for 30 minutes at 52°C and submitted to autoradiography.

#### Detection of Molecular Variants as Conformational Polymorphisms

Each of the nine translated exons of the human LPL gene was enzymatically amplified (Mullis, et al., 1987) from genomic DNA on a Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CT) basically following the method of Orita et al. (1989). Oligonucleotide sequences used as primers are shown in Table 4. The reaction mixture contained 5.2 pmol of each primer, 2 nmol of each dNTP, 0.1 µg of genomic DNA, 1 µCi of [ $\alpha$ -<sup>32</sup>P]dCTP (3000Ci/mmol, 10 mCi/ml, Amershan) and 0.05 units of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT) in 10 µl of amplification buffer. This mixture was subjected to the iterative application of 10 reaction cycles each including denaturation at 95°C for 1 minute, primer-annealing (at 48°C for exon 1, at 43°C for exons 8 and 9, and at 54°C for other exons) for 1 minute, and primer-extension at 72°C for 1 minute. For all exons, 20 subsequent cycles were applied with primer-annealing performed at 60°C. After amplification, 50 µl of 0.1% SDS/10 mM EDTA was added in the mixture. An aliquot was withdrawn and mixed with equal volume of 95% formamide dye, boiled for 2 minutes and applied (2 µl/lane) to a 5% polyacrylamide gel containing 0.5 X TBE, with or without 10% glycerol. Electrophoresis was performed at 300 to 500V for 16 to 20 hours at room temperature or at 4°C. The gel was dried and autoradiographed with or without intensifying screen for 3 to 7 hours.

Table 4. Synthetic Oligonucleotides Used for Enzymatic Amplification of Exons 1-9 from Total Genomic DNA.

Exon	Primer Sequence
1.....	5'-GAAGGATCCAGGGTTGATCCTCATTACTGTTT-3' <sup>1</sup> 5'-AAGGAATTCAGGGGAGTTTGCGCG-3' <sup>1</sup>
2.....	5'-ACTGGATCCTCAAGCAACCCTCCAGTTAACC-3' <sup>2</sup> 5'-GATGAAGCGAATTCCTCTTCCCCAAAGAGCCTCC-3' <sup>1</sup>
3.....	5'-AGCGGATCCAAGCTTGTGTCATCA-3' <sup>1</sup> 5'-GATGAAGCGAATTCATAAGTCTCCTTCTCCCAGT-3' <sup>1</sup>
4.....	5'-CAAGGATCCGGCAGAAGCTGTAAGCACCTTCA-3' <sup>2</sup> 5'-GATGAAGCGAATTCAGACCAACGAAATTGCTTT-3' <sup>1</sup>
5.....	5'-TACGGATCCCATGCGAATGTCATACGAATGG-3' <sup>2</sup> 5'-AGTGAATTCGAAGCTACTGAGTAGGACATTGGG-3' <sup>2</sup>
6.....	5'-ATCGGATCCATCTTGGTGTCTCTTTTTTACC-3' <sup>1</sup> 5'-GATGAAGCGAATTCCTATTTACAACAGTCTCCAG-3' <sup>1</sup>
7.....	5'-ACAGGATCCATGTTTCGAATTTCC-3' <sup>1</sup> 5'-GATGAAGCGAATTCGATGACCGCCCCCAGAGCTA-3' <sup>1</sup>
8.....	5'-CCAGGATCCAATTTATTGCTT-3' <sup>1</sup> 5'-GATGAAGCGAATTCAGGAAGAAAATACATTTAATT-
3' <sup>1</sup>	
9.....	5'-CGTGGATCCTATTCACATCCATTT-3' <sup>1</sup> 5'-GATGAAGCGAATTCGTCAGCTTTAGCCCAGAATG-3' <sup>1</sup>

1. Derived from published exon boundaries (Deeb and Peng, 1989).

2. Derived from additional sequence information obtained by partial sequencing of cosmid cRHL2A (Hata et al., 1990b).

### DNA Sequencing

All translated exons for which a mobility shift was identified were sequenced by one or more of several methods:

(1) Direct sequencing from genomic DNA using single-stranded DNA template generated by asymmetrical amplification. A segment spanning exon 5 of the LPL gene was enzymatically amplified by the polymerase chain reaction and the resulting product was purified by spin-dialysis on a Centricon 100 column (Amicon, Danvers, MA). An aliquot (1  $\mu$ l) was used to generate single-stranded DNA by asymmetric amplification (Saiki et al., 1988). Sequencing was performed by application of a standard chain-termination protocol (Sanger and Coulson, 1975) with the enzyme Sequenase Version 2.0 (U.S. Biochemicals).

(2) Direct sequencing of double-stranded DNA excised from gels by way of a Thermocycling protocol. Bands of altered mobility revealed by electrophoresis of amplified DNA fragments spanning exons 1 and 9 of human LPL were excised from the dried gel, suspended in 100  $\mu$ l of H<sub>2</sub>O and vortexed. After incubation at 37°C for 1 hour, 1  $\mu$ l of each aliquot was subjected to enzymatic amplification. The sense- and antisense-primers included at their 5'-end a sequence complementary to the M13 universal (5'-TGTA AACGACGGCCAGT-3') and reverse (5'-CAGGA-AACAGCTATGACC-3') priming sites respectively. The reaction mixture was subjected to 30 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. Each

amplified product was spin-dialyzed on a Centricon 100 column (Amicon, Danvers, MA).

Direct sequencing of double-stranded DNA was performed on an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA) using fluorescent M13 primers, Taq polymerase, and a thermocycling protocol supplied by the manufacturer (ABI protocol, 1990). In brief, 12  $\mu$ l of a 40  $\mu$ l volume of amplified product were submitted to iterative extensions on a DNA thermocycler. Ten cycles were applied, defined by the following conditions: denaturation at 95°C for 30 seconds, annealing at 60°C for 1 second and extension at 70°C for 1 minute. This was followed by 10 additional cycles of denaturation (95°C for 30 seconds) and extension (70°C for 1 minute). The repeated application of several rounds of denaturation, reannealing and extension should increase the frequency with which termination occurs with incorporation of ddNTP rather than dNTP as would occur when template reannealing prevents further extension.

