Two novel point mutations in the lecithin:cholesterol acyltransferase (LCAT) gene resulting in LCAT deficiency: LCAT (G^{873} deletion) and LCAT ($Gly^{344} \rightarrow Ser$)

Kengo Moriyama,* Jun Sasaki,[1,]* Fumiko Arakawa,* Noboru Takami,* Eiichi Maeda,† Akira Matsunaga,* Yoichi Takada,* Keiichi Midorikawa,§ Tetsuro Yanase, Gen Yoshino,†† Santica M. Marcovina,§§ John J. Albers,§§ and Kikuo Arakawa***

Department of Internal Medicine and Biochemistry,* School of Medicine, Fukuoka University, 45-1, 7-chome Nanakuma, Jonan-ku, Fukuoka, 814-01, Japan; Department of Internal Medicine,† Kobe University, School of Medicine, 7-5, Kusunoki-cho, Chuo-ku, Kobe, 650, Japan; Midorikawa Clinic,§ 5-17, 5-chome Nakasu, Hakata-ku, Fukuoka, 810, Japan; Fukuoka Red Cross Hospital,** 1-1, 3-chome Okusu, Minami-ku, Fukuoka, 815, Japan; Department of Laboratory Medicine,†† Toho University, School of Medicine, 21-16, 5-chome Omorinishi, Ota-ku, Tokyo, 143, Japan; and Department of Medicine,§§ Northwest Lipid Research Laboratories, University of Washington School of Medicine, Seattle, WA 98103

Abstract We investigated the genetic defects in two patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency. Their clinical manifestations including corneal opacities, anemia, proteinuria, and hypoalphalipoproteinemia were identical for familial LCAT deficiency. Their LCAT activities and the cholesterol esterification rate (CER) were nearly zero, and their LCAT masses were below 10% of normal control values. Sequence analysis of the amplified DNA of case 1 revealed one base deletion of G at base 873 (first position of Val^{264}) in exon 6, leading to a premature termination by frameshift. Sequence analysis of amplified DNA of case 2 revealed a single G to A converting Gly (GGT) to Ser (AGT) substitution at residue 344. When COS-1 cells were transfected with these mutants, LCAT activity in the medium was nearly zero, and the LCAT mass was undetectable (< 0.01 μg/ml). In contrast, LCAT activity in the medium of COS-1 cells, transfected with wild-type LCAT, was 1.7 nmol/h per ml and the LCAT mass was 0.09 μg/ml. The LCAT mass in the cell lysates of the mutants was less than 12% of control for case 1 and 18% of control for case 2. Northern blot analysis of the mRNA of COS-1 cells transfected with the mutants showed the same amounts of LCAT mRNA as compared with wild-type LCAT. Biosynthesis of mutant LCATs was analyzed by pulse-chase and immunocytochemistry in transfected baby hamster kidney cells. SDS-PAGE/fluorography demonstrated that wild-type LCAT was synthesized as a high-mannose type of 56 kDa, which was very slowly converted to a mature form of 67 kDa and was secreted into the media. In contrast to the wild-type LCAT, the mutant precursors were not processed into the mature form but slowly degraded along with chase times. On steady and continuous labeling in the case of wild-type LCAT, the mature 67 kDa form was observed in both the cell lysate and media, whereas no mature form was detected in the cell lysates and media which were transfected mutant LCATs. These data suggest that the mutant LCATs are actually synthesized in an amount comparable to that of wild-type, but they are slowly degraded without being processed into the mature form. The immunocytochemistry revealed that mutant LCATs were mainly retained in the endoplasmic reticulum. These data suggest that these two mutations may disrupt the mutant LCATs' transport from the endoplasmic reticulum into Golgi apparatus, resulting in LCAT deficiency.—**Moriyama, K., J. Sasaki, F. Arakawa, N. Takami, E. Maeda, A. Matsunaga, Y. Takada, K. Midorikawa, T. Yanase, G. Yoshino, S. M. Marcovina, J. J. Albers, and K. Arakawa.** Two novel point mutations in the lecithin:cholesterol acyltransferase (LCAT) gene resulting in LCAT deficiency: LCAT (G^{873} deletion) and LCAT ($Gly^{344} \rightarrow Ser$). *J. Lipid Res.* 1995. **36:** 2329–2343.

Supplementary key words hypoalphalipoproteinemia • gene analysis • restriction fragment length polymorphism • intracellular processing

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; CER, cholesterol esterification rate; HDL, high density lipoprotein; ER, endoplasmic reticulum; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; BHK, baby hamster kidney; Endo H, endo-β-N-acetylglucosaminidase H; WGA, wheat germ agglutinin.

[1]To whom correspondence should be addressed.

Lecithin:cholesterol acyltransferase (LCAT E.C.2.3.1.43) catalyzes the transfer of fatty acid from the C-2 position of lecithin to the 3-hydroxy group of cholesterol, promoting its esterification (1). This enzyme is synthesized in the liver and is secreted in the plasma where it is present in association with high density lipoproteins (HDL) (2). Cholesteryl esters formed by LCAT are incorporated primarily into HDL particles and are transported in plasma in association with HDL or transferred to VLDL or LDL by the cholesteryl ester transfer protein (3).

