Lipoprotein lipase genotypes for a common premature termination codon mutation detected by PCR-mediated site-directed mutagenesis and restriction digestion

J. Stocks, J. A. Thorn, and D. J. Galton

Department of Human Genetics and Metabolism, 51-53 Barts Close, St. Bartholomew's Hospital, London EC1A 7HL, United Kingdom

Abstract We have developed a procedure for the determination of a common mutation in exon 9 of the human lipoprotein lipase (LPL) gene. The mutation is due to a C-G transversion which creates a premature termination codon (Ser^{447}-Ter) and results in a truncated LPL molecule lacking the C-terminal dipeptide SER-GLY. The mutation can be detected by polymerase chain reaction (PCR) amplification of exon 9 using a modified 3' amplimer that produces a 140 bp product containing a site for the restriction enzyme Hinf-1 in the presence of the mutation (G allele). The G allele was in strong linkage disequilibrium with a Hind-III restriction fragment length polymorphism (RFLP) allele in intron 8. Genotype determinations for the mutation can be performed by PCR amplification of genomic DNA, digestion with Hinf-1, and analysis of the products by polyacrylamide gel electrophoresis. The allelic frequency of the Ser^{447}-Ter mutation in normal male Caucasian controls was 0.11. The frequency of the mutation was lower in a group of subjects with primary hypertriglyceridemia compared to normolipidemic controls.—**Stocks, J., J. A. Thorn, and D. J. Galton.** Lipoprotein lipase genotypes for a common premature termination codon mutation detected by PCR-mediated site-directed mutagenesis and restriction digestion. *J. Lipid Res.* 1992. **33:** 853–857.

Supplementary key words lipoprotein lipase • exon 9 • Ser^{447}-Ter mutation • G allele • C allele

Human lipoprotein lipase (LPL) is a rate-determining enzyme for the clearance of chylomicrons and very low density lipoproteins from the circulation. A defect in the catabolism of these triglyceride-rich lipoproteins due to complete or virtual deficiency of the enzyme results in gross hypertriglyceridemia and the syndrome of Type I hyperlipoproteinemia. Several cases of this rare autosomal recessive disorder arise from mutations in exons 4 and 5 of the gene resulting in amino acid substitutions close to the highly conserved catalytic site of the enzyme (1–3), whilst others arise from gene insertions or deletions (4). However, relative deficiencies of LPL may also be a factor in some of the more common forms of hypertriglyceridemia (Types IV and V) which in many individuals are characterized by a reduced rate of clearance of triglyceride-rich lipoproteins from the circulation (5).

Some cases of hypertriglyceridemia may have genetic determinants, eg., they may arise from the inheritance of LPL gene variants with altered catalytic activities or binding properties that may predispose to hypertriglyceridemia. This is supported by recent reports using two restriction fragment length polymorphisms (RFLP) as LPL gene markers (a Pvu-II RFLP in intron 6 of the gene and a Hind-III RFLP in intron 8). These studies have shown associations of the LPL Hind-III alleles with both hypertriglyceridemia (6) and low HDL levels and also with premature coronary atherosclerosis (7). In addition Heinzmann et al. (8) have found differences in high density lipoprotein (HDL) cholesterol levels with LPL haplotypes defined by these same RFLPs in a group of subjects with cardiovascular disease. This raises the possibility that common genetic variants of LPL are involved in common forms of hypertriglyceridemia with low HDL levels and thus may contribute to premature coronary artery disease.

The coding regions of the LPL gene are split into 9 exons (9–11). Recently a mutation in exon 9 of the LPL gene has been reported (3, 12) in which a C-G transversion results in a premature termination codon (Ser^{447}-Ter). This gives rise to a truncated mature protein lacking the two carboxyl-terminal amino acids (SER-GLY) that are normally present. The mutant LPL retains catalytic activity towards triacylglycerol emulsions (3). This mutation is

Abbreviations: LPL, lipoprotein lipase; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; HDL, high density lipoprotein.

located 630 bp downstream of the Hind-III RFLP site previously studied.