(3) Sequencing of cloned DNA segments. In some instances, DNA segments were sequenced after cloning into the M13mp18 vector. These segments were generated by enzymatic amplification of genomic DNA using 100 pmol of each pair of primers and 2.5 units of Taq polymerase in 100  $\mu$ l of amplification buffer, with conditions as reported earlier. Since the primers used for amplification were augmented with recognition sites for the endonucleases BamHI and EcoRI, each amplified fragment could be directionally cloned into the M13mp18 vector after restriction

enzyme digestion. After bacterial transformation and plating, 6 independent clones were isolated, and single-stranded DNA was produced directly from plaques suspended in distilled water by asymmetric enzymatic amplification (Saiki et al., 1988), using the M13 universal and reverse primers. Automated analysis of DNA sequence was performed by means of an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA).

#### Allele-Specific Oligonucleotide Hybridization

To verify the presence and test for the zygosity of the mutation identified by sequencing, genomic DNA from the subjects analyzed was submitted to enzymatic amplification for specific exon fragments. Fifteen  $\mu\text{l}$  (1/7th) of the amplification products were denatured in 200  $\mu\text{l}$  of 0.4 N NaOH/25 mM EDTA solution and 90  $\mu\text{l}$  were spotted in duplicate onto a nylon membrane (Bio Trace<sup>TM</sup> RP, Gelman Sciences, Ann Arbor, MI) using the BIO-DOT SF apparatus (BIO-RAD, Richmond, CA). After neutralization with 90  $\mu\text{l}$  of 3 M sodium acetate (pH 5.4), DNA was cross-linked by STRATALINKER (STRATAGENE, La Jolla, CA). Each membrane was hybridized in 50 mM sodium phosphate, pH 7.2/0.9 M NaCl/1 mM EDTA/0.5% SDS at 42°C with <sup>32</sup>P-end labeled oligonucleotide probes. Three distinct base substitutions, located in exon 1, 5 and 9 respectively, were studied by this method. The base substitutions and the oligonucleotide sequences used in this study are summarized in Table 5. After hybridization for 2 hours at 42°C, the membranes were washed in 6x SSC ( 1 x SSC = 0.15 M NaCl/15 mM

Table 5. Synthetic Oligonucleotides Used as Probes for Slot-Blot Hybridization.

Exon	Probe	Oligonucleotide Sequence
1	Wild Type	5'-AGTGAATTTAG <u>G</u> TCCCT-3'
	Mutant	5'-AGGGAC <u>C</u> TAAATTCACT-3' 5'-AGTGAATTTA <u>A</u> GTCCT-3'
5	Wild Type	Ser Ile Gly Ile Glu Lys 5'-AGC ATT <u>G</u> GA ATC CAG AAA-3'
	Mutant	<u>G</u> AA Glu 5'-TTTCTGGATT <u>C</u> CAATGCTT-3' 5'-AAGCATTG <u>A</u> AATCCAGAAA-3'
9	Wild Type	Lys Lys Ser Gly 5'-AAG AAG <u>T</u> CA GGC TGG-3'
	Mutant	<u>T</u> GA stop 5'-TAAGAAGT <u>C</u> AGGCTGGT-3' 5'-TAAGAAGT <u>G</u> AGGCTGGT-3'



Na citrate, pH 7.0) at 1 to 2°C below the melting temperature computed for each probes, and submitted to autoradiography.

### In Vitro Mutagenesis

Mutagenesis was carried out as described by Kunkel (1985) with some modification, using a Muta-Gene M13 in vitro mutagenesis kit, version 2 (BIO-RAD). At first, wild type LPL cDNA was released from the clone pLPL35 after digestion with SalI (Wion et al., 1987), and the SalI sites were converted to EcoRI sites using EcoRI linkers. After digestion with EcoRI, this fragment was cloned into the EcoRI site of M13mp18 (Figure 5). Oligonucleotide containing the 839 nucleotide mutation (5'-TCGA-AGCATTGAAATCCAGAAACCAG-3') (exon 5) and 1595 nucleotide mutation (5'-TA-AGAAGTGAGGCTGAAAC-3') (exon 9) were used as mutagenic primers.

The template for in vitro mutagenesis was prepared by propagation on the dut, ung double mutant bacterium CJ236, leading to the incorporation of Uracil at positions normally occupied by Thymine. A complementary strand was synthesized with the T7 DNA polymerase and closed circles were formed by the T4 DNA ligase. This DNA was transformed into *E. coli* DH5 $\alpha$ F', a strain proficient in uracil N-glycosylase activity, leading to efficient inactivation of the uracil-containing strands, and thereby yielding the non-uracil-containing survivor available for replication. Mutant plaques were selected by oligonucleotide hybridization (Figure 6). DNA of M13 phage in the replicative form (RF) was isolated by an alkaline lysis miniprep method (Birnboim, 1983), digested with EcoRI and inserted into the EcoRI site of the eukaryotic expression vector pMT2 (Kaufman et al., 1989). After transformation of *E. coli* DH5 $\alpha$ , 6 to 12 colonies were

