

Regulatory mutations in the human lipoprotein lipase gene in patients with familial combined hyperlipidemia and coronary artery disease

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Abstract We previously reported a compound heterozygote [T(-39)C/T(-93)G] in the human lipoprotein lipase (LPL) gene promoter in one out of 19 patients with familial combined hyperlipidemia (FCHL) and reduced post-heparin plasma LPL levels. The T(-39)C substitution resulted in 85% decrease in LPL promoter activity. Further screening of Caucasian patients with FCHL, coronary artery disease (CAD), and of unselected Caucasian subjects revealed four additional LPL promoter variants. Among the same 19 FCHL patients with reduced LPL levels, we found one heterozygote for a G(-53)C substitution. Among 115 CAD patients, we found five heterozygotes and one homozygote for the T(-93)G substitution and one heterozygote for a CC insertion between +13 and +19 of the 5' untranslated region. In a group of 183 unselected subjects, three heterozygotes with the T(-93)G substitution were found. The G(-53)C substitution led to approximately 70–75% decrease in promoter activity as assayed by transient transfections of THP-1 (macrophage-like) and C2C12 (myotube-like) cells. The T(-93)G substitution resulted in reduction of promoter activity by approximately 40–50%. The CC insertion between +13 and +19 caused a decrease in promoter activity by 20% in THP-1 and 50% in C2C12. Substitutions at -79 and -95, which had no effect on promoter function, were also discovered in the population samples studied. ■ The finding of two promoter mutations (-39 and -53) among 19 FCHL patients with diminished LPL, but not among the other groups of subjects, suggests a potential role of regulatory mutations of the LPL gene in the development of dyslipidemia in FCHL.—Yang, W.S., D.N. Nevin, L. Iwasaki, R. Peng, B.G. Brown, J.D. Brunzell, and S.S. Deeb. Regulatory mutations in the human lipoprotein lipase gene in patients with familial combined hyperlipidemia and coronary artery disease. *J. Lipid Res.* 1996. **37**: 2627–2637.

Supplementary key words promoter • 5'-untranslated region • LPL • FCHL

Lipoprotein lipase (LPL) catalyzes the hydrolysis of core triglycerides of chylomicrons and very low density lipoproteins. Free fatty acids are generated as the result of this hydrolysis and can be either taken up and utilized as a source of energy or re-esterified for storage in adipose tissue. Independent of its lipolytic activity,

LPL also facilitates the uptake of a variety of lipoproteins by many different cell types through receptor-dependent or receptor-independent pathways (for review, see ref. 1). LPL is synthesized by many tissues, including adipose tissue, skeletal and cardiac muscle, lactating mammary gland, and macrophages. The expression of LPL in these tissues is regulated by various physiological factors such as fasting/feeding, hormones, and pro-inflammatory cytokines (for review, see ref. 2). More than 40 different LPL structural mutations have been identified in patients with familial LPL deficiency, a rare autosomal recessive disorder characterized by fasting chylomicronemia, extreme hypertriglyceridemia, eruptive xanthoma, and recurrent pancreatitis (3, 4). The frequency of carriers of structural mutant alleles was estimated to be 1 in 500 individuals in the general population (4). There is evidence to suggest that heterozygosity for LPL deficiency may also underlie some common lipid disorders, such as familial combined hyperlipidemia (FCHL), thereby contributing to the pathogenesis of atherosclerosis (5–10).

Familial combined hyperlipidemia (FCHL) (11–13), a common inherited lipid disorder with a prevalence rate of 1–2% in the general population, was observed in at least 10–20% of the patients with premature coronary artery disease (CAD) (11, 14). FCHL is characterized by an increase of plasma apoB concentration as well as elevated plasma triglyceride and/or cholesterol in multiple individuals of the affected family (15). It has been proposed that FCHL is heterogeneous and oligogenic in etiology (7, 16). Despite intensive investigation, the genetic basis for FCHL still remains elusive (15).

Abbreviations: LPL, lipoprotein lipase; FCHL, familial combined hyperlipidemia; CAD, coronary artery disease; VLDL, very low density lipoprotein; apo, apolipoprotein; nt, nucleotide.

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Wilson, Edwards, and Chan (5) reported that the lipid and lipoprotein profiles of heterozygous relatives of an LPL-deficient proband in an extended pedigree resembled those of patients with FCHL (5). Babirak, Brown, and Brunzell (7) found that 20 of 56 (36%) FCHL patients had reduced levels of post-heparin plasma LPL activity and mass in the same range as did obligate heterozygotes of LPL deficiency. Taken together, these results suggest that heterozygosity for LPL deficiency may contribute to the development of FCHL. Two studies have been conducted to address this question. Nevin, Brunzell, and Deeb (17) found no structural mutation affecting LPL catalytic activity among 20 FCHL patients who had half the normal levels of LPL activity, while Gagné et al. (18) observed one LPL mutant allele, Gly188Glu, among 31 French Canadian FCHL patients with unknown LPL levels.

Owing to the rarity of LPL structural mutations among FCHL patients, we previously hypothesized that mutations in regulatory sequences of the LPL gene might be associated with this disorder. In an initial screen of 20 FCHL patients who had diminished LPL levels, one was found to be a compound heterozygote for [T(-39)C/T(-93)G] substitutions (19, 20). The -39 substitution was located within an Oct-1 binding site and decreased the promoter activity by at least 85% in transient transfection experiments. In view of this finding, we systematically searched for additional regulatory sequence variants among FCHL patients as well as among patients with CAD; unselected medical students and unselected subjects served as controls. We show that LPL regulatory sequence variants are not rare and might be one of the factors that contribute to the development of FCHL and atherosclerosis.