In order to evaluate the possible role of this exon 9 mutation in the genetics of dyslipoproteinemias and coronary heart disease, we have developed a simple and rapid polymerase chain reaction (PCR) procedure for detecting the LPL variant. The C-G transversion does not create or abolish a restriction site. However, by using a modified 3' amplimer for the PCR procedure, which creates a Hinf-1 restriction site in the presence of the G allele (Ser^{447}-Ter), individuals can be directly genotyped for this mutation by amplification of their DNA followed by digestion with Hinf-1 and analysis of the products by polyacrylamide gel electrophoresis. We have determined the frequency of this LPL variant in normolipidemic control subjects and subjects with primary hypertriglyceridemia and have also examined the gametic association with the Hind-III RFLP alleles in intron 8.

MATERIALS AND METHODS

Subjects

Male Caucasian controls (age 40–65 years, median age 53 years) were recruited from a Health Screening Clinic. Fasting blood samples were obtained at the visit and serum cholesterol, triglycerides, and HDL cholesterol levels were measured using standard procedures. Individuals with fasting triglycerides >2.0 mmol/l or cholesterol >7.0 mmol/l were excluded. Caucasian hypertriglyceridemic subjects (age 38–65 years, median age 54 years) were attending the lipid clinic at St. Bartholomew's Hospital. Primary hypertriglyceridemia was diagnosed on the basis of at least three fasting pretreatment lipid measurements with triglycerides >2.5 mmol/l. Mean levels of cholesterol were 7.8 mmol/l, triglycerides 4.2 mmol/l, and HDL cholesterol 0.90 mmol/l.

Isolation of DNA

DNA was isolated from frozen EDTA whole blood by a modified sucrose lysis procedure as previously described (13). The DNA was redissolved in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.6) and stored at −20°C.

Oligonucleotides

The primers for PCR were synthesized by standard β-cyanoethyl phosphoramidite chemistry using a Biotech BT 8500 DNA synthesizer. The oligonucleotides were purified on a Sephadex G-25 column. The primer sequences for the LPL exon 9 mutation were derived from published sequence data (12) and, in the case of intron 9 sequence, from data supplied by K. Oka (personal communication).

Primers were 5' upstream 5' CATCCATTTTCTTCCACAGGG 3'; 3' downstream 5' AGTCTGGTGAGCATTCTGGGCTA 3'. Primer sets for the Hind-III polymorphism, 5' upstream 5' GATGTCTACCTGGATAATCAAAG 3'; 3' downstream 5' CTTCAGCTAGACATTGCTAGTGT 3'; were described previously (14).

Amplification of genomic DNA

Amplification was performed on a Perkin Elmer 480 Thermal cycler. DNA (0.2–0.5 μg) was amplified in a 25-μl reaction mixture containing 20 mM Tris-HCl, pH 8.8, 4 mM $MgCl_2$, 200 μM each of dATP, dTTP, dCTP, dGTP, 0.5 μM each primer, and 0.5 units Amplitaq (Perkin Elmer Cetus) DNA polymerase. The mixture was overlaid with mineral oil. Tubes and buffer solutions were sterilized by autoclaving. The master reaction mixture was irradiated with ultraviolet light on a 320 nm UV transilluminator for 10 min prior to the addition of Taq polymerase and aliquoting to PCR tubes containing the genomic DNA to minimize contamination (15). Blanks containing no added DNA were run with each set of amplifications. The amplification cycle was: 5 min denaturation at 96°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 7 min extension at 72°C. PCR for the Hind-III RFLP was performed using the same conditions.

Digestion with Hinf-1 and electrophoresis of digests

Two μl of 10 × restriction buffer (500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.9) and 10 units Hinf-1 (New England Biolabs) were added to a 20-μl aliquot of the amplification product and the samples were incubated for 2 h at 37°C. After the addition of 3 μl stop dye, they were electrophoresed in 10% nondenaturing polyacrylamide gels in TBE buffer (89 mM Tris-borate–2 mM EDTA, pH 8.3) at 400 volts for 90 min. DNA was visualized by staining with ethidium bromide.