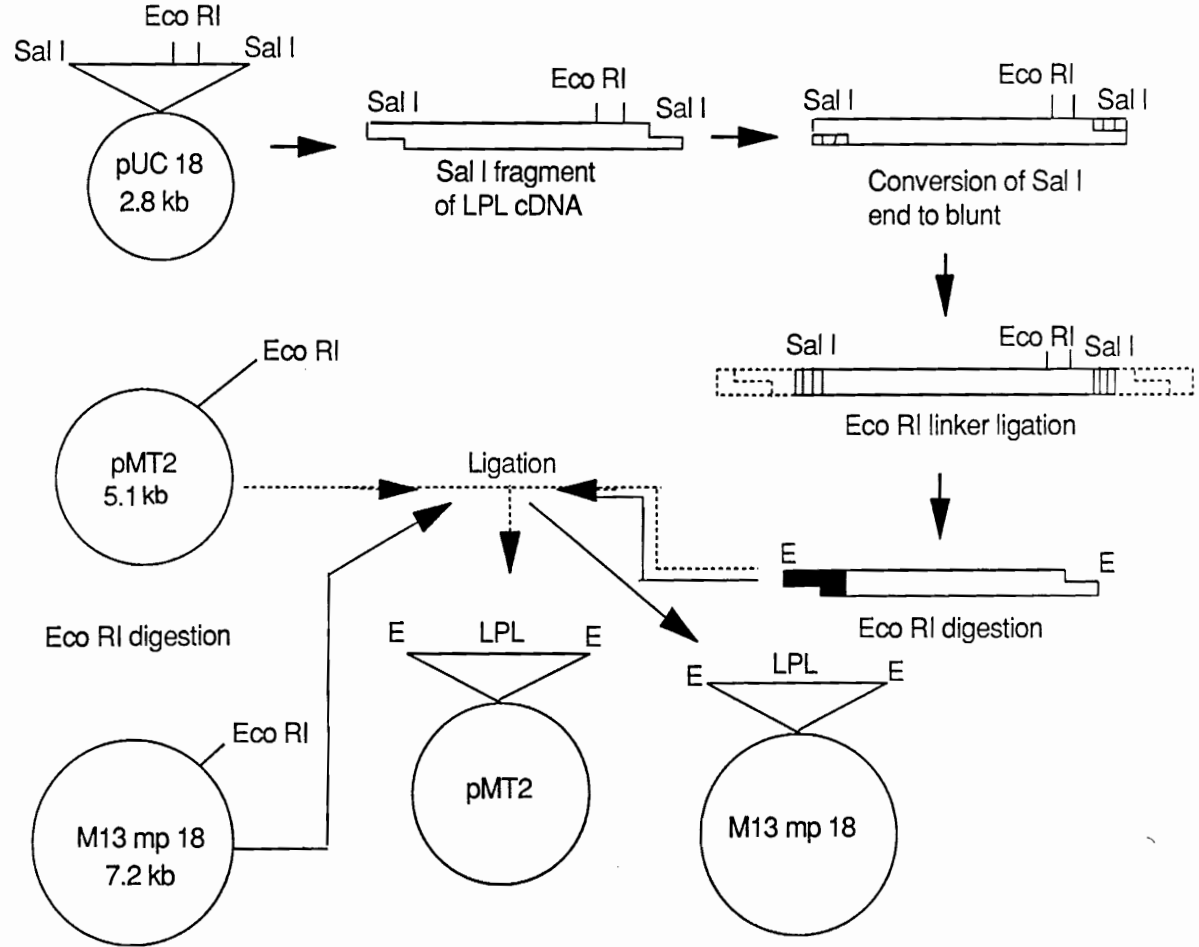


Figure 5. DNA constructions.

The clone of LPL cDNA with expression vector pMT<sub>2</sub> will be used as a wild-type control of transfection whereas the clone of LPL cDNA with phage vector M13mp18 will be used for preparing uracil-containing template.

Uracil-Containing M13 Recombinant

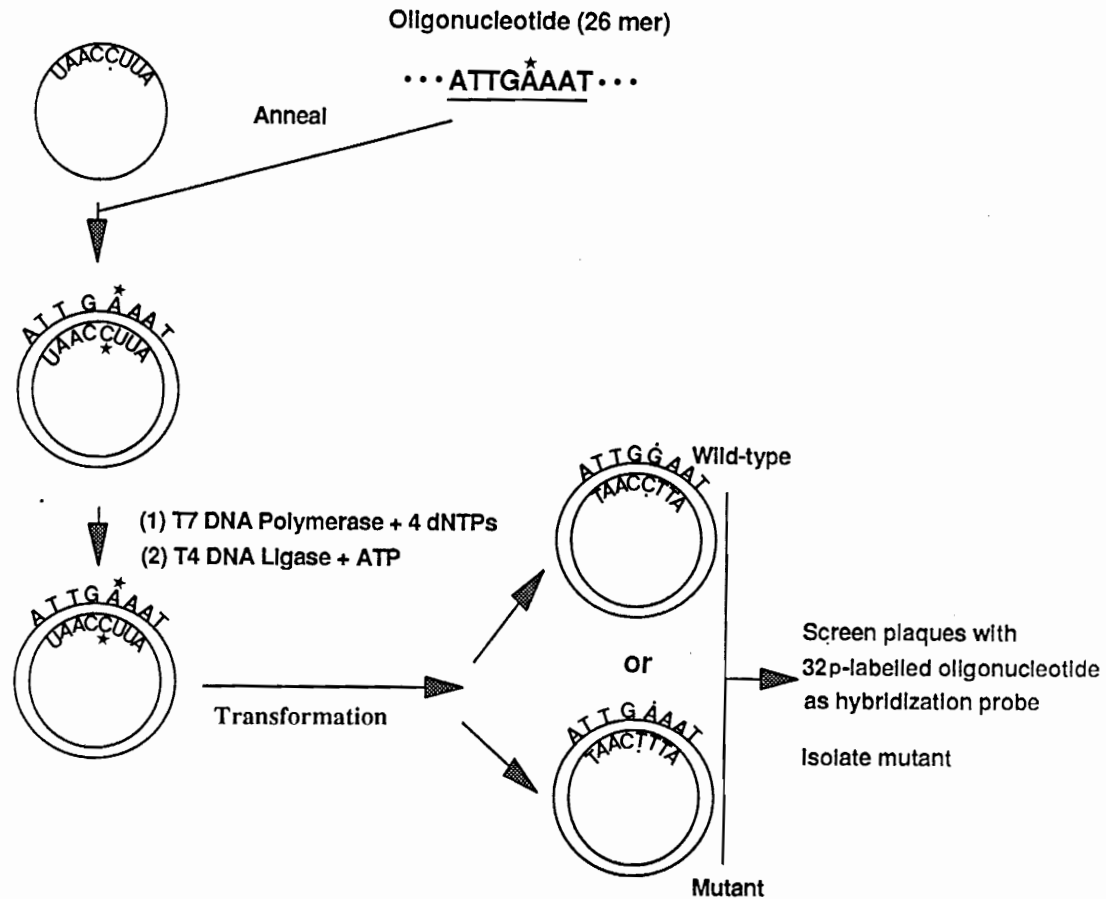


Figure 6. Oligonucleotide-directed mutagenesis of 195 (Gly→Glu) mutation. Asterisks indicate mismatched bases while dots show two types of progeny after transformation.

picked for culturing to provide DNA. Proper orientation of insert was determined by digestion with the restriction enzyme PvuII. Sequencing analysis of the inserts confirmed that either the G to A substitution at position 839 or the C to G substitution at position 1595 were the only change introduced in the mutant construct.