SUBJECTS AND METHODS

Subjects

The subjects screened for LPL promoter variants were Caucasians recruited from the Seattle area. These included 19 subjects with FCHL and half-normal levels of post-heparin plasma LPL activity and mass, 20 subjects with FCHL and normal levels of LPL, and 115 patients with angiographically proven CAD. As a control, DNA samples of groups of 120 unselected medical students and another 63 unselected subjects at the University of Washington were screened. One of the 20 subjects with FCHL and low LPL described in previous reports is non-Caucasian and is not included in this report (17, 19, 20). The lipoprotein profile and post-heparin plasma LPL activity and mass for all FCHL patients have been reported in detail (7). The post-heparin

TABLE 1. Sequence of the primers used for PCR and sequencing

Primers	Position	Sequence
FP-3	+45	5'-GCAGCTTTCCCTTGAGGAGGAGGAAG-3'
R18	+20	5'-cacc aa gcttGAGGGGAATCGACTCTGAC-3'
R17	-103	5'-cg gg taccTGAATTTAGGTCCCTCCCCC-3'
R6C	-144	5'-CAGGGTTGATCCTCATTACTG-3'
R11	-519	5'-GCTGATGCATCTTGCCAATGTTA-3'
R12	-325	5'-CAGCTAAACTTCCCTCCTTGG-3'

The LPL gene sequences in primers are in upper case. The sequences in lower case in R17 and R18 are spacer sequences and restriction sites. The *Kpn*I site in R17 and the *Hind*III site in R18 are in bold. The numbers under Position indicate the location of the most 5' nucleotide of the LPL gene sequences of each primer in the LPL gene.

plasma LPL activity and mass of the subject with the -53 substitution are 61 nmol/min per ml and 42 ng/ml, respectively. The LPL activity and mass of the subject carrying -39/-93 substitutions are 85 nmol/min per ml and 73 ng/ml, respectively. The mean LPL activity and mass of 127 normal subjects are 214 ± 86 nmol/min per ml and 221 ± 109 ng/ml, respectively. Informed consent was obtained according to the procedures approved by the University of Washington Human Subjects Review Committee.

Single-strand conformation polymorphism (SSCP), direct sequencing of amplified DNA, and restriction analysis

Genomic DNA was prepared from peripheral blood leukocytes by the proteinase K/phenol method on an Applied Biosystems model 340A nucleic acid extractor according to the manufacturer's protocol. A 564 bp segment of the LPL promoter extending from nucleotide (nt.) -519 to +45 (according to the numbering in ref. 21) was amplified by the polymerase chain reaction (PCR) using primers R11 and FP-3 (Table 1), digested into three fragments with *Sau*3AI and subjected to SSCP analysis essentially as described (19, 20). The previously identified promoter variant at position -39 was screened for as follows: A 156 bp fragment from nt. -103 to +45 was amplified with primers R17 and FP-3 (Table 1), followed by electrophoresis on a 1X MDE gel (J.T. Baker Inc., Phillipburg, NJ) as previously described (19, 20). As a positive control, the PCR product amplified from genomic DNA of the patient who carried the nt. -39 mutation was used in this SSCP analysis.

Direct sequencing of the PCR-amplified DNA was essentially as described (22). Direct sequencing of the CC insertion gave overlapping patterns staggered by two nucleotides. The sequence of this mutation was confirmed using the cloned fragment as template.

Presence of the -53 and -93 substitutions was confirmed by restriction enzyme analysis as follows. A 370 bp fragment (from nt. -325 to +45) was amplified with

primers R12 and FP-3, using total genomic DNA as template (Table 1). Amplified DNA was subjected to *Sau3A* I and *Apa* I digestion, respectively for screening for the -53 and -93 substitutions, prior to electrophoresis on a 3% agarose gel.

Cloning of the LPL promoter sequences into the luciferase reporter vector

Wild-type and variant promoter sequences amplified by PCR were cloned into the luciferase reporter vector, pXP1 (ATCC #37576, ref. 23). In order to clone the variant alleles with the base substitutions at nt. -53 and -79, primers R17 and R18 (Table 1) were used to amplify a segment of the LPL promoter extending from nt. -103 to +20. The PCR condition and the cloning procedure were essentially as described (19). A second set of primers, R6C and R18 (Table 1) was used to amplify a segment from nt. -144 to +20 for cloning the alleles with the sequence variation at nt. -93 or -95. As primer R18 has a *Hind* III site at its 5' end, the amplified fragment was digested with *Hind* III, purified by electrophoresis, and cloned into the *Sma* I and *Hind* III sites of pXP1 vector. A third set of primers, R17 and FP-3 (Table 1) was used to amplify a fragment from nt. -103 to +45 for cloning the variant with dinucleotide insertion between nt. +13 and +19. This fragment was cloned into the *Sma* I site of pXP1. The cloned variants and their corresponding wild-type LPL promoter segments were verified by sequencing. Plasmid DNA for transfection experiments was prepared using a Qiagen Plasmid Kit (Qiagen Inc., Chatsworth, CA).

Cell culture and transient transfection

THP-1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 2 mM L-glutamine (24). Transfection of THP-1 cells was performed by the DEAE-dextran method basically as described (19, 25). Induction of the LPL promoter activity with PMA (phorbol 12-myristate 13-acetate) and/or dibutyryl-cAMP (dbcAMP) plus isobutyl methylxanthine (IBMX) was reported previously (19). Cell harvest, luciferase (26), β -galactosidase (27), and Bradford assay for protein concentration (28) have been described in detail (19).

The mouse myoblast cell line, C2C12-F3 (29) was maintained in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and 2 mM L-glutamine (30). The cells were transfected using the calcium phosphate method (31). Six hours prior to transfection, the cells were plated at a density of approximately 80% confluence in a 22-mm plate with 1 ml of medium. The DNA/calcium phosphate co-precipitate was prepared as follows. Fifty μ l of 0.248 M calcium chloride containing 2 μ g of luciferase reporter plasmid DNA and 0.5 μ g of co-transfecting lacZ reporter plasmid

DNA (pnCMVlacF) was added dropwise to 50 μ l of 2 \times HBS (50 mM HEPES, pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4), with constant vortexing. The DNA/calcium phosphate solution was incubated at room temperature for 30 min and then added dropwise to the cells. After overnight incubation at 37°C with 5% CO_2 , the medium containing DNA/calcium phosphate was replaced with 1 ml fresh culture medium. The cells were incubated for another 24 h before harvest for luciferase, β -galactosidase and Bradford assays.