The first case of familial LCAT deficiency was described by Norum and Gjone in 1967 (4). Additional patients have since been identified (5–12). Familial LCAT deficiency is associated with one or more of the following abnormalities: corneal opacities, mild hemolytic anemia, renal involvement including proteinuria and uremia, and premature atherosclerosis (13, 14). This deficiency is characterized by a decrease in the plasma level of esterified cholesterol and an increase in the plasma level of unesterified cholesterol, which is caused by the low or absent LCAT activity in circulating plasma (4).

The human LCAT gene is divided into six exons. It codes 416 amino acid residues, with a hydrophobic leader sequence of 24 amino acids (15, 16). The LCAT sequence includes four potential N-glycosylation sites (Asn-X-Ser/Thr) at positions 20, 84, 272, and 384 of the 416-residue protein moiety. The mature LCAT protein is a single glycoprotein with a relative molecular mass of 63000–69000 (17) containing 24% carbohydrate of which up to 7% is sialic acid (18). The initial transfer of dolichol-linked, glucose-capped high-mannose assemblies to polypeptide asparagine residues, and finally the modification of these units into the oligosaccharide sequences of mature protein, occurs first in the endoplasmic reticulum (ER) and subsequently within the Golgi apparatus (19, 20). Within the ER, sequential glucosidase activities generate uncapped high-mannose chains. Subsequent metabolic steps involve mannose trimming by specific glycosidases in the ER and Golgi, followed by the addition of hexose, hexosamine, and sialic acid residues in the Golgi compartment. The structure and function of the carbohydrate moiety of LCAT were determined by using several glycosidases in reaction with the isolated plasma protein or by using specific inhibitors of glycoprotein assembly with cultured cells secreting LCAT activity (21). However, little is known about the biosynthesis of LCAT. A number of molecular defects associated with LCAT deficiency have recently been identified (22–27). These include missense mutations (22–25), a single nucleotide insertion (27), an in-frame GGC insertion (23), nonsense mutation in codon 83 (26), and two compound heterozygotes (25, 26), all found in patients with familial LCAT deficiency. Taramelli et al. (22) reported molecular analysis of $Arg^{147} \rightarrow Trp$ mutant involving expression study and Northern blot analysis. Klein et al. (25) reported the in vitro expression of $Try^{147} \rightarrow Asn$ mutant. However, the mechanism of how these mutations may influence their LCAT masses and activities by expression study has not been investigated.

In this report, we investigated the structure, function, and biosynthesis of LCAT in two Japanese patients with novel familial LCAT deficiency.

METHODS

Informed consent for blood sample analysis was obtained from the patients.

Case 1

Case 1 was a 33-year-old Japanese man with a low level of HDL cholesterol, corneal opacities, proteinuria, and anemia. He had corneal opacities since childhood.

In 1989, at the age of 28, he was hospitalized for treatment of proteinuria (2–2.5 g/day) and hematuria. Hypoalphalipoproteinemia was detected at that time. He showed no signs of cardiac failure or coronary heart disease. Although the plasma level of creatinine and creatinine clearance were normal at the time of that admission, a renal biopsy showed moderate mesangial proliferative glomerulonephritis with segmental sclerosis. He was treated with dipyridamole for the next 5 years. His level of plasma total protein decreased from 7.3 g/dl to 4.6 g/dl over that period. At the time of the second admission, 5 years after the first, the patient was found to be anemic and nephrotic syndrome was diagnosed (total protein: 4.4–4.8 g/dl, proteinuria: 4–5 g/day). His plasma creatinine level was 1.1 mg/dl. A renal biopsy showed advanced membranous glomerulonephritis with extensive foam cell accumulation in glomeruli. A blood smear showed target cells. The pedigree of case 1's family appears in **Fig. 1(A)**. His sister (II-4) also had corneal opacities.

Case 2

Case 2 was a 45-year-old Japanese man with low level of HDL and total cholesterol, corneal opacities, chronic renal failure, and anemia. He had corneal opacities since childhood. Some of his family members also had corneal opacities (II-6, II-7) (Fig. 1(B)). He had no symptoms until the age of 34 years when he experienced fatigability. He was admitted to the hospital because of proteinuria and leg edema at age of 35 years. On admission, the patient was anemic and proteinuria and hematuria were detected. His renal function was slightly decreased

