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Research Article

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Molecular Studies on Primary Lipoprotein Lipase (LPL) Deficiency

One Base Deletion (G^{916}) in Exon 5 of LPL Gene Causes No Detectable LPL Protein due to the Absence of LPL mRNA Transcript

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Abstract

We have systematically investigated a genetic defect resulting in a primary lipoprotein lipase (LPL) deficiency in a proband TN and his affected brother SN, both manifesting familial hyperchylomicronemia. Neither LPL activity nor immunoreactive LPL mass was detected in postheparin plasma from the two patients. Immunocytochemical and biosynthetic studies on the proband's monocyte-derived macrophages with rabbit anti-human LPL antiserum revealed that no immunochemically detectable LPL protein was found in either the cells or culture medium, whereas LPL having a molecular mass of 61 kD was detected in normal cells. No detectable LPL mRNA was identified from poly(A)$^+$RNA of the proband's macrophages by Northern blot analysis, and grossly visible LPL gene rearrangement was not observed by Southern blot analysis. Sequence analysis of polymerase chain reaction-amplified LPL gene exons detected one base deletion of G (first position of Ala^{221}) at base 916 in exon 5 which leads to a premature termination by a frameshift. This mutation, designated as LPL_{Arita} and resulting in the loss of an AluI restriction enzyme site, was newly identified. We further analyzed the LPL gene from the two patients and their family members by digestion with AluI. Both patients were homozygous for LPL_{Arita} allele, while their spouses did not have this mutation. As genetically expected, their children were all heterozygous for LPL_{Arita}. We conclude that primary LPL deficiency in the proband was caused by a lack of enzyme synthesis due to the absence of LPL mRNA resulting from one base deletion of G in exon 5, and that heterozygous LPL_{Arita} deficient subjects show almost half value of control LPL mass. (*J. Clin. Invest.* 1992. 89:581-591.) Key words: lipoprotein lipase • hyperlipoproteinemia • molecular genetics • deficiency • type IV hyperlipidemia

Introduction

Lipoprotein lipase (LPL,[1] EC 3.1.1.34) is a glycoprotein enzyme that anchors to the capillary endothelial cell surface with a proteoglycan chain and plays a key role in hydrolyzing the triglycerides of chylomicrons and very low density lipoproteins at the first step in their metabolism (1, 2). In these reactions, LPL requires apo C-II as an essential cofactor and produces chylomicron and VLDL remnants, and nascent high density lipoprotein. The resulting lipoprotein remnants are thought to be further catabolized by hepatic triglyceride lipase (HTGL) (3, 4). Both enzymes are released into the circulation after intravenous injection of heparin. Recently, both lipases were purified from human postheparin plasma (PHP) and characterized (5, 6). Human PHP-LPL is catalytically active in a monomeric form and its apparent mol wt is 61,000 (6).

Type I hyperlipoproteinemia is a rare autosomal recessive disorder characterized by severe fasting hypertriglyceridemia including massive accumulation of chylomicrons, normal or marginally increased VLDL, and by decreased low density lipoprotein and high density lipoprotein levels in plasma (7–9). Clinically, this disease is usually detected by repeated episodes of abdominal pain, pancreatitis, xanthomatosis, and hepatosplenomegaly. The disorder has been reported to be mainly caused by primary LPL deficiency (8, 9), primary apo C-II deficiency (8, 9), or a circulating inhibitor for LPL (9). In biochemical studies on patients with the primary LPL deficiency as one of the causes, the analysis of LPL activity (8–10) and quantitation of immunoreactive mass (11, 12) in PHP samples are performed as important measures to estimate the pathogenesis of the disease; these measures usually indicate low or absent LPL activity and mass. LPL enzyme takes on a functionally active form at the capillary endothelium through a number of processes, including biosynthesis by extrahepatic parenchymal cells, secretion, transport, and binding to the endothelium (1, 13). Thus, one or more defects in these processes lead to an abnormality of LPL in PHP, and an individual with such LPL defect(s) is usually diagnosed as exhibiting primary LPL deficiency.

Human LPL cDNA has been cloned and its sequence predicts a molecular wt of 50,394 with 448 amino acid residues in a mature enzyme without sugar moieties (14). The human LPL gene is identified to be ~ 30 kb in length and consists of 10 exons (15, 16). Several groups have reported mutations of the LPL gene in patients with primary LPL deficiency. These are gene rearrangements (17, 18), missense mutations (19–25), nonsense mutations (26), addition of several bases (27), and mutation in exon–intron boundary region (22).

In this paper, we describe in detail a genetic defect of LPL gene in patient TN and his brother SN, both with fasting hyperchylomicronemia, and their family members. Both TN and SN

The results from this study were presented at the October 1988 meeting of the Eighth International Symposium on Atherosclerosis, Rome, and appeared in abstract form (Ikeda, Y., A. Takagi, T. Yamamura, and A. Yamamoto. 1988. Poster Sessions Abstract Book, p. 397).

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1. *Abbreviations used in this paper:* AEC, 3-amino-9-ethylcarbazole; EIA, enzyme immunoassay; HTGL, hepatic triglyceride lipase; LPL, lipoprotein lipase; PCR, polymerase chain reaction; PHP, postheparin plasma; SIIA, selective immunoinactivation assay.

were diagnosed as having a primary LPL deficiency by detecting neither LPL activity nor immunoreactive LPL mass in PHP using the selective immunoinactivation assay (SIIA) (6, 12) and the sandwich enzyme immunoassay (EIA) (12). Biochemical studies revealed neither immunochemically detectable LPL protein nor LPL mRNA from monocyte-derived macrophages of the patient TN. Both patients were homozygous for the LPL gene mutation (LPL_{Arita}) which was newly identified to be one base deletion of G at base 916 in exon 5, leading to no synthesis of LPL protein due to the absence of LPL mRNA. The patient's children were all heterozygotes carrying one allele of LPL_{Arita}.

Methods

Materials. Human PHP-LPL and human PHP-HTGL enzymes were purified to homogeneity from human PHP according to the method described previously (6). Pure human PHP-LPL (7.08 μmol/h per ml; sp act, 45 mmol/h per mg [12]) was used as an enzyme source to check whether or not the patient's plasma contained functionally active apo C-II and inhibitors for LPL as indicated in the text. A monospecific, polyclonal antiserum and monoclonal antibody were raised in rabbits and mice against human PHP-LPL and human PHP-HTGL as previously reported (6, 12). An HLC601 LPL cDNA probe was prepared from human THP-I (monocytic leukemia cell line) macrophage cDNA library as reported (28). HLG37-D containing the 3′-noncoding region of the LPL gene was isolated from the human gene library constructed in a lambda dash vector provided by Dr. K. Hiraga, Department of Biochemistry, Toyama Medical and Pharmaceutical University School of Medicine, and was used as a probe.

Subjects and clinical data. The N-family pedigree studied here is shown in Fig. 1. It consists of four siblings (I-1, -3, -5, and -6 in Fig. 1), two (TN and SN) of whom manifest type I hyperlipoproteinemia due to fasting hyperchylomicronemia. Consanguinity was identified in their parents, the father's grandmother being the mother's cousin. Proband TN and his spouse KN, and his brother SN and SN's spouse KN, were not consanguineous. The two patients TN and SN had recurrent episodes of abdominal pain and pancreatitis and were noted to have hepatomegaly, while they did not show clear evidence of diabetes mellitus, alcoholism, obesity, or other possible causes of secondary hyperlipidemia. The patients' family members did not show a history of impaired glucose tolerance as evidenced by oral glucose tolerance tests.