RESULTS

LPL regulatory sequence variants and their frequency among patients and controls

SSCP analysis was used to detect sequence variants in a 515 bp region extending from nucleotide (nt.) -496 of the promoter to +19 of exon 1 of the LPL gene. DNA from 39 FCHL patients (19 with diminished post-heparin plasma LPL activity and 20 with normal levels), 115 patients with coronary artery disease (CAD), and 183 unselected ostensibly normal subjects was analyzed. Among the 19 FCHL patients with low LPL, one was heterozygous for an Asp9Asn substitution and three were heterozygous for a two amino acid truncation (17). These alterations in the coding sequence are known not to affect catalytic activity of LPL (32, 33).

Among the 19 FCHL patients with reduced LPL activity, we found three with novel LPL promoter variants (Table 2), in addition to the previously reported compound heterozygote with the [T-39)C/T(-93)G] substitutions (19, 20). One was a heterozygote for a G \rightarrow C substitution at nt. -53 (Fig. 1 and Fig. 2) and two were heterozygotes for a G \rightarrow T substitution at nt. -95. Two of the 20 FCHL patients who had normal LPL levels were found to have variant alleles in this region of the promoter, one being heterozygous for a T \rightarrow G substitution at nt. -79 (Table 2, Fig. 1) and the other being heterozygous for the G \rightarrow T substitution at nt. -95 (Table 2, Fig. 1).

Among the 115 CAD patients, we found 6 who carried the T \rightarrow G substitution at nt. -93 (one homozygote and 5 heterozygotes) (Table 2, Fig. 1 and Fig. 3), two who were heterozygotes for the G \rightarrow T substitution at nt. -95 (Table 2, Fig. 1), and one who was heterozygous for a dinucleotide (CC) insertion into a stretch of 5 C's between nt. +13 and +19 of the 5' untranslated region of the exon 1 (Table 2, Fig. 1 and Fig. 4).

Among the unselected controls (120 medical students and 63 other subjects), three were heterozygous for the T \rightarrow G substitution at nt. -93 (Table 2, Fig. 1 and 3) and one was heterozygous for the G \rightarrow T substitution

TABLE 2. The frequency of individuals with the LPL regulatory sequence variants

Position	Sequence Change	Frequency #(%)			
		FCHL and 1/2 LPL (n = 19)	FCHL and NLPL (n = 20)	CAD (n = 115)	Controls (n = 183)
-95	G → T	2 (10.5) ^a	1 (5.0) ^b	2 (1.7) ^c	1 (0.5) ^d
-93	T → G	1 (5.3) ^e	0	6 (5.2) ^f	3 (1.6) ^g
-79	T → G	0	1 (5.0) ^h	0	0
-53	G → C	1 (5.3) ⁱ	0	0	0
-39	T → C	1 (5.3) ^j	0	0	0
+13; +19	+2C	0	0	1 (0.9) ^k	0

FCHL, familial combined hyperlipidemia; 1/2 LPL, half normal levels of LPL; NLPL, normal levels of LPL; CAD, coronary artery disease; n, number of individuals. The identification numbers (DNA samples) of the subjects carrying each of the substitutions are as follows: ^a2C1613, LC3038; ^bLC1583; ^c1C247, 1C347; ^dM1126; ^eLC3040; ^fCI2227, 1C274, 1C275, 1C293, 1C320, 1C342; ^gM640, M1097, HC2422; ^hCI2422; ⁱLC1256; ^jLC3040; ^k1C464.

at nt. -95 (Table 2, Fig. 1). The T to G substitution at nt. -93 was of special interest, because it seemed to be relatively common. The allele frequency of this variant was 0.026 (1/38) in FCHL patients who had low LPL, 0.030 (7/230) in CAD patients and 0.008 (3/366) in unselected controls. The difference in allele frequency between the CAD group and controls was borderline in statistical significance (one-tailed $P = 0.044$, two-tailed $P = 0.051$, Fisher's exact test). It is notable that the -93 substitution and Asp9Asn are in complete linkage disequilibrium ($n = 10$).

Promoter activity of the LPL regulatory sequence variants

The functional consequences of the regulatory sequence variants were investigated by transient transfection

of the human monocytic leukemic cell line THP-1 and the mouse myoblast cell line C2C12 with plasmid DNA containing wild-type or variant LPL promoter sequences driving expression of the luciferase reporter gene. We have shown that the proximal 103 base pairs of the human LPL promoter that contain a TATA-like box, CCAAT box, Oct-1 and Sp1 binding sites have maximal basal promoter activity in THP-1 and approximately 25% of maximal basal promoter activity in C2C12 cells (W-S. Yang and S.S. Deeb, unpublished results). Therefore, all the LPL promoter constructs in this study contain at least the proximal 103 base pairs of the LPL promoter and 20 base pairs of the 5' untranslated region. A plasmid with the CMV promoter/enhancer driving expression of the lacZ reporter gene was co-transfected into the cells in order to correct for trans-