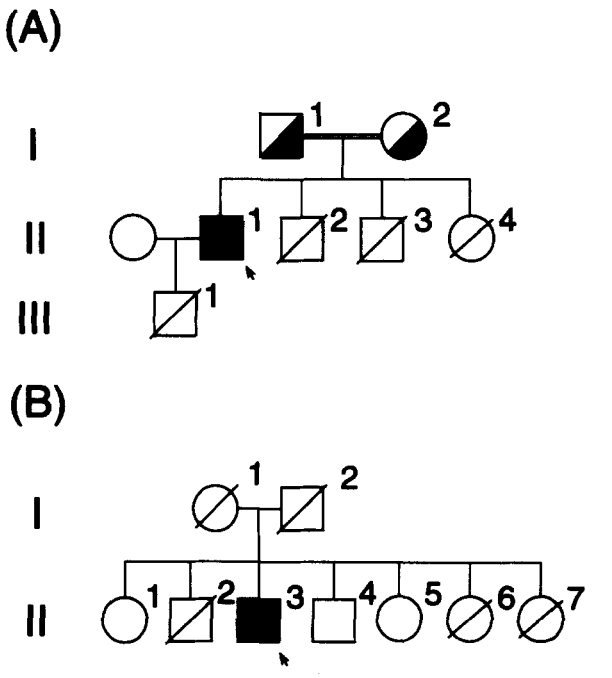

Fig. 1. (A) Pedigree of case 1 and his family: ■, LCAT deficiency homozygote; ◪ ◒, LCAT deficiency heterozygote; ◪ ∅, not studied. Arrow indicates the proband. (B) Pedigree of case 2 and his family. The open squares indicates a normal man; the open circle indicates a normal woman; ■, LCAT deficiency homozygote; ◪ ∅, not studied. Arrow indicates the proband. These data are taken from reference 28 with some modifications.

at this time (creatinine: 1.2 mg/dl, creatinine clearance: 63 ml/min). A blood smear showed target cells. His clinical features were described by Midorikawa et al. (28).

Plasma lipoprotein analysis

Blood samples were collected in tubes containing Na_2 EDTA after a 12-h fast. Lipoproteins were isolated by sequential ultracentrifugation. The following density fractions were obtained: VLDL (d < 1.006 g/ml), IDL (1.006–1.019 g/ml), LDL (1.019–1.063 g/ml), HDL_2 (1.063–1.125 g/ml), and HDL_3 (1.125–1.21 g/ml). Plasma cholesterol and triglyceride values were determined by enzymatic methods (29). HDL cholesterol was quantified by the heparin-manganese precipitation method (29). Apolipoproteins were measured by the single radial immunodiffusion method (30).

Determination of LCAT activity and cholesterol esterification rate (CER)

The enzyme activities of serum and wild-type and mutant LCAT gene products were determined by using a proteoliposome as a substrate. Egg yolk phosphatidylcholine–cholesterol liposome was prepared by ethanol injection. Each assay contained 2.25 nmol 7α-[^{3}H]cholesterol (22 Ci/mmol) and 4.5 μg apoA-I. The molar ratio of cholesterol to phosphatidylcholine was 1:6. Esterification rates were measured over a 4-h period at 37°C using 0.039 ml plasma or culture medium containing recombinant LCAT added to a mixture 0.7 mM EDTA, 4 mM β-mercaptoethanol, and 1% bovine serum albumin (essentially fatty acid-free) to a final volume of 0.0625 ml. The reaction was stopped by adding 0.625 ml chloroform–methanol 2:1 to the assay, and incubating the preparation for 2 h at room temperature to extract lipids. Labeled cholesteryl esters were separated by thin-layer chromatography on silica gel layers (E. Merck, Germany). The silica gel was extracted with petroleum ether–diethyl ether–acetic acid 20:0.6:0.2 and the amount of radioactivity was determined by liquid scintillation spectrometry.

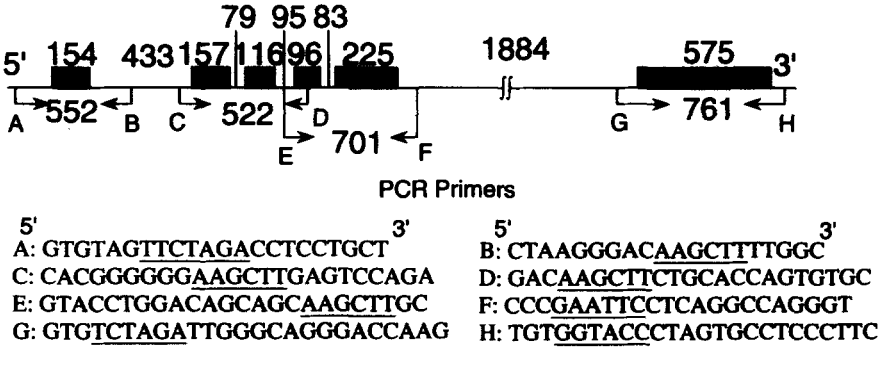

Fig. 2. Location and sequence of the oligonucleotide primers used for PCR. Upper panel: genomic structure of the human LCAT gene. Six exons represented by black boxes are interrupted by five introns, designated by lines. The arrows indicate the location of the primers and the direction of the PCR. The numbers indicate the length of the PCR-generated products in base pairs and the length of introns and exons of the LCAT gene. Lower panel: primer sequences. Incorporated restriction sites for the restriction enzymes are underlined.