RESULTS

Genotyping for the mutation

A map of the LPL gene showing the position of the mutation is given in **Fig. 1** and the strategy for detection of the G allele (Ser^{447}-Ter mutation) is shown in **Fig. 2**. The primer set amplifies part of exon 9 and part of intron 9 and encompasses the mutation that is located 10 nucleotides upstream of the 3' end of exon 9. The modified primer contains a T in place of the G normally present in either allele. In the presence of the G allele, the amplified DNA then contains a site for Hinf-1, but does not contain a site in the presence of the C allele. Amplification of human genomic DNA with the amplimer set produces a

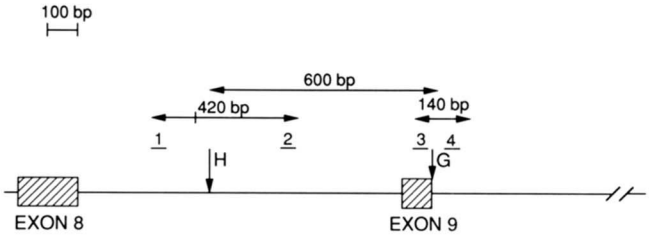

Fig. 1. Map of the 3' end of the lipoprotein lipase gene showing the position of the Ser^{447}-Ter mutation and the Hind-III RFLP site. The Hind-III site is indicated (H) and the G-C transversion in exon 9 is indicated (G).

product of 140 bp. In the presence of the G allele restriction, digestion with Hinf-1 produces a product of 118 bp and 22 pb. **Fig. 3** shows a ethidium bromide-stained polyacrylamide gel showing an individual homozygous for the G allele with a band at 118 bp, individuals homozygous for the C allele with a band at 140 bp, and heterozygotes with bands at 140 and 118 bp. The 22 bp fragment is not visible.

Gametic association of the mutation and Hind-III RFLP alleles

The Ser^{447}-Ter mutation is located 630 bp from a polymorphic Hind-III restriction site located in intron 8 (Fig. 1). Linkage disequilibrium between the Hind-III RFLP alleles and the Ser^{447}-Ter mutation was examined in 72 individuals genotyped for both the RFLP and the mutation. The Ser^{447}-Ter mutation (G allele) was in strong linkage disequilibrium with the Hind-III RFLP H1 allele. Using a maximum likelihood estimation (16) shows a significant departure from random association (chi-square 26.35 1 df $P < 0.000001$). Estimated haplotype frequencies were HIG (Ser^{447}-Ter) 0.097, HIC 0.215, and H2C 0.687.

Plasma lipid and HDL levels in control subjects with the mutation

Table 1 shows fasting serum lipid values of control males with different exon 9 genotypes. There was no statistically significant difference in mean cholesterol, triglyceride, or HDL levels among those individuals heterozygous for the Ser^{447}-Ter mutation compared to those homozygous for common allele. Only two individuals homozygous for the mutation were found in the control group.

Frequency of the mutation in controls and subjects with hypertriglyceridemia

Allelic frequencies (**Table 2**) in normal Caucasians (302 chromosomes) were (0.109) for the G allele and (0.891) for the C allele. The genotype distribution was in Hardy-Weinberg equilibrium.

Comparing the genotypic frequencies at this locus in subjects with hypertriglyceridemia compared to normolipidemic controls demonstrated a significantly lower frequency of the Ser^{447}-Ter mutation in subjects with primary hypertriglyceridemia compared to normolipidemic controls (Table 2).