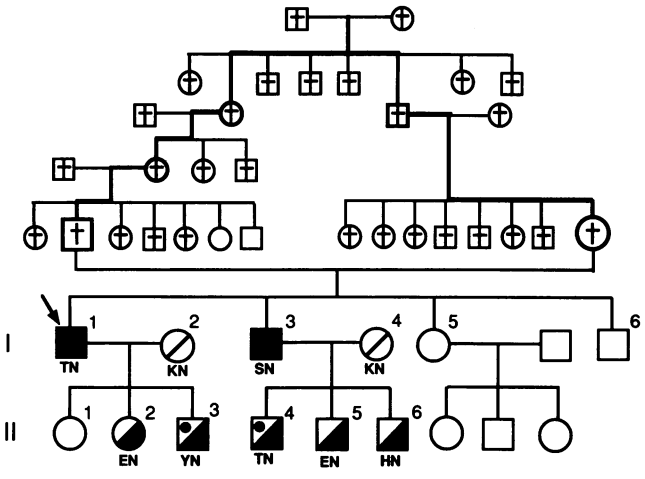

Figure 1. Pedigree of N-family. Proband TN is indicated by an arrow. ■, Type I hyperlipidemic subject who is homozygous for the LPL_{Arita} allele resulting in no LPL protein synthesis; ◪, normolipidemic subject who is heterozygous for LPL_{Arita}; ◪, type IV hyperlipidemic subject who is heterozygous for LPL_{Arita}; ◫, normal; □, not studied; [†], deceased subject. Expressions of phenotype and genotype are derived from the results obtained from Table I and Figs 2, 3, 5, 7, and 8.

40 healthy subjects (20 males and 20 females) aged between 23 and 69 with a mean±SD of 39.1±11.2 years were studied as a control group. Their total triglyceride and cholesterol concentrations were 70.0±22.3 mg/dl and 187.0±24.8 mg/dl (mean±SD), respectively.

Analysis of plasma lipid, lipoprotein, and apolipoprotein. Plasma was prepared from blood samples collected in tubes containing EDTA-2Na (1 mg/ml) after the subjects fasted overnight. Chylomicron and VLDL fractions were obtained from plasma by a quantitative ultracentrifugation at a solvent density of 1.006 g/ml (29). Triglyceride and cholesterol in plasma, chylomicron and VLDL fractions were measured by the enzymatic method described previously (30). Apo A-I, A-II, B, C-II, C-III, and E were measured by a single radial immunodiffusion assay (Daiichi Pure Chemicals Co., Tokyo, Japan) according to the supplier's directions.

Measurement of LPL and HTGL activities and immunoreactive masses in plasma. Blood samples, from subjects after an overnight fast, were collected in EDTA-2Na tubes before and 10 min after the injection of heparin (30 U/kg of body weight) for determination of LPL and HTGL activities as well as masses. Total PHP lipolytic activity, LPL and HTGL activities in PHP were measured with substrate of gum arabic-emulsified tri[9,10-^{3}H]olein (sp act, 8.93 μCi/μmol) by SIIA using rabbit anti-human PHP-LPL antiserum and anti-human PHP-HTGL antiserum as previously reported (6, 12). The unit of enzyme activity was expressed as micromoles of free fatty acid released per hour per milliliter of enzyme solution unless otherwise mentioned.

The sandwich EIA for LPL mass quantitation was performed by using two distinct types of anti-human PHP-LPL mAbs that recognize different epitopes on the LPL molecule. The immunoreactive mass of LPL in PHP was specifically measured using a β-galactosidase-labeled anti-human PHP-LPL mAb (1(1)D2B2) as an enzyme-linked mAb, an anti-human PHP-LPL mAb (2(10)F8F9) linked with the bacterial cell wall as an insolubilized mAb, and pure human PHP-LPL (0, 100, 200, 300, and 400 ng/ml) as a standard as previously reported (12). Immunoreactive mass of HTGL in PHP was also measured by the sandwich EIA using the combination of two distinct types of mAbs, such as a β-galactosidase-labeled anti-human PHP-HTGL mAb (2(4)F12C12) and an anti-HTGL mAb linked with the cell walls (1(11)A3H3) (12). Mass value was expressed as nanograms per milliliter of plasma.

Isolation and culture of monocyte-derived macrophages. Mononuclear cells were prepared from 100 ml of the heparinized blood obtained from the proband, his family, and normal subjects with Ficoll-Paque (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) according to the supplier's directions. The pooled cells were washed twice with RPMI 1640 medium supplemented with penicillin and streptomycin (Flow Laboratories Inc., McLean, VA) and suspended in the same RPMI 1640 medium containing 10% heat-inactivated autologous human serum (growth medium). In the case of sera obtained from hyperlipoproteinemia, they were further processed to remove lipoprotein by an ultracentrifugation and supplemented into the medium as lipoprotein-depleted sera. The cells were plated into 60-mm plastic culture dishes ($2–4 \times 10^7$ cells/dish) to isolate monocytes according to the supplier's directions (Japan Immunoresearch Laboratories Co., Takasaki, Japan). After 1 h incubation, nonadherent cells were removed and adherent monocytes were harvested with PBS containing fetal calf serum and EDTA. The harvested monocytes were plated into 35-mm dishes (1.5×10^6 cells/dish) or 100-mm dishes (2×10^7 cells/dish) containing 1 ml or 10 ml of the growth medium. The dishes were maintained for 8 d at 37°C in a 5% CO_2 atmosphere. The growth medium was replaced every day with new growth medium. Monocyte-derived macrophages cultured for 8 d were used in each experiment unless otherwise mentioned.

Assay of LPL activity derived from macrophages. The medium was harvested from the macrophages cultured in the 35-mm dishes containing 1 ml of growth medium at the time indicated in the text. The cells were harvested with 3 ml of RPMI 1640 medium containing 10%

FCS and protease inhibitors (0.1 mM PMSF, 0.1 mM leupeptin, 0.01 mM pepstatin A, and 0.6 μM aprotinin). The cell suspensions were centrifuged at 1,800 *g* for 10 min and the cell pellets were dissolved in 0.1 ml of 20 mM phosphate buffer, pH 7.4, containing 10% glycerol, 1 mM EDTA, 0.15% *n*-octyl-D-thioglucoside, heparin (50 μg/ml), and the same protease inhibitors as above. The cell suspension was sonicated three times for 1 min at intervals of 1 min in an ice bath. LPL activity in the medium and cell lysate (0.1 ml each) was quantitated under the same method described above. For determination of cell proteins, cells were solubilized with 1 ml of 0.1 N NaOH solution and the protein concentrations were measured by the method of Lowry et al. (31).

Immunocytochemical procedures. Monocyte-derived macrophages (1.5×10^6 cells/dish) in 35-mm dishes were fixed for 20 min with methanol containing 0.3% H_2O_2, followed by blocking with 5% normal goat serum/PBS solution for 20 min. The cells were incubated for 1 h with rabbit anti-human PHP-LPL antiserum (1/1,000) diluted by 1.5% normal goat serum/PBS solution (dilution buffer). After 1 h incubation, the cells were incubated for 0.5 h with 1 ml of biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) in the dilution buffer, reacted with 1 ml of avidin/biotinylated horseradish-peroxidase complexes (Vector Laboratories) in the dilution buffer, and then stained for 20 min with 0.02% of 3-amino-9-ethylcarbazole (AEC)/0.01% H_2O_2 in 50 mM acetate buffer. In control experiments, the cells were incubated with nonimmune rabbit serum (1/1,000) dissolved in the dilution buffer, followed by the subsequent procedures mentioned above. After AEC reaction, nuclei were stained with Mayer's hemalum solution. Photographs were taken using an Olympus (Vanox S) light microscope. Immunocytochemical detection of apo E in macrophages was also performed with rabbit anti-human apo E antiserum (a gift from Daiichi Chemical Co., Tokyo) under the same methods described above.