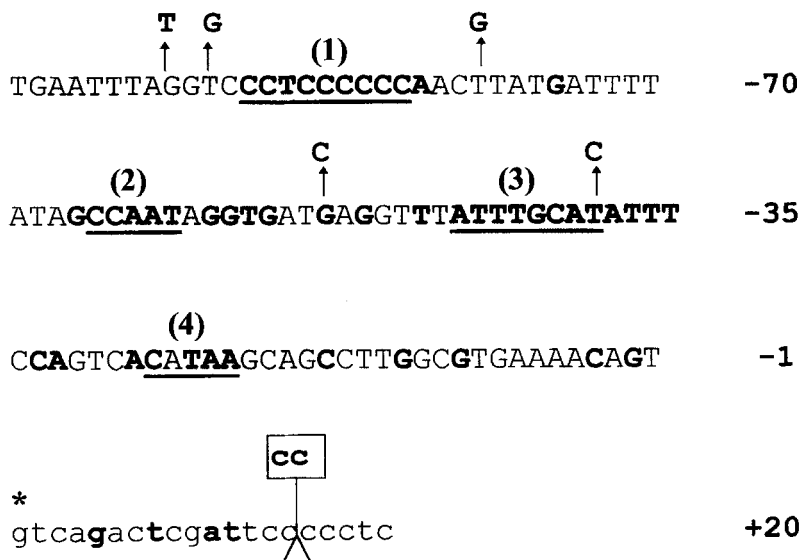


Fig. 1. Sequence of the proximal promoter of the human LPL gene. The underlined sequences are (1) inverted GA box, (2) CCAAT box, (3) Oct-1 binding site, and (4) TATA-like box. The major transcription start site is indicated with an asterisk. Exon 1 sequence is shown in lower case. The nucleotides in bold are conserved among the human, mouse, and chicken LPL promoters.

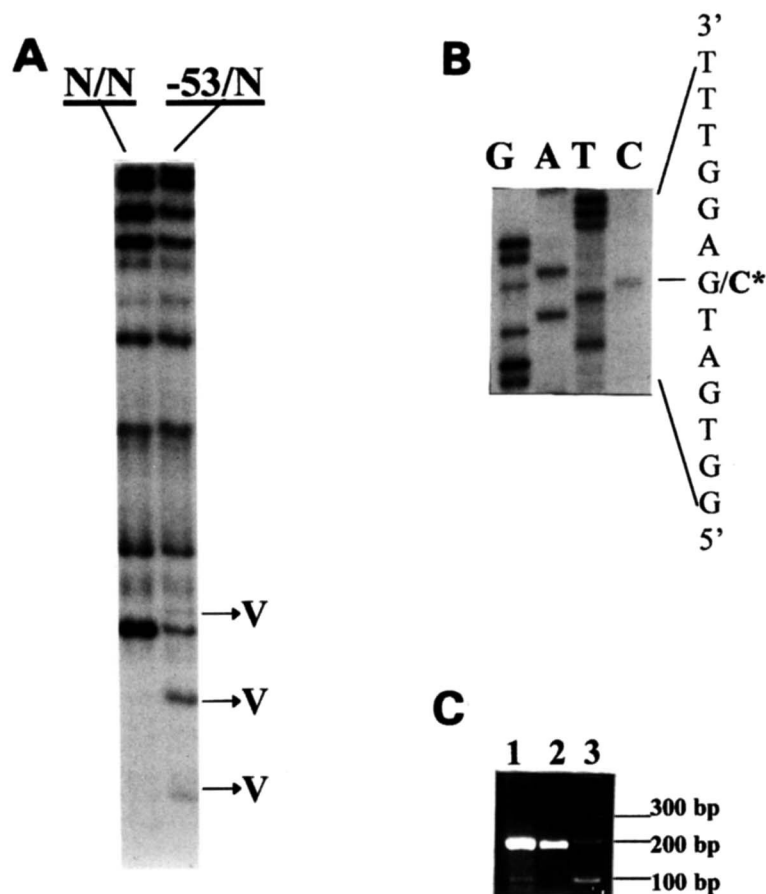


Fig. 2. Detection of the G→C substitution at nt. -53. (A) Autoradiograph of an SSCP gel showing migration patterns of single-strand DNA fragments of a subject with wild-type alleles (N/N) and of a heterozygote for the G→C substitution at nt. -53 (-53/N). Variant bands are indicated by arrows. A 564 bp fragment extending from nt. -519 to nt. +45 was PCR amplified and digested with *Sau3A* I prior to electrophoresis on a 5% nondenaturing acrylamide gel. (B) Autoradiograph of a gel showing the sequence of a subject heterozygous for a G→C substitution at -53 (indicated by an asterisk). Sequencing was performed on PCR amplified DNA using total genomic DNA as template. (C) Photograph of an agarose gel showing migration patterns of PCR-amplified DNA fragments of subjects heterozygous for the -53 substitution (lane 1) and homozygous for wild-type alleles (lane 2), along with 100 bp ladder (lane 3). A 370 bp fragment (from nt. -325 to +45) was amplified, using total genomic DNA as template, then digested with *Sau3A* I prior to electrophoresis on a 3% agarose gel. The wild-type allele shows two fragments of 180 and 190 bp. The mutant allele shows three fragments of 81, 99, and 190 bp.

fection efficiency. Both THP-1 and C2C12 cell lines were shown to express endogenous LPL (24; S.S. Deeb and R. Peng, unpublished observation). Transient transfection assays in THP-1 cells were performed under four different conditions: 1) in the presence of PMA alone; 2) in the presence of dbcAMP plus IBMX; 3) in the presence of both PMA and dbcAMP plus IBMX; and 4) in the absence of both. PMA is known to induce THP-1 cells to differentiate into macrophage-like cells and to markedly activate transcription of the LPL gene (24). Cyclic AMP is also known to induce transcription of the LPL gene in these cells (S.S. Deeb et al., unpublished observation). The promoter activity in C2C12 was assayed after partial differentiation (approximately 50%) into myotubes.

G→C substitution at nucleotide -53. Plasmid constructs containing the LPL promoters from nt. -103 to +20 with wild-type or variant sequences were transfected into THP-1 and C2C12 cells. This substitution reduced the LPL promoter activity to approximately 30% of wild-type in THP-1 under all four different conditions described above, and to approximately 25% of wild-type in C2C12 cells (Fig. 5). Nucleotide -53 is located between the CCAAT (nt. -65 to -61) and Oct-1 (nt. -46

to -39) motifs and is conserved among the human, mouse, and chicken LPL promoters (Fig. 1) (34, 35). This G→C substitution at -53 creates a new *Sau3A* I site (5'-GATC-3', -56 to -53) (Fig. 2C).