### Transient Expression in Cultured Mammalian Cells

COS-1 cells were plated at an initial density of  $1.0 \times 10^6$  per 10 cm dish and maintained in 5 ml of Dulbecco's modified Eagle's medium with 10% FCS before transfection. They were transfected in triplicate with plasmid DNA (6  $\mu\text{g}/\text{dish}$ ), following the DEAE-dextran method described by Selden<sup>(21)</sup> with some modification. One hour after DNA addition, chloroquine was added to the medium at a final concentration of 80  $\mu\text{M}$ . After 4 hours of incubation at 37°C, the cells were treated with dimethyl sulfoxide (DMSO). The 5 ml of 10% DMSO/PBS was added to each plate and incubated for 2 minutes at room temperature. After aspirating the DMSO, each plate was then washed with 5 ml of 1x PBS. Afterwards, 5 ml of complete medium was added to each plate and incubation was carried out at 37 °C . Twenty-four hours after transfection, serum-free, heparin-containing (5 U/ml) medium was substituted and the culture was allowed to continue for another 48 hours. Conditioned medium and cells were collected to assay LPL activity and immunoreactivity.

### Assay of LPL Mass and Activity

LPL mass was determined by a sandwich enzyme-linked immunoadsorbent assay using purified bovine LPL as the standard (Iverius and Östlund-Lindqvist, 1976). LPL

activity in conditioned medium or cell lysates was determined by monitoring the hydrolysis of triacylglycerols in an emulsion composed of a radiolabeled triolein substrate and Intralipid as an emulsifier, as described by Iverius et al. (1985,1986). Both assays were performed in the laboratory of Dr P.H. Iverius at the Veterans Affairs Hospital, Salt Lake City.

#### Affinity Chromatography on Heparin-Sepharose

Analytical heparin-Sepharose chromatography was performed at 0°C using an FPLC system (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ ) equipped with a FRAC-100 fraction collector, a HR 5/10 column, and a 10 ml Superloop for sample loading. The column (2 ml) was packed with heparin-Sepharose CL-6B, prepared as described previously (Iverius et al., 1971, 1976), submerged in a cylinder of ice-water and equilibrated with 0.15 M NaCl/0.1% (v/v) Triton X-100/0.01 M Na-phosphate buffer (pH 7.5). Samples were loaded from a loop at a flow rate of 0.2 ml/min, after which the column was washed with 19.5 ml of the equilibration buffer. Elution was performed with an 8 ml linear gradient of 0.15 to 1.5M NaCl in 0.1% (v/v) Triton X-100/0.01 M Na-phosphate buffer (pH 7.5) at a rate 0.05 ml/min. Finally, the column was stripped with 6 ml of 2 M NaCl in the same buffer at a rate of 0.2 ml/min. Fractions of 0.5 ml were collected throughout the procedure in tubes chilled by packing ice into the fraction collector carousel.

## RESULTS

It is hypothetically proposed that molecular variants of the LPL gene can account for either LPL deficiency, as documented by lack of LPL activity in the postheparin plasma of subjects K2042, or for a subset of more common forms of primary hypertriglyceridemia, as observed in the other three subjects under study. Mutational events that might affect the gene range from major genomic rearrangements, such as large insertions or deletions, to single nucleotide substitutions. Both of these extremes have been previously observed in the LPL gene of LPL deficient subjects.

### Probing Total Genomic DNA for Major Rearrangements

Major rearrangements in a gene can be detected by probing genomic DNA after digestion with restriction endonucleases. This is of particular relevance here, given the report of a common internal duplication in the LPL gene of LPL deficient patients (Devlin et al., 1990).

Total genomic DNA was digested with the restriction endonucleases PstI and StuI, electrophoresed, transferred to nylon membranes, and hybridized to a radiolabelled probe containing the entire coding region of the LPL cDNA (pLPL35, a gift from Dick Lawn; Wion et al., 1987). The results are presented in Figure 7. None of the 5 subjects exhibited any obvious molecular defect.