TABLE 1. Biochemical characteristics of the homozygous and heterozygous individuals from families with LCAT deficiency

Family	Total Cholesterol	Free Cholesterol: Total Cholesterol	Triglyceride	HDL Cholesterol	ApoA-1	Cholesterol Esterification Rate	LCAT Activity	LCAT Mass
	mg/dl		mg/dl	*mg/dl*	*mg/dl*	*nmol/hr/ml*	*nmol/hr/ml*	*μg/ml*
Case 1	244	0.95	833	17.0	29.7	2.27	0	0.43
Father	173	0.35	77	31.0	120.0	ND	367.6	2.55
Mother	224	0.33	139	40.2	151.8	ND	420.1	3.30
Case 2	177	0.94	178	6.9	35.2	0.59	0	0.51
Control	187 ± 30 (14)	0.27 ± 0.01 (14)	127 ± 71 (14)	49 ± 13 (14)	149 ± 22 (14)	51.2 ± 5.3 (3)	722.8 ± 25.8 (9)	5.7 ± 1.0 (19)[a]

Control values represent mean ± SD (number of subjects); ND, not determined.
[a]Data from reference 23.

Radiolabeled plasma was equilibrated with 7α-[^{3}H]cholesterol at 4°C and then added to a pre-cooled 24-well plate containing 7α-[^{3}H]cholesterol. The mixture was incubated at 4°C for 18 h. Radiolabeled plasma was then incubated at 37°C for 6 h and the incubation was terminated by adding 0.45 ml chloroform–methanol 2:1 (v/v). Labeled cholesterol and cholesteryl ester were separated by thin-layer chromatography as described above. The radioactivity associated with cholesterol and cholesteryl ester was determined by liquid scintillation counting.

LCAT mass

The LCAT mass was measured by a radioimmunoassay using a polyclonal antibody and ^{125}I-labeled LCAT as previously described (31).

DNA preparation

Genomic DNA was isolated from 100 μl of peripheral blood as described previously (32). Peripheral blood (100 μl) was incubated at 60°C with 400 μl of a cell lysis buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100), 5 μl of proteinase K (10 mg/ml), and 25 μl of 10% SDS. The supernatant was extracted with phenol–chloroform 1:1 saturated with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and chloroform. The supernatant (20 μl) was used as the polymerase chain reaction (PCR) template.

Oligonucleotides

Synthetic oligonucleotide primers based on the published manual were made on a DNA synthesizer (Model 380B, Applied Biosystems, Inc., Foster City, CA) using the phosphoramide method.

DNA amplification by the PCR

PCR was performed using a modified protocol obtained from Perkin-Elmer Cetus Instruments (Norwalk, CT). Twenty pmol of each primer was mixed in 100 μl of a reaction mixture consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (W/V) gelatin, and 20 mM each of dATP, dCTP, dGTP, and dTTP. The mixture was then incubated at 96°C for 10 min and

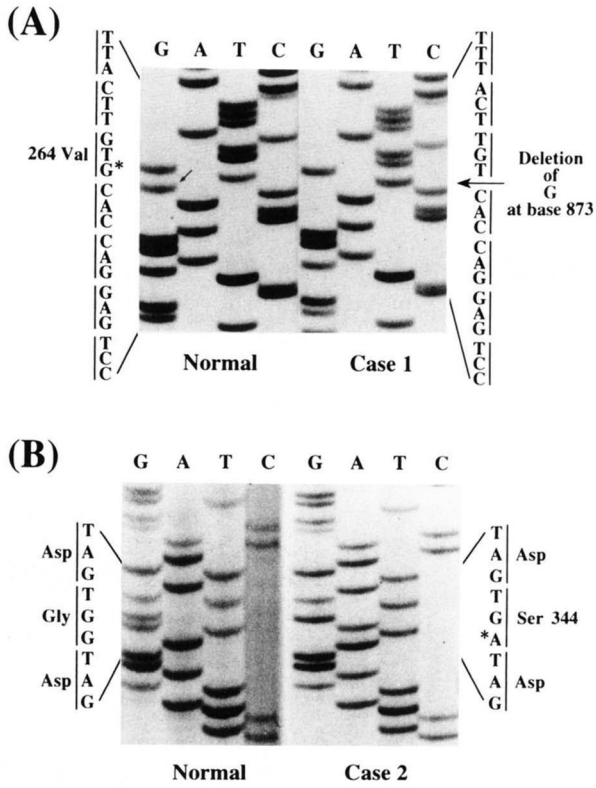

Fig. 3. Nucleotide sequences of a part of a sense strand from exon 6 from a normal subject and case 1 (A) and 2 (B). In A, the deletion of a G at base 873 is indicated by the arrow (right lanes). The position of the base G in the normal sequence is indicated by the arrow (left lanes). In B, the normal LCAT allele (left lanes) contains the sequence GGT coding for amino acid 344, glycine, of the mature protein. In the LCAT deficiency allele (right lanes) the sequence AGT is indicated by the asterisk. This codon represents serine.