Labeling of macrophages in culture. The monocyte-derived macrophages in the 35-mm dishes were washed twice with PBS and then incubated for 1 h in 1 ml of labeling medium (methionine-depleted Eagle's minimum essential medium containing 10% dialyzed FCS). The medium was replaced with 1.5 ml of fresh medium containing 100 μCi of L-[^{35}S]methionine (Amersham Corp., Arlington Heights, IL). The dishes were incubated at 37°C for the time indicated in the text. The medium (1.5 ml) was harvested and mixed with 0.15 ml of 0.1 M Tris buffer (pH 7.4) containing 0.1 M EDTA, 5% Triton X-100, and 2.5% SDS. The mixture was subjected to immunoprecipitation. The cells were first washed twice with PBS and solubilized with 2 ml of the lysis buffer (10 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.25% SDS, and 1% unlabeled methionine) as described in our previous papers (32, 33). The cell lysate was centrifuged at 356,000 *g* for 10 min using an Ultracentrifuge (TL-100; Beckman Instruments, Palo Alto, CA) and the supernatant was subjected to immunoprecipitation.

Immunoprecipitation of radiolabeled LPL and electrophoresis. The culture medium and cell lysate solubilized in the lysis buffer were mixed with 7 μl of rabbit anti-human PHP-LPL antiserum or 10 μl of rabbit anti-human apo E antiserum. The mixtures were incubated for 12 h at 4°C. The immunocomplexes were recovered by adding 100 μl of *Staphylococcus aureus* cell suspension (10%, wt/vol) (Bethesda Research Laboratories, Gaithersburg, MD) followed by centrifugation at 1,700 *g* for 5 min (32, 33). The pellets were washed three times with 2 ml of 10 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, and 1.0% deoxycholate. The washed immunoprecipitates were solubilized in 25 μl of 0.1 M Tris buffer (pH 6.8) containing 3% SDS, 37% glycerol, 33 mM dithiothreitol, and 0.019% bromophenol blue and boiled for 5 min (32). The sample mixtures were centrifuged at 4,000 *g* for 10 min, and then the supernatant was subjected to a slab SDS-PAGE with 10% gel according to the method of Laemmli (34). The gel was stained with Coomassie blue, destained, and treated for fluorography with Autofluor (National Diagnostics, Inc., Somerville, NJ) (33). The radiolabeled protein bands were visualized on Kodak XAR-5 x-ray film after exposure at −80°C. The subunit molecular mass was estimated using the following marker proteins: phosphorylase B (94 kD), bovine serum albumin (68 kD), ovalbumin (46 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (21 kD), and lysozyme (14 kD).

Northern and Southern blot analysis. Total cellular RNA was prepared from macrophages, cultured in the 100-mm dishes, of normal subjects and proband TN. Cells were lysed in 4 M guanidine isothiocyanate buffer and centrifuged through a cesium chloride gradient to isolate total RNA (35). Poly(A)$^+$RNA was then isolated from the total RNA by oligo (dT)-cellulose column chromatography (35). An aliquot of 6.8 μg of poly(A)$^+$RNA was denatured in formaldehyde/formamide and electrophoresed in a formaldehyde-containing 1.0% agarose gel (35). RNA was transferred to a membrane of GeneScreen Plus (Dupont-NEN, Boston, MA) overnight, and hybridized with ^{32}P-labeled HLC601 LPL cDNA (32-1643) containing the complete coding region of human LPL cDNA (28) or ^{32}P-labeled chicken β-actin cDNA (36). Prehybridization, hybridization, and washing were performed according to the standard procedure (35).

Genomic DNA was isolated from peripheral mononuclear cells of normal subjects, the two patients (TN and SN) and their family members as described (35). Southern blot analysis was performed on DNA separated by 1.0% agarose electrophoresis after digestion with the appropriate restriction enzymes, followed by transfer to a GeneScreen Plus membrane and hybridization with ^{32}P-labeled HLC601 or ^{32}P-labeled HLG37-D (1629-3154 of human LPL cDNA) according to the standard procedure (35). The blotted sheets were exposed to Kodak XAR-5 film for several days at −80°C with intensifying screens.

Cloning and sequencing of the genomic DNA of proband TN. DNA containing each exon and exon-intron boundary, and the 5′ upstream region of the LPL gene were enzymatically amplified from the genomic DNA of proband TN by a DNA Thermal Cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) using 20 pmol of each pair of primers described below and 5 U of *Taq* DNA polymerase (37, 38). The reaction mixtures were denatured at 94°C for 10 min before addition of *Taq* DNA polymerase. The polymerase chain reaction (PCR) was performed with 1 min denaturation at 94°C, 1 min primer annealing at 55°C, and 2 min extension at 72°C. Amplified DNA was purified by a Geneclean Kit (BIO 101, Inc., La Jolla, CA) according to the supplier's directions. The purified DNA was digested with PstI and BamHI, and cloned into the Bluescript SK II M13(−) vector (Stratagene Inc., La Jolla, CA), while DNA blunted with T4 DNA polymerase was also cloned into the same vector. DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (39).

Primers were synthesized by a DNA synthesizer (380 A; Applied Biosystems, Inc., Foster City, CA) and are listed below: DNA including 5′ upstream region, and each exon and exon-intron boundary was amplified with Exon-1F (5′-GCCGGATCCAAATGGAATCATACAATATGTGTCTTTTG-3′), Exon-1R (5′-CGGGTCTGCAGGTGGAGGGTAGTTT-3′), Exon-2F (5′-GGTGGATCCAACCCTCCAGTTAACCTCATATCCAA-3′), Exon-2R (5′-GTGCTGCAGCACCACCCCAATCCACTCTTCCCCA-3′), Exon-3F (5′-ACCGGATCCTAGGTGGGTATTTTAAGAAAGCTTGTG-3′), Exon-3R (5′-GAGCTGCAGCACTGCTTTGGACACATAAGTCTCC-3′), Exon-4F (5′-GAGGGATCCGCAGAACTGTAAGCACCTTCATTTTC-3′), Exon-4R (5′-GGTCTGCAGTTCACCTCTTATGATAAGACCAACGAA-3′), Exon-5F (5′-GGAGGATCCAAATTTACAAATCTGTGTTCCTGCTTTTT-3′), Exon-5R (5′-GCCCTGCAGGATAAGAGTCACATTTAATTCGCTTCTA-3′), Exon-6F (5′-AGAGGATCCTTCTGCCGAGATACAATCTTGGTGTC-3′), Exon-6R (5′-AGGCTGCAGGACTCCTTGGTTTCCTTATTTACAACA-3′), Exon-7F (5′-GGCGGATCCATAAAGATTGATCAACATGTTCGAATTTC-3′), Exon-7R (5′-TATCTGCAGGGGACTGGTGCCATGATGACCGCCC-3′), Exon-8F (5′-GCCGGATCCGATCTCTATAACTAACCAAATTTATTGCT-3′), Exon-8R (5′-TCCCTGCAGTGGGGGTCTAAAGTGAAGGAAGAAAA-3′), Exon-9F (5′-GAAGGATCCTTGTTCTACATGGCATATTCACATCCA-3′), Exon-9R (5′-TAACTGCAGAGCTCAGGATGCCCAGTCAGCTTTA-3′), Exon-10F

Table I. Plasma Lipoprotein Lipid Concentration of N-Family Members

Number*	Subject's name	Age‡	Sex	Triglyceride§ Total	Triglyceride§ d < 1.006 fraction	Cholesterol§ Total	Cholesterol§ d < 1.006 fraction	Cholesterol§ LDL	Cholesterol§ HDL	Apo C-II§
Proband TN family										
I-1	Proband TN	59	M	1899	1784	207	164	26	18	10.0
I-2	Spouse KN	55	F	134	94	195	25	109	48	3.3
II-2	Daughter EN	27	F	126	84	211	21	140	46	3.3
II-3	Son YN	24	M	296	223	216	54	114	43	6.5
Proband's brother SN family										
I-3	Brother SN	58	M	2028	1931	270	220	30	14	9.6
I-4	Spouse KN	57	F	88	48	258	10	177	63	3.9
II-4	Son TN	34	M	228	163	196	29	112	38	7.4
II-5	Son EN	32	M	126	68	197	17	134	38	4.9
II-6	Son HN	27	M	128	88	198	18	125	47	4.3

Plasma of N-family members was fractionated by an ultracentrifugation procedure. Triglycerride and/or cholesterol of the fraction (d < 1.006) containing chylomicron and/or VLDL, LDL, and HDL were measured as described in the experimental procedures. Apo C-II was quantitated by single radial immunodiffusion.
* Number is identical to the subject number described in Fig. 1. ‡ Age is shown in years. § Concentration of triglyceride, cholesterol, and apo C-II is expressed as milligrams per deciliter.