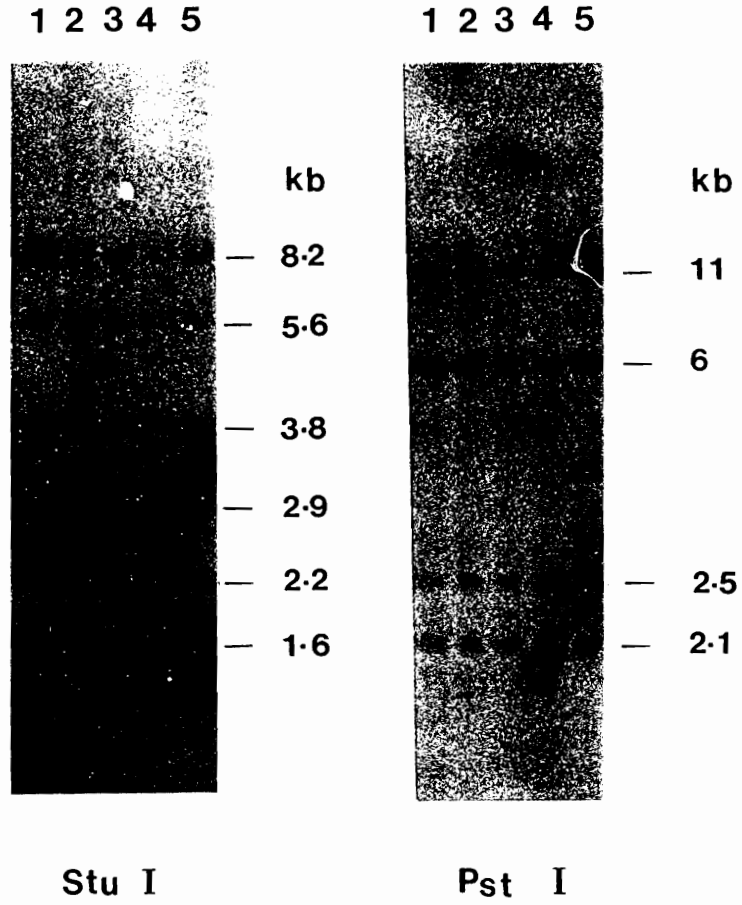


Figure 7. Experimental results from Southern-Blot hybridization. The total genomic DNA of 5 study subjects was digested with the restriction enzyme Stu I and Pst I. None of the subjects has major gene rearrangement.  
Lane 1: K2039, Lane 2: K2040, Lane 3: K2041  
Lane 4: K2042, Lane 5: control

### Detection of Single Nucleotide Substitutions

Minimal alterations such as nucleotide substitutions eventually require DNA sequencing for their characterization. Given the total size of the LPL gene, approximately 30,000 nucleotides, direct examination of the entire gene is not practical. There are, however, a variety of procedures which allow to screen segments of genomic DNA for the presence of such variants.

The approach followed in this work relies on the detection of molecular variants through mobility shifts revealed upon electrophoresis of single-stranded DNA under nondenaturing conditions. Single-stranded DNA is known to present ordered structures in solution, although such structures are much less stable than those adopted by double-stranded DNA. It has been shown that even a single nucleotide substitution can induce a conformational change of single-stranded DNA which can be detected as a mobility shift upon electrophoresis under nondenaturing conditions (Kanazawa et al., 1986). This was developed into a method, called by the authors "single-stranded conformation polymorphism (SSCP)," for the direct detection of base substitutions in DNA segments by Orita et al. (1989). A given DNA segment is amplified by the polymerase reaction, labelled during this process through incorporation of a radioactive nucleotide, denatured by heat in the presence of formamide, electrophoresed on a polyacrylamide gel under nondenaturing conditions, and the latter is subjected to autoradiography.

This method has been used to examine the nine coding exons and corresponding splice junctions of the LPL gene in five study subjects. The primers used for this purpose are presented in Table 3. As the conformations adopted are quite sensitive to conditions such as pH, ionic strength, temperature and solvent, four distinct electrophoretic



conditions were tested. Results obtained are summarized in Figure 8 and Table 6.

Three distinct mobility shifts were observed. In subject K2039, a mobility shift was observed in exon 1 when electrophoresis was performed in the cold room in the presence of glycerol. In subject K2041, another mobility shift was observed in exon 9 under two experimental conditions. Subject K2042 presented a third electrophoretic variant in exon 5 of the gene.

#### Identification of Molecular Variants by DNA Sequencing

To identify the sequence variation at the origin of the observed mobility shifts, each observed electrophoretic variant was subjected to DNA sequencing. Three distinct protocols were applied.

In the first method, direct sequencing by the chain termination method is performed on DNA segments produced by enzymatic amplification from total genomic DNA. A drawback of this method, however, is that it may be difficult to identify molecular variants present in the heterozygous state.

The second method, developed in this laboratory by Hata et al. (1990a), exploits the electrophoretic separation to purify and sequence an individual allele. Each band representing a mobility shift was excised from the gel, resuspended in distilled water, and subjected to enzymatic amplification with unique primers extended with universal M13 direct and reverse primers. After purification of the amplified DNA segment on a spin column, an aliquot of double-stranded DNA was sequenced on an automated instrument (ABI373A) by the chain termination method using a newly developed thermocycling protocol (ABI protocol, 1990).

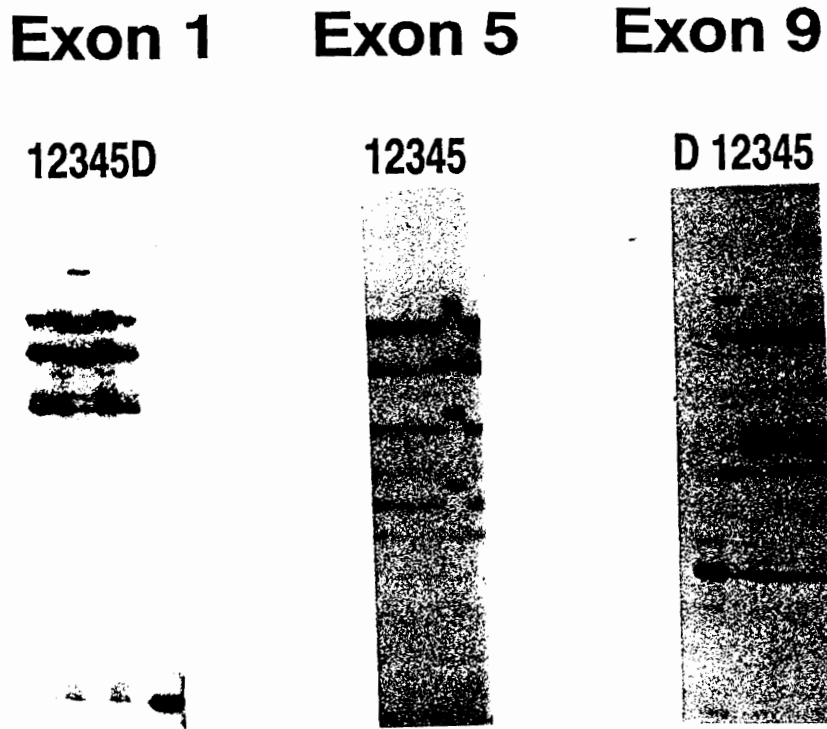


Figure 8. Experimental results from conformational polymorphism. All nine translated exons were examined by this analysis. Only sample 3 (K 2041) has band displacements in exon 1; sample 4 (K 2042) has band mobility differences in exon 5; sample 1 (K 2039) has distinct band shifts in exon 9. D: non-denatured, double stranded DNA used as a distance control.