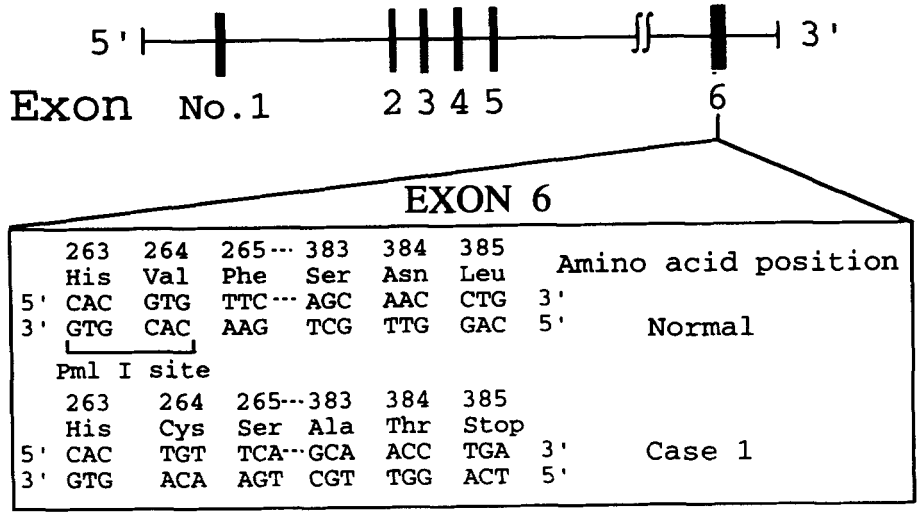

Fig. 4. Nucleotide and amino acid sequences of a section of exon 6 from a normal subject and case 1. Amplified DNA with one base deletion, which led to a premature termination in exon 6 by frameshift. Nucleotide sequences and amino acid numbers are based on the human LCAT cDNA sequence (15).

centrifuged briefly. One unit of Ampli Taq DNA polymerase (Perkin-Elmer Cetus) was added to the mixture, which was then sealed in 100 μl of mineral oil. Primer pairs (**Fig. 2**) were subjected to 35 PCR cycles with a Model PJ 1000 Thermalcycler (Perkin-Elmer Cetus) as follows: denaturation at 96°C for 30 sec, annealing at 65°C for 30 sec, and polymerization at 72°C for 1 min.

DNA sequence analysis

PCR-amplified DNA was purified with Geneclean (Bio 101. La Jolla, CA). A 1-μl sample of the first reaction mixture was re-amplified using an unequal ratio (100/1) of the same primers (33). Single-stranded DNA from the second PCR was sequenced by the dideoxynucleotide chain termination method (34) using Sequenase (United States Biochemical Co., Cleveland, OH). Regions with strong secondary structures were sequenced after being subcloned into the vectors pUC118 and pUC119. All sequences were determined on both strands, and the region across a mutation was sequenced on separately amplified DNAs in duplicate.

PmlI and HphI digestion study

Because both mutations of case 1 and case 2 lost restriction sites for PmlI and HphI, respectively, we performed restriction fragment length polymorphism. To identify the one base deletion in the LCAT gene in case 1, we amplified the 271 bp segment of the LCAT gene comprising the deletion at base 873 in exon 6. The upstream primer was 5′ TGGCGTGGCCTGAGGACCAC 3′, which was matched to bases 1109 to 1128 in exon 6. The downstream primer was 5′ GTGTCATCACCATCCTCATA 3′, which is complementary to bases 1360 to 1379 in exon 6. Nucleotide numbering is based on the published sequence for LCAT cDNA (15). PCR products were purified by Geneclean (Bio 101, La Jolla, CA) and digested with the restriction enzyme PmlI according to the manufacturer's instructions (Takara Shuzo, Kyoto, Japan) and then electrophoresed on an 8% polyacrylamide gel. To confirm the point mutation, DNA from case 2 was amplified by PCR using a primer pair consisting of 5′ GACCTGCACTTTGAGGAAGGCT 3′ [bases 1189–1210] and 5′ CTGAAACATAGCCATCAGGGC 3′ (complementary to bases 1613–1633). PCR products (445 bp) were purified by Geneclean and digested with restriction enzyme HphI (New England Biolabs, Inc., Beverly, MA) according to the manufacturer's instructions and then electrophoresed on an 8% polyacrylamide gel.

Cloning of LCAT cDNA

Poly(A)$^+$ RNA fractions were prepared from healthy human liver and used for construction of a human liver cDNA library according to a previously described method (35). The resulting cDNA library was screened by the hybridization method (36). Poly(A)$^+$ RNA was reverse transcribed with oligo (dT) primers using a first-strand cDNA synthesis kit (Pharmacia LKB, Uppsala, Sweden) and then amplified by PCR using the primers 5′ GCTGGAATGGGGCCGCCCGGC 3′ [bases 262–282] and 5′ CCTCCACCAGCCTGCGAGCTT 3′ (complementary to bases 814–835) as hybridization probe. Nucleotide numbering is based on the published sequence of LCAT cDNA (15). We screened 3×10^5 clones; 12 clones were subjected to a second screening. The two cDNA clones with the longest insert (1.5 kb)