T→G substitution at nucleotide -93. Plasmid constructs containing the LPL promoters from nt. -144 to +20 with wild-type or variant sequences were transfected into THP-1 and C2C12 cells. This T→G substitution reduced the promoter activity to approximately 60% of wild-type in THP-1 under the four different conditions described above and to approximately 50% of wild-type in C2C12 (Fig. 6). The nucleotide T at -93 is not conserved among the human, mouse, and chicken LPL promoters. In fact, it is G instead of T at this position in both mouse and chicken LPL promoters (Fig. 1) (34, 35). This T→G substitution at -93 destroys an *Ava* II site (5'-GGTCC-3', from nt. -95 to -91) and creates an *Apa* I site (5'-GGGCCC-3', from nt. -95 to -90). The presence of this substitution in subjects carrying this variant was also confirmed by restriction analysis (Fig. 3C).

Dinucleotide (CC) insertion between nucleotide +13 and +19 in the 5' untranslated region of exon 1. Plasmid constructs containing the LPL promoters from nt. -103 to

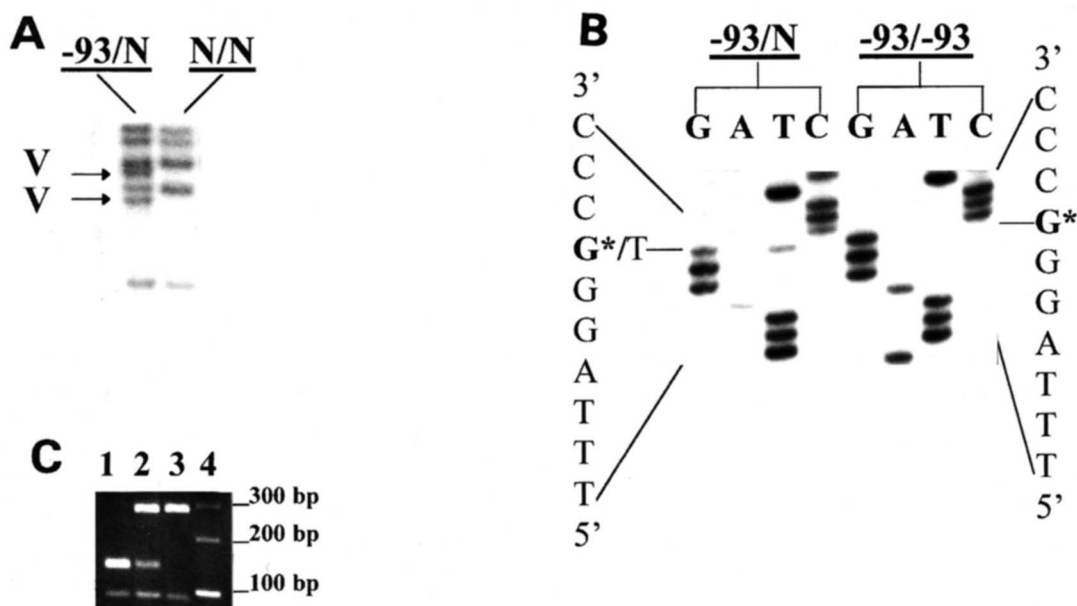


Fig. 3. Detection of the T→G substitution at nt. -93. (A) Autoradiograph of an SSCP gel showing migration patterns of single-strand DNA fragments from a subject with normal alleles (N/N) and from a heterozygote for the T→G substitution at nt. -93 (-93/N). Variant bands are indicated by arrows. SSCP was performed as described in Fig. 2. (B) Autoradiograph of a gel showing the sequence of two subjects, one heterozygous (-93/N) and the other homozygous (-93/-93) for the T→G substitution at -93 (indicated by an asterisk). Sequencing was performed on PCR amplified DNA using total genomic DNA as template. (C) Photograph of an agarose gel showing migration patterns of PCR-amplified DNA fragments of subjects homozygous for the -93 substitution (lane 1), heterozygous for the -93 substitution (lane 2), and homozygous for wild-type alleles (lane 3), along with 100 bp ladder (lane 4). A 370 bp fragment (from nt. -325 to +45) was amplified, using total genomic DNA as template, then digested with *Apa* I prior to electrophoresis on a 3% agarose gel. The wild-type allele shows two fragments of 90 and 280 bp. The mutant allele shows three fragments of 90, 99, and 181 bp.