Table 6. Analysis of Conformational Polymorphism and Results.

Exon	K 2039				K 2040				K 2041				K 2042				Control			
	Gly (+)		Gly (-)		Gly (+)		Gly (-)		Gly (+)		Gly (-)		Gly (+)		Gly (-)		Gly (+)		Gly (-)	
	4C	22C	4C	22C	4C	22C	4C	22C	4C	22C	4C	22C	4C	22C	4C	22C	4C	22C	4C	22C
1	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Gly (+) -- Polyacrylamide gel with 10% glycerol

Gly (-) -- Polyacrylamide gel without glycerol

+ : Mobility shift detected

- : No mobility shift

The third method relied on subcloning of DNA segments amplified from total genomic DNA. Primers were designed to include recognition sequences of restriction endonucleases. This allowed to clone the amplified segment into the M13mp18 vector. After bacterial transformation, identification of insert-containing plaques, and generation of single-stranded DNA by asymmetric amplification from such plaques, sequencing was performed by the chain termination method following a conventional protocol. To allow for heterozygosity as well as misincorporation artifacts which may have occurred during the amplification steps, several independent clones were sequenced for each target.

For each segment at the origin of the observed mobility shifts, a single nucleotide substitution was observed (Figure 9). The exon 1 variant identified in subject K2039 by the second protocol occurred in the noncoding region located upstream of the first translated codon. This variant has been identified previously in this laboratory as a common polymorphism, and the subject was heterozygous for this mutation. No further analysis of this variant was undertaken.

The exon 9 variant was also identified by application of the second protocol. It is a single transversion of a cytosine to a guanine at position 1595, which introduces a termination codon at the position normally encoding a serine at residue 447 of the mature enzyme. The consequence of this mutation is the production of a truncated protein lacking two residues at the carboxy terminus of the protein. This variant was previously identified in this laboratory (Hata et al., 1990a) and shown to occur in 30% of Caucasian subjects tested in Utah. The electrophoretic pattern revealed in the subject corresponded to the heterozygous state. Transient expression of this mutation in cultured mammalian cells yielded an enzyme similar to wild type in both activity and affinity for heparin.

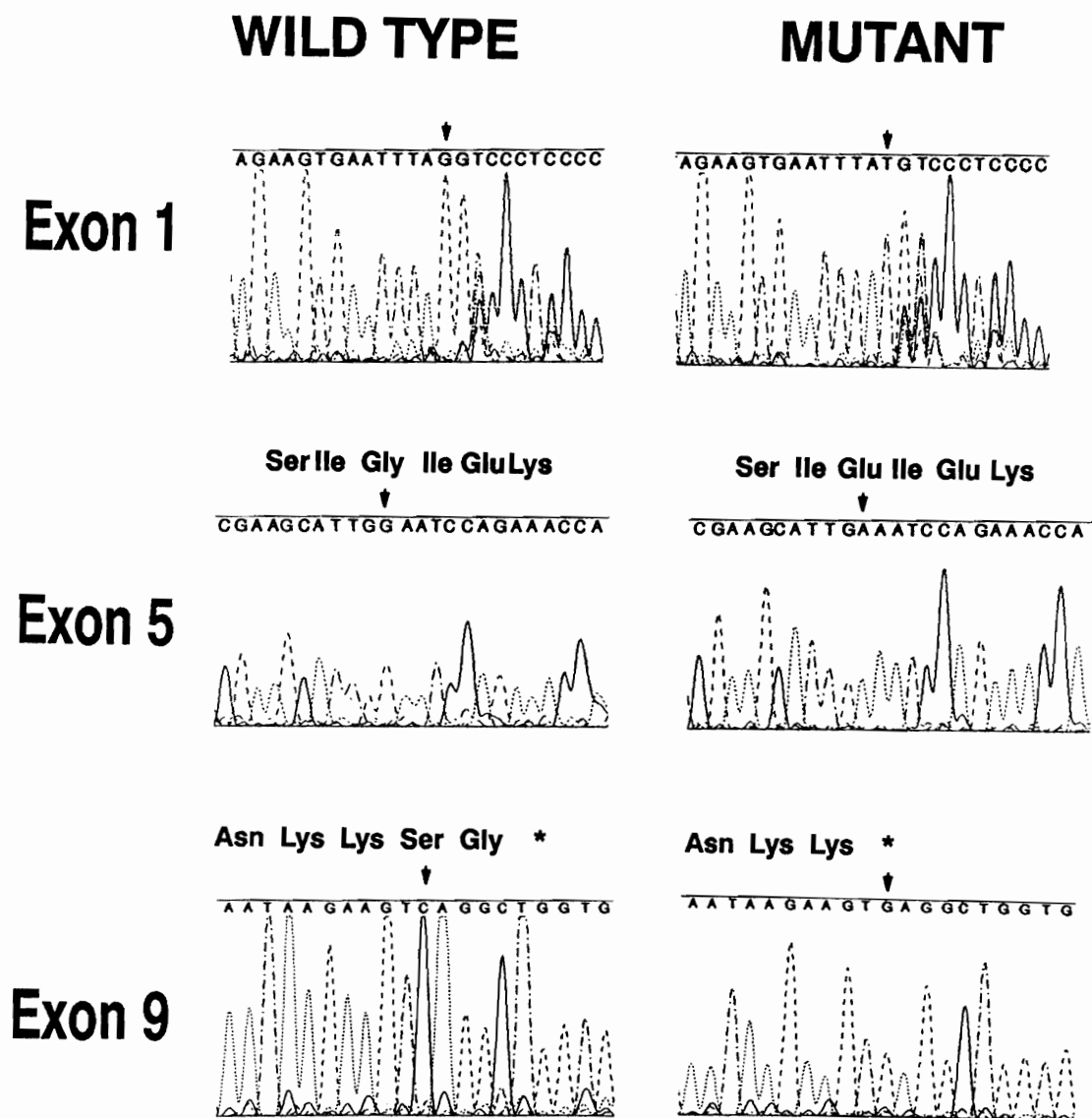


Figure 9. Comparison of normal nucleotide sequence with mutant sequences found in exons 1, 5, 9, of the LPL gene. Arrows point the base change from normal to mutant. Asterisks indicate the stop codon.