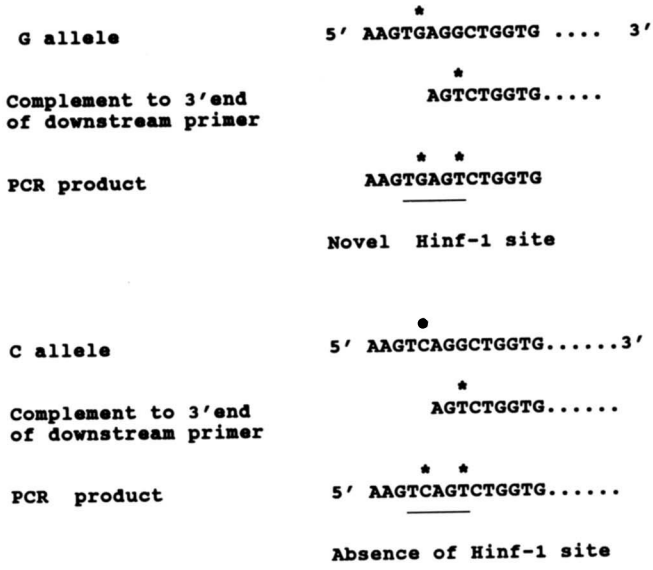

Fig. 2. Strategy for detection of the G allele (Ser^{447}-Ter mutation) in exon 9 of the lipoprotein lipase gene by site-directed mutagenesis using a modified primer. Introduction of T(*) in place of G in the modified downstream primer creates a new Hinf-1 site when the G allele (*) is present but not with the C(*) allele. The sequence given is to the 3' end of exon 9. In the common (C) allele, the A of the termination codon is the first nucleotide of exon 10. NB: the sequence of the complement to the downstream primer is given. The enzyme Hinf-1 has the recognition sequence GA(N)TC.

DISCUSSION

The premature termination codon mutation (Ser^{447}-Ter) in exon 9 reported by Hata et al. (12) and by Fustinella

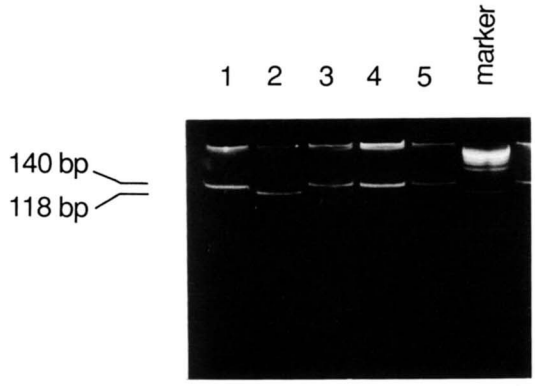

Fig. 3. Polyacrylamide gel electrophoresis of human genomic DNA amplified by PCR using primers as described in Fig. 2 and digested with Hinf-1. 20 μl of PCR product was digested with 10 units enzyme for 2 h at 37°C, applied to 2 mm 10% PAGE gel in 1 TBE, and electrophoresed for 90 min at 400 volts. Gels were stained with ethidium bromide. Genotypes are track 1:CC,2:GG,3:GC,4:CC 5:CC.

TABLE 1. Fasting lipid and HDL values in normal Caucasian male subjects with the Ser^{447}-Ter mutation

LPL Exon 9 Genotype (n)	Cholesterol	Triglyceride	HDL-Cholesterol
		mmol/l	
All (149)	5.59 ± 1.05	1.31 ± 0.46	1.23 ± 0.30
CC (120)	5.44 ± 1.00	1.33 ± 0.43	1.24 ± 0.37
GC (29)	5.43 ± 0.92	1.27 ± 0.41	1.30 ± 0.34

Results are means ± SD. Comparison of values by two-sample *t*-test shows no significant difference. The two individuals with the genotype GG are not included. Individuals with the genotype GC are heterozygotes for the Ser^{447}-Ter mutation.

et al. (3) is the first report of a relatively common protein polymorphism of human lipoprotein lipase. The procedure we have developed, using a modified amplimer for PCR and restriction digestion with Hinf-1 to detect the mutation, provides a rapid and direct method of analysis of the variant without the need for blotting, probe labeling, and hybridization required with the allele-specific oligonucleotide (ASO) procedure originally described.