(5′-GAAGATAATAAATTGCCCTTTTTCCTG-3′), and Exon-10R (5′-GCCAACAACAAATCATACATCATTTTA-3′). The primers were designed on the basis of the 5′-upstream DNA sequence (16), the intron–exon boundaries (40), and cDNA sequence (14).

Restriction enzyme digestion of PCR-amplified DNA. Genomic DNA obtained from two patients (TN and SN), their family members, and control subjects were amplified by the PCR using a pair of primer exon-5F and primer exon-5R. The amplified DNA was digested with 50 U of AluI (Gibco Laboratories, Bethesda, MD) overnight at 37°C. The resulting DNA fragments were analyzed on a 4% NuSieve agarose gel (FMC BioProducts, Rockland, ME). DNA bands were visualized by staining with ethidium bromide.

Results

Plasma lipid, lipoprotein, and apolipoprotein profiles of N-family

As shown in Table I, proband TN (I-1) and his brother SN (I-3) exhibited fasting hyperchylomicronemia with triglycerides

Table II. LPL and HTGL Activities and Masses in Preheparin Plasma and PHP of N-Family Members and Normal Subjects

Number*	Subject's name	LPL Preheparin plasma Mass	LPL PHP Activity	LPL PHP Mass	HTGL PHP Activity	HTGL PHP Mass
		ng/ml	*U*	*ng/ml*	*U*	*ng/ml*
Proband TN family						
I-1	Proband TN	ND‡	ND‡	ND‡	12.8	610
I-2	Spouse KN	11.8	8.3	164	21.0	1339
II-2	Daughter EN	26.6	6.6	127	16.8	625
II-3	Son YN	19.2	6.0	118	23.6	1363
Proband's brother SN family						
I-3	Brother SN	ND‡	ND‡	ND‡	14.1	785
I-4	Spouse KN	15.3	7.1	162	30.8	1896
II-4	Son TN	14.9	5.1	112	57.0	3624
II-5	Son EN	27.6	5.6	136	30.6	1657
II-6	Son HN	17.8	5.2	111	42.1	2367
Normal (n = 40)		34.2±9.9§	9.5±3.0§	203±43§	21.6±9.2§	1210±532§

Both enzyme activity and immunoreactive mass were quantitated by the SIIA and sandwich-EIA, and units of activity and mass are defined under experimental procedures.
* Number is the same as in Table I. ‡ ND, not detectable. § Mean±SD.

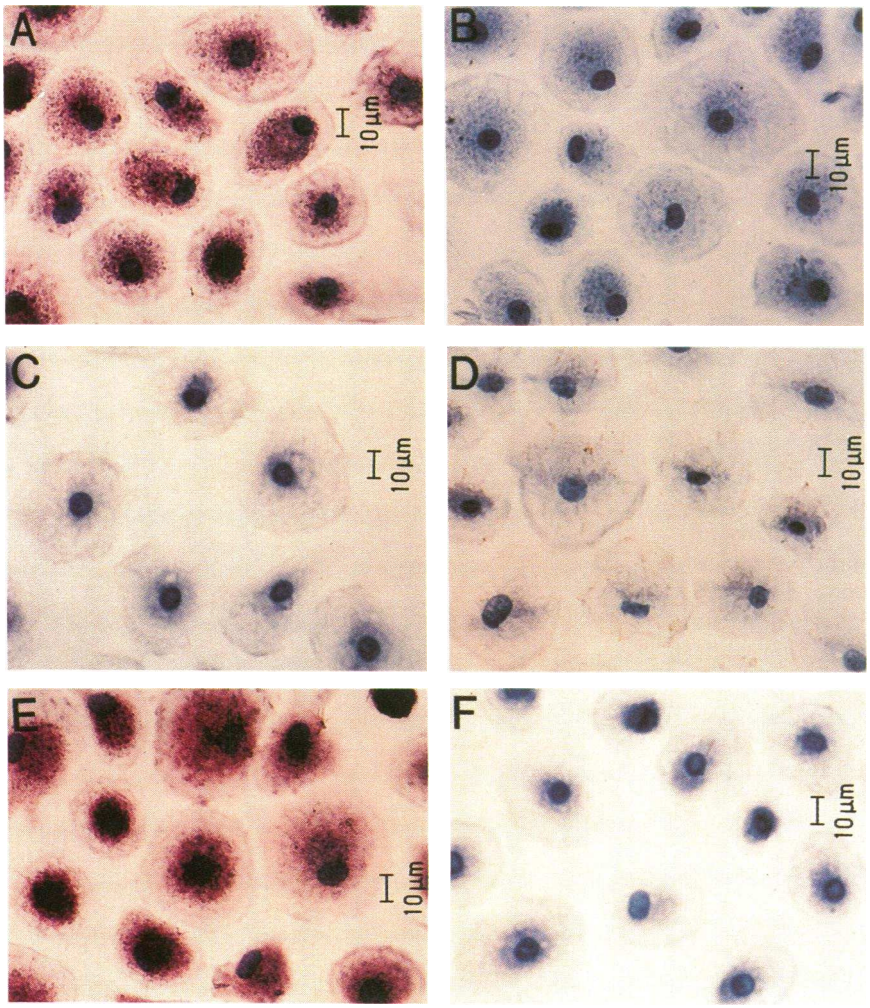

Figure 2. Immunocytochemical detection of LPL and apo E in cultured macrophages. Macrophages were fixed with methanol containing 0.3% H_2O_2. After the fixation, cells were immunostained for LPL with rabbit anti-human PHP-LPL antiserum (*A* and *C*) and for apo E with rabbit anti-human apo E antiserum (*E*) as described in the experimental procedures. Red color shows LPL (*A*) and apo E (*E*) proteins stained by the AEC substrate. Control experiments were carried out with nonimmune rabbit serum (*B, D,* and *F*). *A* and *B,* normal macrophages; *C, D, E,* and *F,* macrophages of proband TN.

> 1,500 mg/dl, and low cholesterol concentration of both low density and high density lipoproteins. The patients manifested type I hyperlipoproteinemia as a phenotype based on the plasma lipoprotein pattern as reported (7, 8). Two of their children (II-3 and -4) exhibited type IV hyperlipoproteinemia as a phenotype reported (7, 10). The patient's spouses (I-2 and -4) and the other children (II-2, -5, and -6) displayed normolipidemia. Apo C-II mass values for N-family members were within or more than normal range (3.4±1.3 mg/dl, mean±SD) and the biological function of apo C-II was determined to be normal by the observation that two patients' plasma activated pure human PHP-LPL enzyme in vitro.