Individual K2042, who presented typical LPL deficiency, was the offspring of a consanguineous marriage. Furthermore, the electrophoretic pattern exhibited by this subject, with an apparent absence of bands of characteristic wild type mobilities, suggested that he might be homozygous for a mutation in exon 5 of the LPL gene. Consequently, direct sequencing was first applied. A guanine-to-adenine transition at nucleotide position 839 was observed. This leads to a missense mutation with the substitution of glutamic acid for glycine at residue 195 of the mature enzyme. The presence of this mutation was confirmed when two clones were sequenced by the third method: both clones contained this mutation. Hybridization of total genomic DNA of the patient with synthetic oligonucleotides specific for wild type and mutant sequences at this position established that the patient was homozygous for this mutation (Figure 10).

#### The 839A Mutation Encodes a Functionally Inactive Enzyme

The functional significance of the amino acid substitution observed in subject K2042 cannot be inferred from the sequence alone. Therefore, the mutation was reproduced in vitro, expressed in cultured cells, and cell lysates and conditioned media were assayed for several criteria of LPL.

The experimental protocols used were presented in the previous section, and therefore they are only briefly outlined here. As LPL is a glycoprotein, a mammalian expression system was used. Mutagenesis was performed by second-strand synthesis on a wild type template initiated by a mutagenic synthetic oligonucleotide which differed from the wild type sequence only with respect to the observed mutation. The wild type strand had been prepared in a deficient bacterial strain, which resulted in the

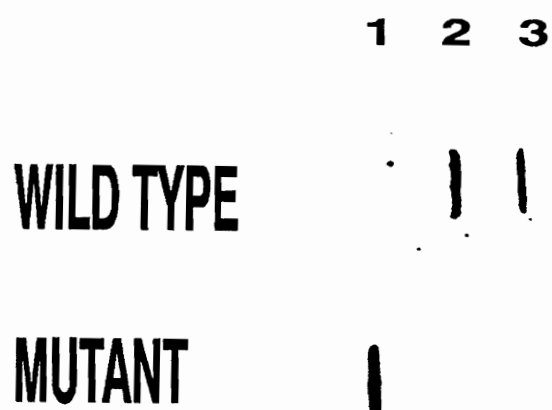


Figure 10. Experimental results from allele-specific oligonucleotide hybridization. Allele-specific oligonucleotide hybridization after enzymatic amplification of a segment of exon 5 from the LPL gene. Column 1 corresponds to sample 4 (K 2042) (homozygous for this mutation). Columns 2 and 3 are normal controls (homozygous for wild type).

incorporation of uracil instead of thymine at a number of positions. As a consequence, when double-stranded heteroduplexes generated at the mutagenic step were propagated in a wild type bacterial strain, the uracil-containing strand was hydrolyzed, yielding a high proportion of plaques containing only mutant phages. Several plaques were selected and the presence of the desired mutation was confirmed by DNA sequencing. The LPL insert of one such phage was released and transferred into the expression vector pMT2, where the LPL gene is placed under the control of the major promoter of Adenovirus. After transfection of cultured monkey kidney cells (COS-1 cells), cell lysates and conditioned media were collected and analyzed by Dr P.H. Iverius, at the Veterans Affairs Hospital. A positive control was provided by parallel transfection with a vector containing the wild type sequence.

The results of such assays are presented in Table 7. Immunoreactive LPL material was detected with a monoclonal antibody for human LPL in cell lysates and conditioned media obtained after transfections with both wild-type and mutant sequences. However, while transfection with the wild-type sequence led to the recovery of active enzyme in both cell lysates and conditioned medium, no such activity could be detected in cells and media of transfection experiments performed with the vector containing the 839A mutation. Preliminary results indicates that the immunoreactive material detected in cells transfected with pMT2-LPL839A has slightly reduced affinity for heparin compared to wild-type enzyme when analyzed by heparin-Sepharose chromatography.

In conclusion, data obtained demonstrate that the 839A mutation leads to the production of functionally inactive enzyme, which accounts for the enzymatic deficiency noted in subject K2042.



Table 7. LPL Activity and Mass in the Medium and Lysate of Transfected COS-1 Cells.

Sample	Medium		Cell Lysate	
	Mass ng/ml	Activity nmol/min/ml	Mass ng/ml	Activity nmol/min/ml
Wild Type	151	73	643	100
195 Mutant	24	0	629	0
Wild Type	200	58	-	-
447 Mutant	183	55	-	-

## DISCUSSION

Four hypertriglyceridemic subjects for the possible presence of mutations in the LPL gene have been examined. No major genomic rearrangement was revealed when total genomic DNA digested with selected enzymes was resolved by electrophoresis, transferred to nylon membranes, and hybridized to a cDNA probe spanning the entire coding region of the gene. Subsequently, genomic segments of DNA enzymatically amplified by the polymerase reaction were submitted to electrophoresis on polyacrylamide gels in an attempt to detect mobility shifts induced by nucleotide substitutions. Three distinct electrophoretic variants were detected, and the sequences of the corresponding DNA segments were determined.

A nucleotide substitution identified in exon 1 of the gene occurs upstream from the translated region. This variant was previously identified in a number of other subjects in this laboratory, and it is likely to represent a common polymorphism. Calculations based on estimates of nucleotide substitution and the neutral theory of molecular evolution predict that one such polymorphism is expected for every 100 to a few hundreds nucleotides in regions devoid of functional significance. Without evidence for any function of the sequence involved, it is reasonable to assume that this common polymorphism is without biochemical significance.