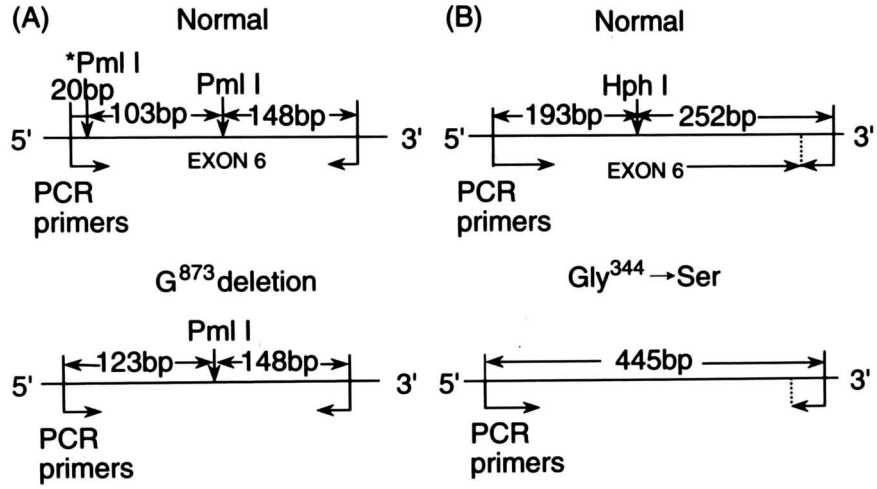

Fig. 5. (A): PCR amplified DNA fragments digested with PmlI. Upper panel: normal control; lower panel: case 1. PCR product of case 1 is missing a PmlI site (indicated by the asterisk) in exon 6 at base 873. (B): PCR amplified DNA fragments digested with HphI. Upper panel: normal control; lower panel: case 2. PCR product of case 2 is missing a HphI site at base 344.

obtained from the library were sequenced by the dideoxynucleotide chain termination method using Sequenase (United States Biochemical Co.).

Construction of expression plasmid and site-directed mutagenesis

cDNA inserts of wild-type LCAT were prepared by digestion of plasmid (pBluescript SK-) with EcoRI, electrophoretic purification, and then inserted into the EcoRI site of the pSG5 expression vector (37). The insert orientation was confirmed by restriction endonuclease mapping. This plasmid was modified by site-directed mutagenesis to encode LCAT molecules by substituting one base deletion of G (first position of Val^{264}) at base 873 and Gly (wild) for Ser (mutant) at position 344 in exon 6. Antisense strand of the plasmid was used as a template for site-directed mutagenesis; 18-mer synthetic oligonucleotides, 5′-GACCACTGTTCATTTCCA-3′ and 5′-GAGGATAGTGATGACACG-3′, were used as a primer for in vitro synthesis of the second strand of the mutant plasmids.

Transfection

COS-1 cells (1×10^6) were seeded into a 100-mm tissue culture plate in Dulbecco's modified Eagle's medium (DMEM: Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) and were incubated at 37°C in 5% CO_2 incubator until the cells were 60% confluent. The following solutions were prepared in 12 × 17 mm polystyrene sterile tubes before transfection:

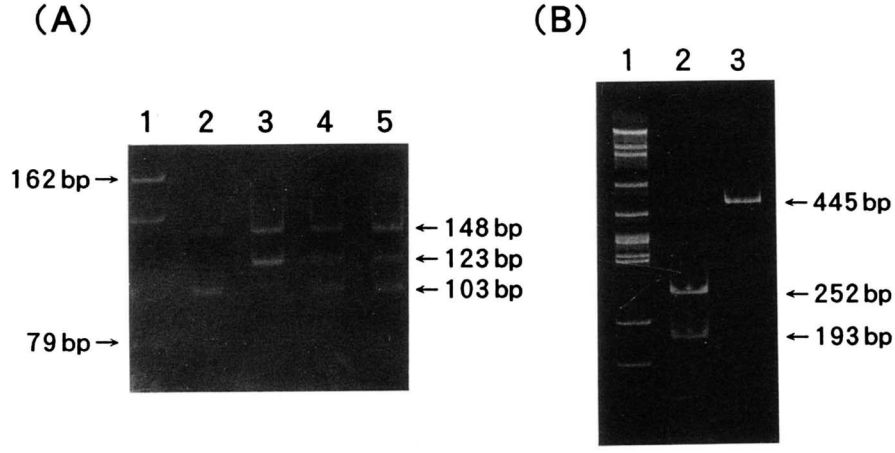

Fig. 6. Eight percent polyacrylamide gel electrophoresis of restriction fragments generated by PmlI (A) and HphI (B) digestion of PCR amplified DNA. DNA bands were visualized by staining with ethidium bromide. (A): case 1. Lane 1, HincII-digested ϕX174; lane 2, normal control; lane 3, proband; lane 4, father; lane 5, mother. (B): case 2. Lane 1, HincII-digested ϕX174; lane 2, normal control; lane 3, proband.