Analysis of LPL and HTGL activities and masses in PHP

Table II shows LPL and HTGL activities and masses in preheparin plasma and PHP from the N-family members and nor-

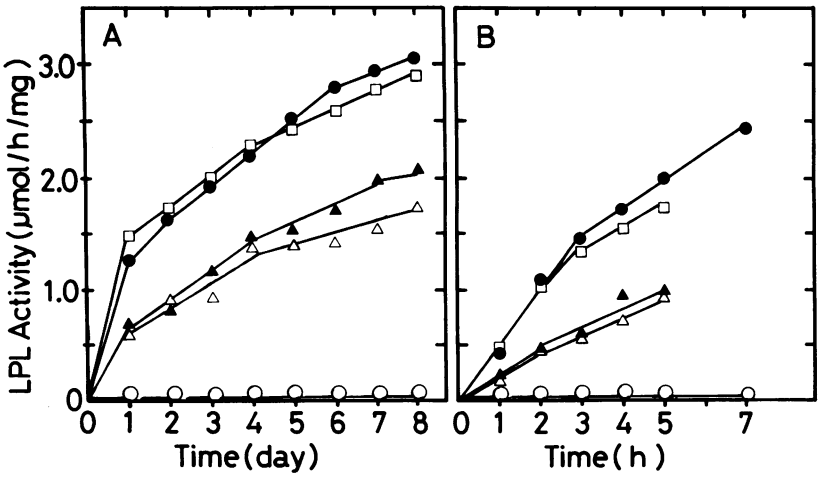

Figure 3. Time course of LPL activity secreted by macrophages of proband TN, his family, and a normal subject. (*A*) Monocyte-derived macrophages were cultured for 8 d in 35-mm dishes containing 1 ml of the growth medium, and LPL activity in the medium was monitored every 24 h as described under the experimental procedures. (*B*) After 8 d incubation of the macrophages, the growth medium was replaced with 1 ml of fresh growth medium. The dishes were incubated for the indicated times under the same conditions as in *A*. LPL activity in the medium was measured. In *A* and *B,* LPL activity is expressed as micromoles of free fatty acids released per hour per milligram of cell proteins. Values shown are average of the duplicate LPL assay from one dish each. ○, proband TN; □, spouse KN; ▲, daughter EN; △, son YN; ●, normal subject.

mal subjects. Both proband TN and his brother SN exhibited neither LPL activities nor mass values in both preheparin plasma and PHP, indicating that they should be diagnosed as having a primary LPL deficiency. The existence of inhibitors for LPL in the two patients' plasma was excluded because the patients' plasma did not inactivate pure human PHP-LPL enzyme activity. Their spouses showed apparent normal LPL activity and mass levels in PHP, whereas LPL activities and mass values for their children were ~ 50% of the mean values of normal subjects. HTGL activities and mass values of all N-family members were within or more than normal range.

Immunocytochemical detection of LPL in macrophages of proband TN and a normal subject

Monocyte-derived macrophages of a normal subject and proband TN were cultured for eight days and fixed with a methanol solution. After the fixation, macrophages were reacted with rabbit anti-human PHP-LPL antiserum or anti-human apo E antiserum. As shown in Fig. 2, LPL proteins in normal macrophages (*A*) were well stained and localized in the perinuclear area in all cells. In the case of proband TN, however, no LPL proteins were detected in any cells (*C*), suggesting that macrophages of proband TN did not have the ability to produce LPL proteins. In contrast, macrophages of proband TN could synthesize apo E proteins and they were clearly detected in the perinuclear area (*E*). With nonimmunized rabbit serum, no LPL proteins in macrophages were stained at all as shown in *B* and *D*, and no apo E proteins in *F*.

Molecular nature of LPL synthesized and secreted by macrophages of proband TN and his family, and a normal subject

LPL activity derived from macrophages. LPL activities secreted from macrophages of proband TN and his family members, and a normal subject were examined after one day each in culture for eight days (Fig. 3 *A*). LPL activities from proband TN were not detected through culture for eight days, while LPL activities from his spouse KN were almost the same as those of the normal subject. LPL activities from daughter EN and son YN were almost half of the normal subject. Furthermore, to avoid the possibility that the secreted proband TN's LPL was inactivated by long incubation (24 h) at 37°C, we examined LPL activities secreted in short incubation (1–7 h) from the macrophages obtained at the eighth day of culture (Fig. 3 *B*). Again, no LPL activities were detected from the proband even in the short time incubation, whereas LPL activities from the proband's family members and a normal subject were linearly accumulated until at least 3 h. In addition, no intracellular LPL activity was observed from the macrophages of the proband, whereas normal macrophages gave intracellular LPL activity of 0.63 μmol/h per mg of cell protein.

Biosynthesis of LPL by macrophages

The molecular nature of LPL synthesized and secreted by macrophages of a normal subject and proband TN was examined by pulse-labeling experiments with [^{35}S]methionine. Macrophages were pulse-labeled for 1, 3, and 5 h. The radiolabeled LPL proteins were immunoprecipitated with rabbit anti-human PHP-LPL antiserum and then analyzed by a slab SDS-PAGE as shown in Fig. 4. In the normal subject (Fig. 4 *A*), a sharp single labeled LPL protein band was detected in cell lysate at three each pulse-labeling time (lanes *8–10*), while LPL protein in culture medium was clearly detected at 3 and 5 h labeling time (lanes *3* and *4*). These bands were ascertained to