Another subject presented a nucleotide substitution in exon 9 of the gene which introduces a premature stop codon. As a consequence, this molecular variant encodes a

truncated protein lacking the last two amino acids normally observed at the carboxy-terminal end of the protein. This variant had also been identified previously in this laboratory, occurring among 30% of Utah subjects of Northern European descent (Hata et al., 1990a). The mutation was reproduced in vitro and expressed in cultured mammalian cells. The truncated protein was similar to the wild type enzyme with respect to the properties examined: enzymatic activity towards a triolein lipid emulsion, immunoreactivity with a monoclonal antibody, and affinity for heparin when analyzed by heparin-sepharose chromatography.

The third variant identified corresponded to a nucleotide substitution in exon 5 of the LPL gene, leading to a single amino acid substitution at position 195 of the mature enzyme, with the presence of glutamic acid instead of glycine. This mutation was present in the homozygous state in the patient. When expressed in cultured cells, this sequence yielded immunoreactive but inactive LPL. Preliminary data indicated that affinity for heparin, as examined by chromatography on an heparin-Superose column, was not impaired in a major way. This patient presented classical LPL deficiency, with no significant LPL activity in post heparin plasma, and he was the offspring of a consanguineous marriage. Therefore, it can be concluded that the mutation identified is responsible for this enzyme deficiency and its accompanying clinical manifestations.

While the molecular defect responsible for LPL deficiency observed in patient K2042 has been identified, the hypothesis that mutations in the LPL gene may account for the more common types of hypertriglyceridemia observed in the other three subjects cannot be confirmed, despite a report documenting the delayed expression of hypertriglyceridemia in individual pedigree members heterozygous for a single amino

acid difference in the LPL gene, the substitution of glutamic acid for glycine at residue 188 (Wilson et al., 1990). There are several possible interpretations for this observation:

(1) Expression of hypertriglyceridemia in heterozygotes for LPL deficiency requires saturation of the reduced clearance rate of such subjects by extraneous factors that were present in the pedigree studied by Wilson et al. (1990) but absent in present series of cases of French origin. These might be diet, habitus, or other genetic determinants as yet unidentified. Another possible interpretation is that not all mutations of LPL lead to the latent defect associated to the mutation noted in that pedigree.

(2) Some mutations present in the DNA segments screened may not have been detected by present electrophoretic screening. To be identified, a nucleotide substitution must induce a conformational change in single-stranded DNA under the experimental conditions used for this screening. When a variety of known substitutions were tested by this method, it was found that about 80% could be detected by this method when four distinct conditions (room temperature or cold room and glycerol or no glycerol) were used (Hata et al., 1990a). When eight known variants of the LPL gene were tested in this laboratory, all but one could be detected under at least one of the four experimental conditions used here.

(3) Mutations may occur in parts of the gene which were not examined in present screening. Analysis of total genomic DNA by

Southern blotting is of limited resolution, and only exons and corresponding intron-exon boundaries were screened for conformational polymorphism. The gene spans over 30 kilobases of genomic DNA, and it is reasonable to assume that various noncoding regions may be affected by mutations of functional significance, whether small insertions or deletions or nucleotide substitutions.

For smaller targets such as the alpha-globin and beta-globin genes, which have been subjected to intense examination in light of their modest sizes (1 and 2 kilobases respectively) and their implication in hemoglobinopathies, a vast array of mutations affecting domains other than exons and splice junctions have been identified (Weatherall et al., 1989), such as mutations affecting the rate of transcription or the stability of mRNA, mutations activating cryptic splice sites in exons or in introns, or mutations at polyadenylation sites.

(4) Other genes may be involved in the etiology of hypertriglyceridemia. These could be regulatory proteins affecting LPL gene expression, or they could be other genes affecting triglyceride metabolism.

(5) Various hormonal and environmental determinants can account for hypertriglyceridemia in the absence of a molecular defect in lipid and lipoprotein metabolism, and these are of more common occurrence than heterozygosity for LPL defects.

Experimental data obtained indicate that the mutation noted in subject 4 (K2042) leads

to the production of inactive enzyme in present in vitro heterologous expression system. Although LPL contains multiple functional domains which, if altered, could conceivably lead to functional deficiency, it is likely that the mutation at residue 195 directly impairs the catalytic activity of the enzyme, at least with respect to lipid emulsions. Figure 4 shows the sequence alignment of human pancreatic lipase and lipoprotein lipase for that region of greatest homology. The recent determination of the three-dimensional structure of human pancreatic lipase by Winkler et al. (1990) has provided clear evidence that this segment of the protein includes the catalytic domain. This domain includes the catalytic triad Ser-Asp-His found in serine proteases. However, the active serine is flanked by hydrophobic residues and the elements of the catalytic triad are embedded into a deep pocket, a structure which may account for the specificity of the enzyme for liposoluble substrates and its activity at lipid-water interfaces. The homology between LPL and pancreatic lipase is greatest in the vicinity of the three elements of the catalytic triad, and therefore it is reasonable to assume that this region of LPL also constitutes its catalytic domain. Residue 195 occurs in this region, and therefore it can be proposed that the mutation noted in subject 4, although preserving the active site, affects the catalytic domain of the enzyme. The precise mechanism by which loss of function occurs as a result remains to be determined.

## CONCLUSION

Present experimental data support the conclusion that a mutation leading to a single amino acid substitution at residue 195 of the mature LPL enzyme, with glutamic acid instead of glycine, results in the production of inactive enzyme in subject K2042 and accounts for this subject's chylomicronemia syndrome. Homology between human lipoprotein lipase and pancreatic lipase and the reported structure of the latter suggests that this mutation occurs within the catalytic site of the molecule. The etiology of the hypertriglyceridemia noted in three other subjects remains unresolved, as no evidence of the functional significance of two other mutations has been found.

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