The functional significance, if any, of this isoform remains to be defined. The LPL gene is comprised of 10 exons spanning 30 kb of chromosome 8 (9–11). Exon 5 codes for the highly conserved region which contains the catalytic site of the enzyme. Exon 6 codes for a region rich in basic amino acids which may constitute a heparin sulfate-glycosaminoglycan binding site, by which the enzyme attaches to the capillary endothelium. The sequence of 14 amino acids at the carboxyl-terminal end of the molecule is highly conserved and the C-terminal dipeptide Ser-GLY is present in all six reported mammalian LPL sequences (17–20) but this region has no defined function. Expression of cloned LPL with the Ser^{447}-Ter mutation in vitro has shown that the truncated isoform of LPL which lacks the terminal dipeptide has normal catalytic activity (3). From our population data it appears to have no overt phenotypic effect. But this not does exclude an influence on LPL secretion, binding to endothelial cells, or its recently reported function as a ligand to the chylomicron remnant receptor (21).

The previous findings of an altered distribution of Hind-III alleles in hypertriglyceridemic subjects compared to normolipidemic controls (6) suggested that one of the Hind-III alleles is in linkage disequilibrium with a putative mutation in the LPL gene, probably at the 3' end of the gene, which can predispose to hypertriglyceridemia. The Hind-III RFLP site is 630 bp from the G-C transversion giving rise to the mutation and would be expected to show strong linkage disequilibrium. From analysis of the combined genotypes of the Ser^{447}-Ter mutation and Hind-III RFLP alleles, it appears that in Caucasians the Ser^{447}-Ter mutation is in strong or absolute linkage disequilibrium with the Hind-III H1 allele, ie., there appear to be three haplotypes H1G, H1C, and H2C. This raises the possibility that the association is derived from the Hind-III H1 allele being a marker for the Ser^{447}-Ter mutation.

In the control sample that we have examined, possession of the Ser^{447}-Ter mutation does not appear to have any marked effect on lipid levels, although there is a tendency to higher HDL levels. However, there is a significant difference in the frequency of the Ser^{447}-Ter mutation in subjects with primary hypertriglyceridemia compared to normolipidemic controls. The Ser^{447}-Ter mutation was present in 11% of hypertriglyceridemic subjects compared to 19% of controls. A similar difference was noted by Hata et al. (12) who found frequencies of 9% and 30%, respectively. This raises the possibility that the Ser^{447}-Ter mutation may have some protective effect against the development of hypertriglyceridemia. This finding may also explain the altered distribution of Hind-III alleles in hypertriglyceridemic subjects. Since the Ser^{447}-Ter mutation is associated with the H1 allele, a decreased frequency of the Ser^{447}-Ter mutation will be accompanied by a decreased frequency of H1 alleles, and a corresponding increase in H2 alleles, which is in accord with the finding we have reported previously (6, 7). Alternatively, it is possible that both the H1 allele and the Ser^{447}-Ter mutation are markers for another mutation in the LPL gene, or of a neighboring gene, with which they are both in linkage disequilibrium. To clarify this, the kinetic properties of the enzyme isolated from posthepa-

TABLE 2. Frequencies of the LPL Ser^{447}-Ter mutation in normal subjects and individuals with hypertriglyceridemia

	LPL: Genotypes			Alleles	
Group	CC	GC	GG	C	G
Controls (n = 151)	120 (79%)	29 (19.2%)	2 (1.3%)	0.891	0.109
All hypertriglyceridemic subjects (n = 102)	91 (89%)	11 (10%)	0[a]	0.946	0.053

Chi-square analysis of genotype frequencies (2 × 2 contingency table CC vs. GC + GG).
[a]Chi-square = 4.18, $P < 0.05$.

rin plasma of individuals homozygous for the Ser^{447}-Ter mutation or the common allele are being studied with regard to lipid binding, heparin release, and activation by apoC-II.

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