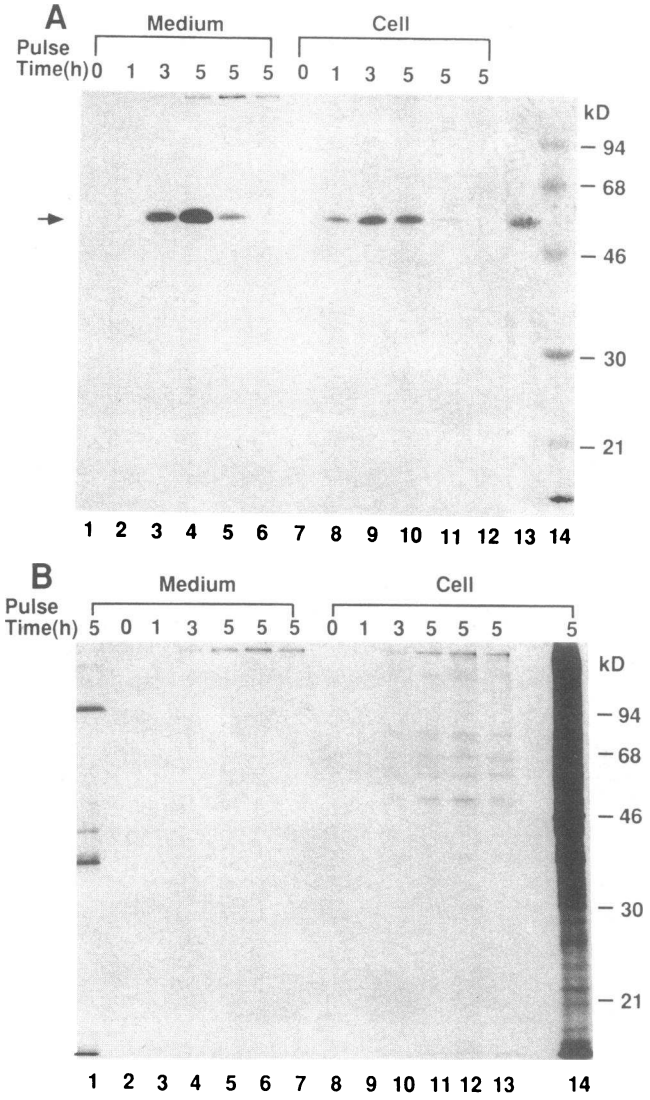

Figure 4. Slab SDS-PAGE of LPL synthesized and secreted by cultured macrophages of a normal subject (*A*) and proband TN (*B*). The macrophages were pulse labeled for 0, 1, 3, and 5 h with [^{35}S]methionine. The labeled products in the medium (1.5 ml) and cell lysate (2 ml) were immunoprecipitated with anti-human PHP-LPL antiserum, and the immunoprecipitates were analyzed on SDS-PAGE with 10% gel, followed by autoradiography. (*A*) Lanes *1–4* and *7–10* are the labeled LPL in the medium and in the cell lysate, respectively, immunoprecipitated with the antiserum. Lanes *5* and *11* are competitive immunoprecipitates from the medium and cell lysate, respectively, to which 5 μg of unlabeled pure human PHP-LPL was added before the addition of the antiserum. Lanes *6* and *12* are control immunoprecipitates with 7 μl of nonimmune rabbit serum. Lanes *13* and *14* are pure human PHP-LPL (10 μg) and marker proteins (5 μg each), respectively, and its picture was taken from the dried gel stained with Coomassie blue. (*B*) Lanes *1* and *14* are the labeled products in the medium (3 μl) and the cell lysate (2 μl), respectively. Lanes *2–5* and *8–11* in *B* correspond to lanes *1–4* and *7–10* in *A*. Lanes *6*, *7* and *12*, *13* in *B* correspond to lanes *5*, *6* and *11*, *12*, in *A*. The x-ray film was exposed for 1 d in *A* and for 4 d in *B*. The arrow mark on the left indicates the position of the LPL polypeptide.

be LPL protein bands by competition and control experiments. The labeled LPL bands diminished in the presence of excess pure human PHP-LPL before the addition of the antiserum (lanes *5* and *11*). Also, these protein bands were not detected by the addition of nonimmune rabbit serum (lanes *6* and *12*). The subunit molecular mass of the labeled LPL (61 kD) in both the culture medium and cell lysate was almost identical to that (61 kD in lane *13*) of pure human PHP-LPL. In contrast, proband TN's macrophages exhibited no detectable labeled LPL protein bands in both culture medium (lanes *3–5* in Fig. 4 *B*) and cell lysate (lanes *9–11* in Fig. 4 *B*) at any of the pulse-labeling times even in four times longer exposure of x-ray film than that of normal macrophage experiment, although the proband's macrophage proteins were well labeled with [^{35}S]methionine (lane *14* in Fig. 4 *B*). In the proband's experiment (Fig. 4 *B*), it should be noted that protein bands visualized in cell lysates labeled for 3 and 5 h (lanes *10* and *11*) were determined to be nonspecific, because those bands were nonspecifically observed in both competition (lane *12*) and control (lane *13*) experiments.

In the proband's family study (Fig. 5 *A*), the LPL protein band from spouse KN (lanes *9* and *11*) was nearly the same as that of the normal subject (lanes *1* and *3*) in its molecular mass (61 kD) and intensity, indicating that spouse KN was normal for the ability to synthesize and secrete LPL. As expected, the labeled LPL protein bands from son YN (lanes *13* and *15*) and daughter EN (lanes *17* and *19*) were approximately half of the amount of LPL from the normal subject in both medium and cell extracts. As a control experiment (Fig. 5 *B*), the molecular nature of apo E synthesized and secreted by macrophages of the normal subject and the proband's family was examined using rabbit anti-human apo E antiserum. All tested members including proband TN showed apo E proteins (odd number lanes in lanes *5–19*) which were the same as that of the normal subject (lanes *1* and *3*) in their molecular mass (38 kD in the medium and 36 kD in the cell lysate) and intensity. Together with the observations from the immunocytochemical studies (Fig. 2), these data demonstrate that proband TN should be determined as having the disability to synthesize the LPL molecule, while the fact that apo E is normally produced in the proband suggests that other proteins might not be affected.

Northern and Southern blot analysis of proband TN

Northern blot analysis of poly(A)$^+$RNA isolated from monocyte-derived macrophages of proband TN, and normal subjects YI and AY was carried out with ^{32}P-labeled HLC601 LPL cDNA. As shown in Fig. 6 *A*, proband TN showed no detectable amount of LPL mRNA (lane *2*), while almost equal amounts of two species of mRNA (3.4 kb and 3.8 kb) were detected in the macrophages of the two normal subjects (lanes *1* and *3*). As a control experiment, the same membrane was treated to remove the HLC601 probe and was rehybridized with a ^{32}P-labeled chicken β-actin cDNA probe. The proband (lane *2*) and two normal subjects (lanes *1* and *3*) yielded almost equal amounts of β-actin mRNA (Fig. 6 *B*).

To examine the gross structure of proband TN's LPL gene, the digestion pattern of genomic DNA with PvuII, BamHI and HindIII was studied by Southern blot analysis with a ^{32}P-labeled HLC601 or ^{32}P-labeled HLG37-D probe. No detectable deletion or insertion in the LPL gene was observed in proband TN (data not shown).

Identification of mutation in LPL gene of proband TN

Each DNA segment encompassing an exon and exon–intron boundary, and the 5′-upstream region of the LPL gene was amplified with the primers as described under the experimental procedures. The amplified DNAs were cloned into Bluescript SK II M13 (−) vector. Five independent clones were isolated for each amplified DNA and sequenced. Only one apparent

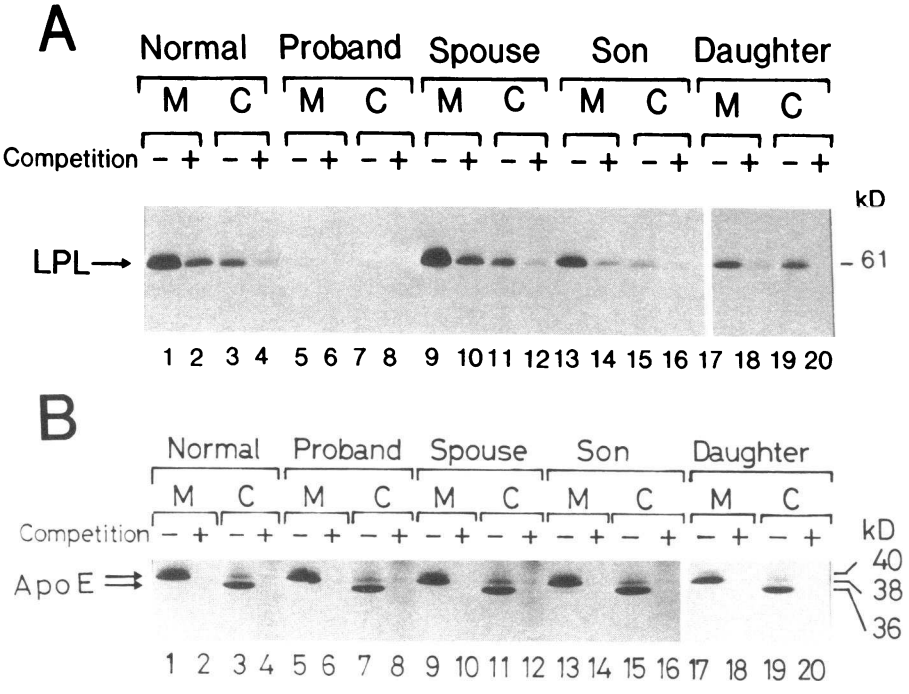

Figure 5. Slab SDS-PAGE of LPL (*A*) and apo E (*B*) synthesized and secreted by macrophages of proband TN and his family. The macrophages were pulse labeled for 5 h with [^{35}S]methionine. (*A*) and (*B*) The labeled products in the medium (*M*) and cell lysate (*C*) were immunoprecipitated with 7 μl of anti-human PHP-LPL antiserum (*A*) or 10 μl of anti-human apo E antiserum (*B*), and were analyzed on SDS-PAGE with 10% gel as described in Fig. 4. Competition experiments were performed with (+) or without (−) 5 μg of unlabeled pure human PHP-LPL or 5 μg of unlabeled pure human apo E before the addition of the corresponding antiserum. Only the relevant portion of the gel fluorogram is shown. Subjects names were listed on the top of each panel.