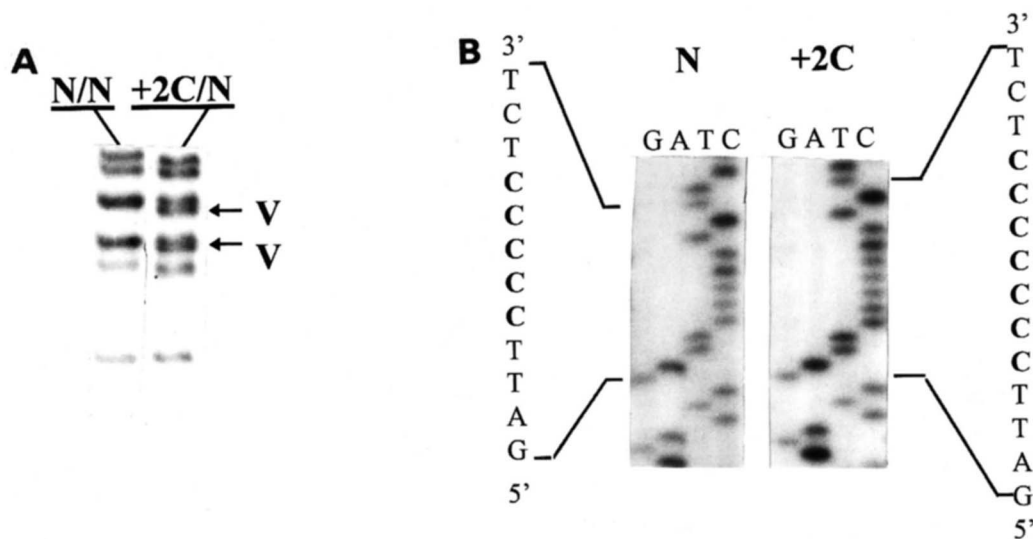


Fig. 4. Detection of the 2 C insertion between nt. +13 and +19. (A) Autoradiograph of SSCP gels showing migration patterns of single-stranded DNA amplified from a normal subject (N/N) and from a heterozygote for the 2C insertion between nt. +13 and +19 of exon I (+2C/N). The variant bands are indicated by arrows. (B) Autoradiograph of gels showing the sequence of a normal allele (N) and a mutant allele with 2C insertion between nt. +13 and +19 (+2C). Sequencing was performed on the pXP1 vector plasmid DNA containing the wild-type or mutant promoter alleles.

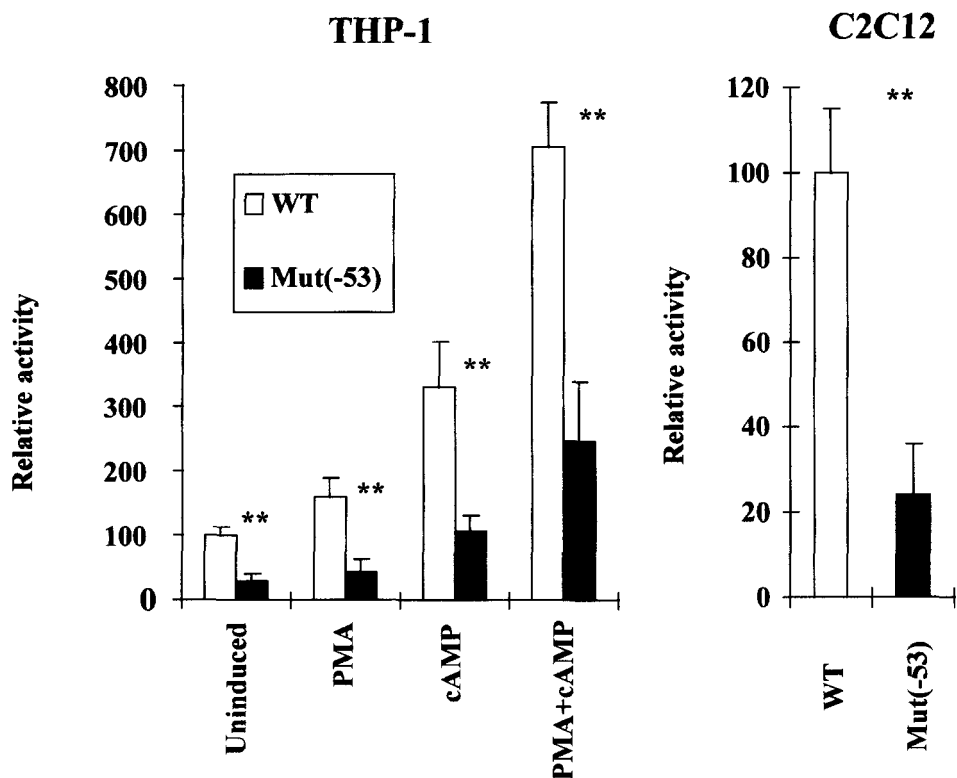


Fig. 5. Activity of the LPL promoter with the G→C substitution at nt. -53. Wild-type and mutant promoters (from nt. -103 to +20) driving expression of the luciferase gene were transfected into THP-1 and C2C12 cells. The activity in THP-1 cells was assayed in undifferentiated, PMA-differentiated, undifferentiated but dbcAMP-IBMX treated, or PMA-differentiated plus dbcAMP-IBMX treated cells. All values were corrected for transfection efficiency. The data using THP-1 represent means and standard errors (SE) of 9 independent transfections from three separate experiments, using two different DNA preparations. The data using C2C12 represent means and SE of 22 independent transfections from 9 separate experiments, using three different DNA preparations. ** $P < 0.01$ (*t*-test)

+45 with wild-type or variant sequences were transfected into THP-1 and C2C12 cells. This dinucleotide insertion reduced promoter activity to approximately 80% of wild-type in THP-1 under the four different conditions described above and to approximately 50% of wild-type in C2C12 (Fig. 7). The stretch of 5 Cs into which the CC insertion took place is not conserved among the human, mouse, and chicken LPL promoters (Fig. 1) (34, 35). This stretch of C's is located in close proximity (8 to 13 nucleotides way) to the major and minor transcription start sites of the human LPL gene (21).

T→G substitution at nucleotide -79 and the G→T substitution at nucleotide -95 did not affect promoter activity. The promoters with these variants were demonstrated to be functionally equivalent to wild-type promoter by transient transfection assays in both THP-1 and C2C12 (for base substitution at -79, $104 \pm 30\%$ of wild-type in THP-1 and $96 \pm 35\%$ of wild-type in C2C12; for base substitution at -95, $110 \pm 17\%$ of wild-type in THP-1

and $97 \pm 5\%$ of wild-type in C2C12). The T at position -79 and the G at -95 are not conserved among the human, mouse, and chicken LPL promoters (Fig. 1) (34, 35).