solution A: 4 μg of plasmid DNA diluted with 400 μl of reduced serum-free medium (OPTI-MEM, Gibco-BRL) and solution B: 40 μl of Lipofectin reagent (Gibco-BRL) diluted with 400 μl of OPTI-MEM. Solutions A and B were mixed and incubated at room temperature for 15 min. The medium in the culture plate was replaced with fresh medium and the plasmid DNA-Lipofectin reagent complex was layered over cells. After 5 h incubation at 37°C in an atmosphere of 5% CO_2, DNA-containing medium was replaced with DMEM/10% FCS for 24 h. The medium was then replaced with fresh serum-free medium (S-clone: Sanko-Junyaku Co. Tokyo, Japan) and the preparation was incubated for 72 h. To obtain intracellular extracts, cells were collected by centrifugation, and cell pellets were suspended in 0.22 M Tris-HCL (pH 8.5) containing 0.2% sodium deoxycholate, 0.008% Nonidet P-40, 0.005% heparin, 1% BSA, and 0.25 M sucrose and sonicated. Aliquots of the medium and the intracellular extracts were maintained at -20°C until used in LCAT assays.

Northern blot analysis

Total cellular RNA was extracted using ISOGEN (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions, with some modifications. Briefly, 1 × 10^6 transfected COS-1 cells were homogenized in 1 ml of a detergent solution (ISOGEN). Homogenates were transferred to test tubes containing 200 μl chloroform. The aqueous phase was removed to another tube containing 500 μl n-isopropanol and stored at -20°C for 2–3 h. The RNA pellet was rinsed with 75% ethanol, dried, and then dissolved in a denaturing buffer containing 6% formaldehyde and 50% formamide. For Northern blot analysis, total RNA was denatured with 50% formamide and 6% formaldehyde. The samples were fractionated on a 1.5% agarose gel. RNA was transferred to a nylon membrane (Hybond-N, Amersham-Japan, Tokyo, Japan). The blots were washed briefly in 2 × SSC, dried, and baked at 80°C for 2 h.

Wild-type LCAT cDNA was labeled by random primer labeling and used as a hybridization probe. After prehybridization at 65°C for 1 h with a hybridization buffer (1 mM EDTA, 0.5 M $NaHPO_4$, 7% SDS), the membrane was hybridized at 65°C for 16–18 h with a hybridization buffer containing the ^{32}P-labeled probe. Radiolabeling of cDNA probe with [^{32}P]dCTP (3000 Ci/mmol, Amersham-Japan) was carried out using a random primer DNA labeling kit (Amersham-Japan). The blots were exposed to X-ray film (Kodak, XAR-5) with an intensifying screen at -80°C. The LCAT mRNA signal was then stripped by washing the filters in boiling water containing 0.1% SDS. The filters were then rehybridized with a radiolabeled β-actin probe.

Cell labeling and immunoprecipitation

Each plasmid was transfected into 1 × 10^6 baby hamster kidney (BHK) cells using Lipofectin as described above. After a 48-h culture, the cells were pulse-labeled with [^{35}S]-methionine (100 μCi/dish) for 30 min in Eagle's minimum essential medium lacking unlabeled methionine and then chased in the methionine-containing medium. At the indicated times of chase, the cells were separated from the medium, washed, and lysed in phosphate-buffered saline containing 1% Triton X-100, 0.5% DOC. The cell lysates and medium were subjected to immunoprecipitation of LCAT. When indicated, tunicamycin (5 μg/ml) (Sigma, St. Louis, MO) was added 30 min before the start of labeling and was included throughout the experiment. The immunoprecipitates were subjected to SDS-PAGE (9% gels), followed by fluorography. Half of the immunoprecipitates prepared from ^{35}S-labeled cells were treated with endo-β-N-acetylglucosaminidase H (Endo H: Seikagaku kogyo, Tokyo). The other half was incubated without enzyme, as a control for proteolysis. Digestions were performed 16 h at 37°C, and the samples were analyzed by SDS-PAGE (9% gels), followed by fluorography. For steady label, transfected BHK cells were steady-labeled for 8 h with [^{35}S]methionine (300 μCi/dish). After 8 h label, cell lysates and media were prepared and subjected to immunoprecipitation of LCAT. Samples were analyzed by SDS-PAGE (9% gel), followed by fluorography.

Immunocytochemistry

Cells grown on coverslips were rinsed in phosphate-buffered saline and fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. After fixation, cells were permeabilized by

TABLE 2. α-LCAT activity and LCAT mass of in vitro expression of wild-type and mutant LCATs

	Cell Culture Medium			Intracellular Extracts		
	Wild-type	G^{873} Deletion	$Gly^{344} \rightarrow Ser$	Wild-type	G^{873} Deletion	$Gly^{344} \rightarrow Ser$
Total α-LCAT activity (nmol/h/ml)	1.66 ± 0.65 (6)	0 (6)	0 (5)			
Total LCAT mass (μg/ml)	0.09 ± 0.01 (6)	< 0.01 (6)	< 0.01 (5)	0.17 ± 0.02 (6)	< 0.02 (5)	0.03 ± 0.01 (6)
Specific α-LCAT activity (nmol/h/μg)	20.0 ± 1.5 (5)	0 (6)	0 (5)			