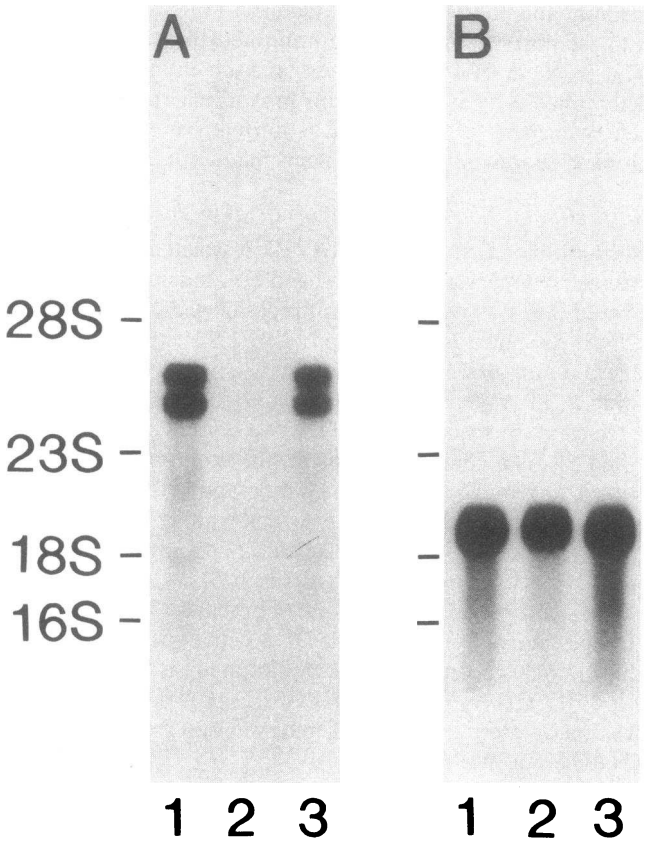

Figure 6. Northern blot analysis of poly(A)$^+$ RNA from macrophages of proband TN and normal subjects. The blot was first hybridized with ^{32}P-labeled HLC601 LPL cDNA (*A*) and after the removal of the HLC601, the same membrane was rehybridized with ^{32}P-labeled chicken β-actin (*B*). In *A* and *B*, all lanes contain poly(A)$^+$ RNA (6.8 μg) isolated from macrophages. Lane *1*, normal YI; lane *2*, proband TN; lane *3*, normal AY.

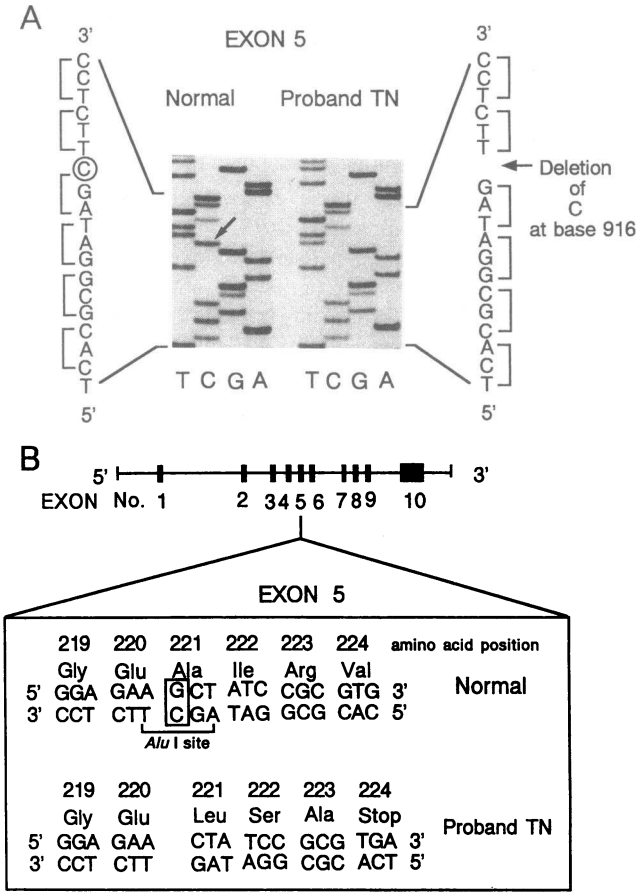

Figure 7. Nucleotide sequence of a part of the antisense strand from exon 5 of a normal subject and proband TN (*A*), and schematic representation of the portion showing one base deletion leading to a premature termination in exon 5 by a frameshift (*B*). In *A*, the deletion of a C at base 916 in the proband's antisense strand is indicated by the arrow. The position of the base C is circled in the normal antisense strand and is also indicated by the arrow on the sequence picture. In *B*, the nucleotide and amino acid sequence of a section of exon 5 from a normal subject and the proband are illustrated, and the deleted base is highlighted by a box in the normal strands. Both nucleotide sequence and amino acid numbers are taken from the human LPL cDNA sequence (14).

nucleotide variant from the normal entire cording exon was identified in exon 5; all five clones possessed one base deletion of G (it is noted as a C base deletion of the antisense strand of exon 5 in Fig. 7 *A*), which is the first position of the GCT codon for alanine at position 221 in a normal allele (Fig. 7 *B*), at nucleotide position 916 in exon 5. The one base deletion generates a stop codon (TGA) at position 224 within exon 5 by a frameshift (Fig. 7 *B*), and resulted in the loss of an AluI restriction enzyme site present in the normal LPL gene (Fig. 7 *B*). This mutation was designated as LPL_{Arita} in connection with the birth place of proband TN. Sequence analysis of the 5'-flanking region of the LPL gene did not show any variants for important consensus sequences in the regulatory regions of gene expression reported (15, 16), and neither were any significant variants detected in the 3'-noncoding region (exon 10).

Analysis of the LPL gene in N-family members by AluI digestion

N-Family members including proband TN and his affected brother SN were examined by AluI digestion of their PCR-amplified DNA from the LPL gene to confirm the carrier status of the LPL_{Arita} allele which was characterized by the loss of an AluI site in exon 5 (Fig. 8 *A*). As shown in Fig. 8 *B*, proband TN (lane *4*), and his brother SN (lane *8*) exhibited a 270-bp band which resulted from the loss of an AluI site, whereas their spouses gave two bands of 189 bp and 82 bp that were derived by the digestion of AluI (lanes *7* and *12*) as the same in normal subjects (lanes *1–3*). Their children showed three bands of 270 bp, 189 bp, and 82 bp (lanes *5, 6, 9, 10,* and *11*). The DNA fragment of 61 bp is produced in common from all samples under the conditions employed. These data indicated that proband TN and his brother SN possessed both alleles of LPL_{Arita}, their spouses did not have the same mutation, and all children were carriers of one allele of LPL_{Arita}. When genomic DNA was directly digested by AluI without PCR amplification, homozygosity of both proband TN and his brother SN, and heterozygosity of their children were essentially confirmed to be the same as those of the PCR amplification experiment (data not shown).