DISCUSSION

The main objective of this study was to determine whether or not LPL promoter mutations underlie the partial LPL deficiency observed in a subset of FCHL patients. In an earlier report, the same FCHL patients were found not to have any LPL structural mutations that affect the catalytic activity (17). We have found three promoter variants (base substitutions at nt. -39, -53, and -93) in the 19 FCHL patients with reduced plasma LPL activity. These variants significantly reduced promoter activity in transient transfection assays in both THP-1 and C2C12 cells. In contrast, the -39

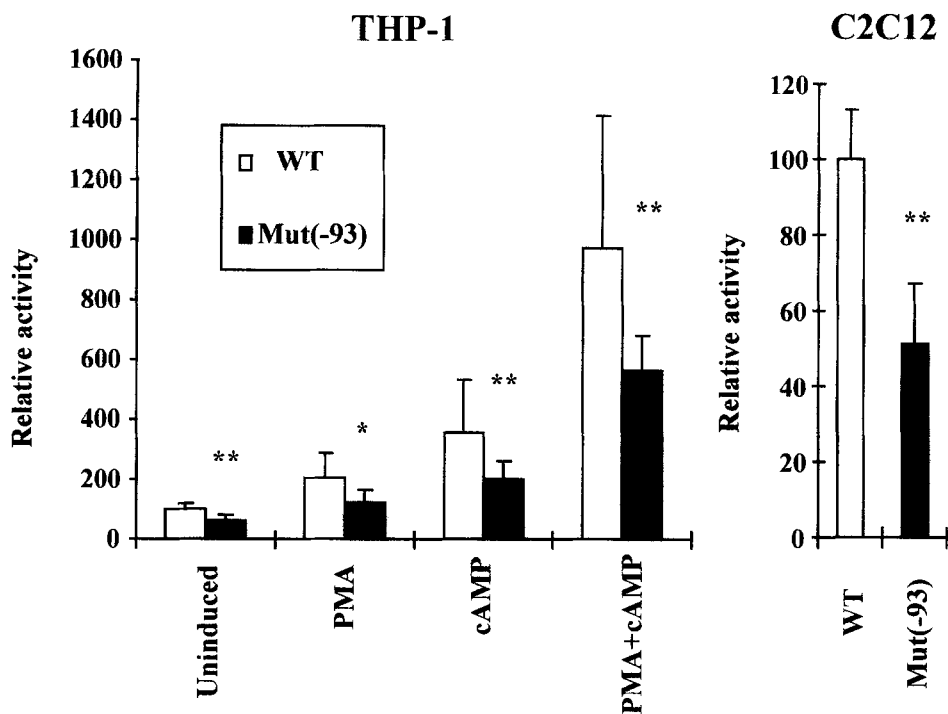


Fig. 6. Activity of the LPL promoter with the T→G substitution at nt. -93. Wild-type and mutant promoters (from nt. -144 to +20) driving expression of the luciferase gene were transfected into THP-1 and C2C12 cells. All values were corrected for transfection efficiency. The data using THP-1 represent means and SE of 15 independent transfections from five separate experiments, using three different DNA preparations. The data using C2C12 represent means and SE of 8 independent transfections of the wild-type promoter and 19 independent transfections of the mutant promoter from 4 separate experiments, using three different DNA preparations. * $P < 0.05$; ** $P < 0.01$ (*t*-test).

and -53 substitutions were not found among 20 FCHL patients with normal LPL levels, 115 patients with CAD, or among 183 control subjects. Taken together, these results suggest that LPL promoter mutations may contribute to the etiology of FCHL, at least in the subset of patients who have reduced post-heparin plasma LPL activity.

Post-heparin plasma LPL activity and mass of the individuals carrying the -53 and -39/-93 substitutions were approximately half of the mean values of normal controls. Correlation between the level of post-heparin plasma LPL activity in carriers of LPL promoter mutations and in vitro promoter activity of these promoter mutations is difficult to establish in this study because of the relatively small number of cases.

The G→C substitution at nt. -53 is located between the CCAAT (nt. -65 to -61) and Oct-1 (nt. -46 to -39) motifs and within a putative Ca²⁺-responsive element, 5'-TGAGGTTT-3' (nt. -54 to -47), similar to that (TGACGTTT) of the *c-fos* gene promoter (36). DNase I protection assays of the human LPL promoter with nuclear extract from differentiated 3T3-L1 adipocytes revealed a footprint in the region extending approximately from nt. -52 to -35 (37). The -53 substi-

tion may affect binding of Oct-1 to the octamer site or of some other transcription factors to the putative Ca²⁺-responsive element. The T→G substitution at nt. -93 lies 2 bases 5' to a totally conserved inverted GA box (5'-CCTCCCCC-3', nt. -91 to -83) (Fig. 1) in the LPL promoter. We have shown that this motif binds the transcription factors Sp1 and Sp3 and is essential for the LPL promoter activity (W-S. Yang and S.S. Deeb, unpublished results). The nucleotides flanking this element may influence binding affinity of Sp1 and Sp3. As the -93 substitutions seems to be relatively prevalent, its impact on lipid metabolism in vivo merits further investigation. The -53 and -93 substitutions create *Sau3A* I and *Apa* I sites, respectively.

The dinucleotide insertion between nt. +13 and +19 of the 5' untranslated region is adjacent to the major and minor transcription initiation sites (21) and, therefore, may interfere with transcription initiation. As it caused moderate reduction in promoter activity, its in vivo significance needs to be further investigated.