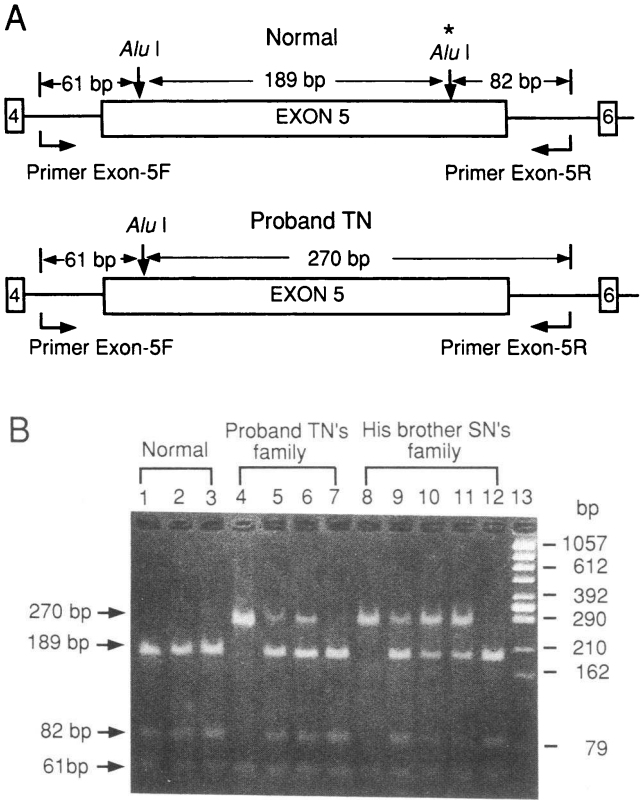

Figure 8. Schematic illustration of the portion of the LPL gene that was PCR amplified by using primer exon-5F and -5R is shown in *A*. The DNA fragments that derived from the digestion with AluI are 189, 82, and 61 bp. The LPL_{Arita} allele of the proband is missing an AluI site (indicated by the asterisk) in exon 5 at base 916 resulting in a DNA fragment of 270 bp. (*B*) NuSieve Agarose gel electrophoresis pattern of the restriction fragments generated by AluI digestion of the PCR-amplified DNA. DNA bands were visualized by staining with ethidium bromide. Lanes *1–3,* normal YI, ZT, and AT; lane *4,* proband TN; lane *5,* daughter EN; lane *6,* son YN; lane *7,* spouse KN; lane *8,* proband's brother SN; lane *9,* son TN; lane *10,* son EN; lane *11,* son HN; lane *12,* spouse KN; lane *13,* ØX-174 DNA digested by HincII.

Discussion

We have systematically studied the molecular basis of primary LPL deficiency in proband TN with type I hyperlipoproteinemia by analyzing LPL activity and immunoreactive mass in PHP, the molecular nature of LPL biosynthesized by monocyte-derived macrophages, and the genetic defect of the LPL gene. Analysis of LPL in PHP from the proband revealed neither detectable activity nor mass, indicating that the proband has a disability to synthesize LPL protein or some defects by which intracellular LPL molecules cannot be secreted or transported, or bound to endothelium. To elucidate which processes are defective in the proband, we used cultured monocyte-derived macrophages as one of the representatives of LPL producing cells (41) instead of adipocytes or muscle cells, because a relatively large amount of monocytes are easily obtained from peripheral blood. Neither LPL activity nor immunoreactive LPL protein was detected in either the culture medium or the cultured macrophages of the proband by biochemical studies, including immunocytochemical and biosynthetic experiments, whereas a catalytically active LPL protein having a molecular mass of 61 kD was identified in normal macrophages. Thus, it is clearly demonstrated that the defect on this type of primary LPL deficiency is the lack of ability to synthesize LPL molecules. Consistent with this finding was the result from the blot hybridization analysis, which demonstrated that no LPL mRNA was detected in the proband's macrophages. In the Southern blot analysis, no significant deletions or additions were detected in the LPL gene from the proband.

Among the several reports on the molecular basis of LPL deficiency (17–27), it has been reported that a gross LPL gene mutation with a 2-kb insertion resulted in no identifiable LPL activity nor mass in PHP (18). In our case, one base deletion of G at base 916 in exon 5 of the LPL gene from proband TN revealed no detectable LPL activity nor immunoreactive mass in PHP. This mutation (LPL_{Arita}) resulted in the loss of an AluI site at the position of G base deletion. The proband was clearly demonstrated to be homozygous for LPL_{Arita} allele because of the absence of normal-sized DNA fragments (189 and 82 bp) from PCR-amplified exon 5 DNA digested by AluI. The LPL_{Arita} allele generates a stop codon at amino acid position 224 within exon 5 through a frameshift, and results in no LPL protein synthesis due to the absence of LPL mRNA instead of a truncated LPL protein synthesis. It therefore seems that occurrence of a premature termination in exon 5 is closely related to the reduced stability of LPL mRNA, although its mechanism remains to be further elucidated. There are two reports about the occurrence of premature termination in the LPL gene of patients with primary LPL deficiency responsible for low LPL activity in PHP (26), or both low LPL activity and mass in PHP (27): one is derived by C to T transition at base 571 in exon 3 (26), and the other is from an addition of 5 bp in exon 3 (27). In both cases, however, there were no experimental observations about the identification of newly synthesized LPL protein or LPL mRNA from the cells producing LPL as reported in detail in this study. Thus, it is unclear whether their mutations result in no LPL protein synthesis or the production of truncated LPL protein. The relationship between premature termination and stability of mRNA has extensively studied on patients with β^0-thalassemia (42, 43). A similar finding to ours has been identified in the β-grobin gene of a patient with β^0-thalassemia: one base deletion of C was detected in exon 2 of β-grobin gene containing three exons, leading to a premature termination within exon 2 by a frameshift, and resulting in no detectable β-grobin mRNA (42, 43). It is interpreted that the total lack of β-globin mRNA resulted from the rapid degradation of the mature β-globin mRNA with a hypothesis that the portion of mRNA distal to the nonsense mutation is hypersusceptible to degradation (43).

In the N-family study, through the digestion test of LPL gene with AluI enzyme, the proband's brother SN was also confirmed to be homozygous for the LPL_{Arita} allele as expected from the observations that he exhibited neither LPL activity nor immunoreactive mass in PHP the same as the proband. In consideration of the reported consanguinity between their parents, these two LPL_{Arita} alleles are likely to be identical by descent. The proband's spouse was determined to be normal, because she was not a carrier of this type of LPL mutation, and her LPL activity and mass in PHP and newly synthesized LPL protein in macrophages were all within the normal range. As genetically expected, the proband's children were all heterozygotes, carrying one allele of LPL_{Arita}, which was in good agree-

ment with the data of approximately half the values of the control in the quantitations of LPL in PHP and LPL protein synthesized by macrophages. In the family of affected brother SN, his spouse was normal and their children were all heterozygous for LPL_{Arita} the same as in the proband's family. The N-family, which does not show a history of symptoms leading to secondary hyperlipoproteinemia, is an informative model to study the relationship between the heterozygosity for LPL deficiency and lipoprotein abnormalities. In fact, two of the tested five children exhibited type IV hyperlipidemia, suggesting that this type of hyperlipidemia is closely related to the heterozygous state for LPL deficiency.

In this paper we have shown an approach to identify molecular defects of LPL in the patients with primary LPL deficiency by systematically investigating the molecular basis of LPL. The combination of two procedures, such as the SIIA and sandwich EIA methods to quantitate LPL activity and mass in PHP, and immunochemical quantitations of LPL in the cultured macrophages, were powerful in estimating defects of the LPL molecule. These data together with LPL gene information (14, 15, 16, 40) led us to finally identify the LPL_{Arita} allele. The sequence of LPL_{Arita} provided by this study should be useful to screen hyperlipidemic patients for the presence of LPL_{Arita} allele based on the primary information of LPL defect obtained by both the SIIA and sandwich EIA methods.

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