As only 515 base pairs of the proximal LPL promoter were examined in this study, it is possible that more of the FCHL individuals with diminished LPL levels will be found to have LPL regulatory mutations. In fact, we

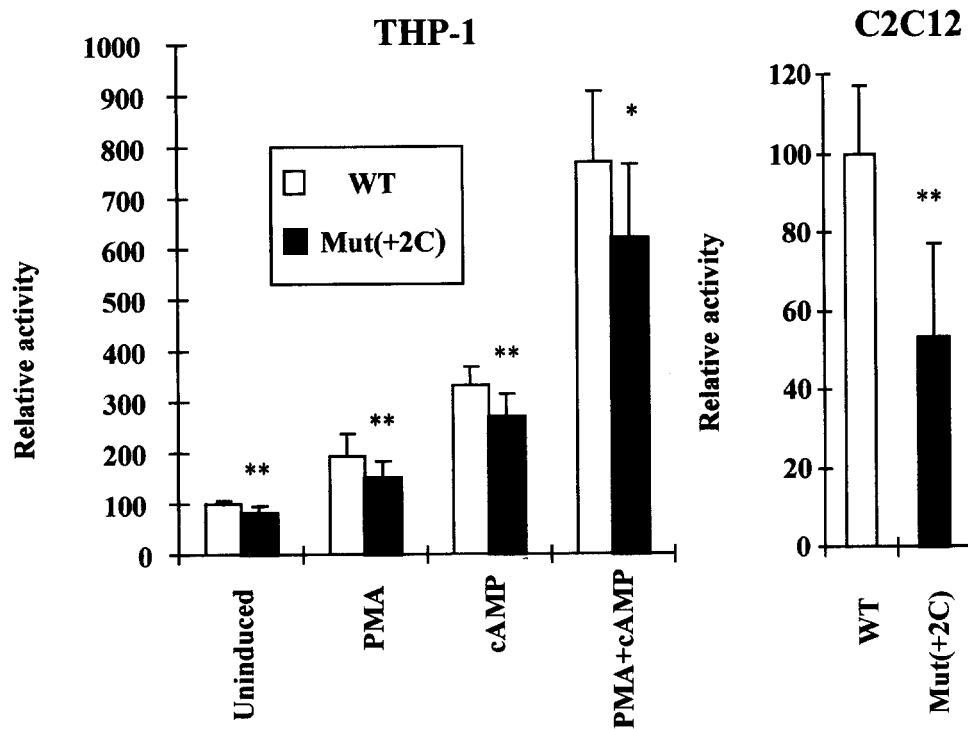


Fig. 7. Activity of the LPL promoter with the 2 C insertion between nt. +13 and +19. Wild-type and mutant promoters (from nt. -103 to +45) driving expression of the luciferase gene were transfected into THP-1 and C2C12 cells. All values were corrected for transfection efficiency. The data using THP-1 represent means and SE of 12 independent transfections from four separate experiments, using two different DNA preparations. The data using C2C12 represent means and SE of 8 independent transfections of the wild-type promoter and 10 independent transfections of the mutant promoter from three separate experiments, using two different DNA preparations. * $P < 0.05$; ** $P < 0.01$ (*t*-test).

and others have identified several candidate enhancer regions in introns and 3' flanking sequences of the LPL gene by DNase I hypersensitivity (38, S.S. Deeb and W.S. Yang, unpublished observation). These regions serve as candidates for further screening for regulatory variants. In addition to enhancer elements, a silencer element (from nt. -169 to nt. -152) has been reported in the promoter of the human LPL gene (39). Mutation in this element may also modulate expression of the LPL gene. No variants were found within this silencer element in this study.

One intriguing question is why three LPL promoter mutations but no LPL structural mutations affecting its catalytic activity were found among the same FCHL patients who had reduced LPL levels (17). It was estimated that the frequency of heterozygous carriers of LPL structural mutant alleles was 1 in 500 individuals in the general population (4). The absence of LPL structural mutations among FCHL patients may simply reflect their scarcity in the population from which the FCHL patients of this study were recruited.

As FCHL is relatively common among patients with CAD, we screened the DNA of 115 such patients for the presence of LPL promoter sequence variants. Among

these, 6 patients (1 homozygote) carried the base substitution at nt. -93, two patients carried the variant with the base substitution at nt. -95; and one patient carried the variant with the dinucleotide (CC) insertion in the 5' untranslated region (Table 2). The frequency of individuals carrying the variant alleles that affected promoter activity in CAD patients was more than 3-fold that in unselected controls (6.1%, 7/115 vs. 1.6%, 3/183; $P < 0.05$, Fisher's exact test). Reymer et al. (9) reported an association of the LPL structural mutation Asn291Ser with reduced levels of HDL-cholesterol in male CAD patients as well as controls, and with reduced HDL and elevated triglyceride levels in male FCHL patients (10). Partial LPL deficiency caused by the promoter mutations may also contribute to the development of dyslipidemia in atherosclerosis.

In addition to the LPL regulatory sequence mutations, the contributions from the other genetic and environmental factors cannot be overemphasized for oligogenic disorders like FCHL and atherosclerosis. Individuals with heterozygous LPL deficiency tend to have relatively normal lipid levels, but are liable to develop dyslipidemia when challenged with secondary factors (8, 10, 40, 41). Likewise, partial LPL deficiency

caused by promoter mutations may not by itself cause obvious metabolic derangement, but may jeopardize the ability of carriers to handle certain metabolic stresses. Therefore, the phenotypic expression of partial LPL deficiency would be expected to be modulated by other genetic and environmental factors, including diet, obesity, insulin resistance, hypertension, diabetes, and medications like estrogen, thiazide diuretics, and β -adrenergic antagonists.

Regulatory mutations have been reported in association with several genetic disorders including β -thalassaemia (42; reviewed in ref. 43), hereditary persistence of fetal hemoglobin (43, 44), hemophilia B (45, 46), retinoblastoma (47), and heterozygous familial hypercholesterolemia (48). Studies of these naturally occurring regulatory mutations not only provide new insight into the pathophysiology of these human disorders, but also offer in vivo models to decipher the complex networks of human gene regulation.

In conclusion, the finding of two promoter mutations (-39 and -53) that profoundly reduced the promoter function only among the 19 FCHL patients with reduced plasma LPL levels, but not among the 20 FCHL patients with normal LPL levels, 115 CAD patients, and 183 unselected ostensibly normal controls, suggests that partial LPL deficiency caused by promoter mutations may be one of the contributing factors for the etiology of FCHL. **BB**

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