After transfected COS-1 cells were incubated for 72 h in serum-free medium, the culture medium and intracellular extract were analyzed for protein mass and activity of wild-type and mutant LCATs. Values represent the mean ± SD (number of experiments).

treatment with 0.05% saponin in phosphate-buffered saline for 15 min. The cells were incubated with rabbit anti-human LCAT serum in permeabilizing buffer for 1 h at room temperature. Coverslips with cells were briefly washed with the same solution, and incubated with rhodamine-labeled goat anti-rabbit IgG (10 μg/ml) for 1 h. After being washed with phosphate-buffered saline, the cells were incubated for 10 min at room temperature with a substrate medium containing diaminobenzidine (0.5 mg/ml) and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.0). In separate experiments, cells that had been fixed and permeabilized as above were also incubated with FITC-conjugated wheat germ agglutinin (WGA) (E-Y Laboratories Inc., San Mateo, CA) (50 μg/ml) in permeabilizing buffer for 1 h at room temperature. All coverslips with attached cells were then mounted on glass slides with glycerin. Specimens were examined with a Nikon light microscope.

RESULTS

Biochemical analyses

Plasma levels of total cholesterol and triglyceride were elevated in case 1, but were normal in case 2. The ratios of free cholesterol to total cholesterol were markedly elevated and HDL cholesterol levels were markedly reduced in both case 1 and case 2 (**Table 1**). Plasma apoA-I, A-II, B, C-II, C-III, and E levels of case 1 proband were 33.0, 8.8, 68.0, 5.1, 9.2, and 10.8 mg/dl, respectively (normal values were 137.2 ± 24.9, 31.8 ± 5.9, 79.1 ± 20.1, 3.4 ± 1.3, 7.5 ± 3.0, and 4.1 ± 1.2 mg/dl, respectively). Case 2 proband's apoA-I, A-II, B, C-II, C-III and E levels were 35.2, 5.0, 60.0, 3.5, 8.4, and 6.5 mg/dl, respectively. Lipid and lipoprotein levels were also elevated in case 1's parents. Both cases 1 and 2 exhibited markedly reduced CER, no LCAT activity, and an LCAT mass that was about one tenth of the mean mass in normal subjects, indicating primary LCAT deficiencies (Table 1). The LCAT activity and LCAT mass in case 1's parents were approximately 50% of the mean values in normal subjects.

DNA sequence analysis

In case 1, each single-stranded DNA from the second PCR was sequenced but clear nucleotide ladders across the mutation were not observed, probably due to the strong secondary structure. When amplified DNAs of exon 6 were cloned into vectors pUC118 and pUC119, 12 independent clones were isolated and sequenced. All 12 clones possessed one base deletion of G, which is the first position of GTG codon for valine at position 264 in the normal allele, at nucleotide position 873 in exon 6 (**Fig. 3A**). The one-base deletion generated a stop codon (TGA) at position 385 by frameshift and resulted in the loss of a PmlI restriction enzyme site present in the normal LCAT gene (**Fig. 4**). No other substitution was found. In case 2, each single-stranded DNA from the second PCR was sequenced; a substitution for G was observed converting Gly (GGT) to Ser (AGT) at position 344 (Fig. 3B). The amplified DNA of exon 6 was cloned into pUC118 and pUC119 and then sequenced to confirm the mutation. The base substitution lost an HphI restriction enzyme site present in the normal LCAT gene. No other substitution was found.

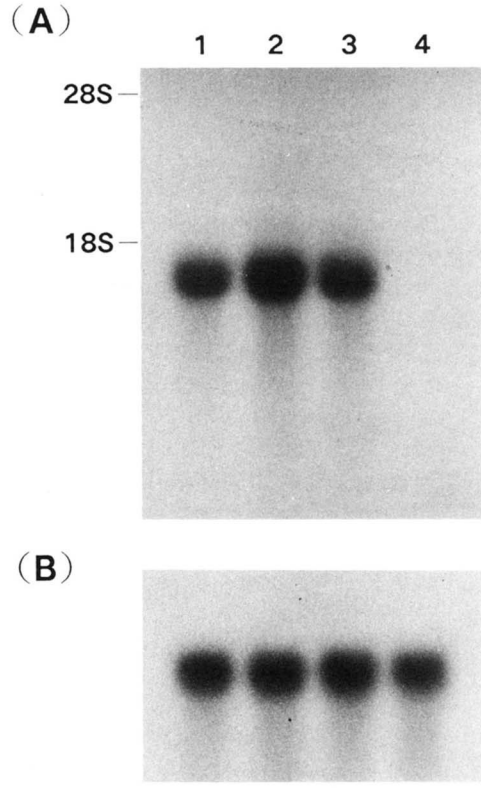

Fig. 7. Northern blot analysis of poly (A)$^+$ RNA from COS-1 cells transfected wild-type and two mutants LCAT cDNA. The blot was hybridized with ^{32}P-labeled LCAT cDNA (A), and after the removal of the LCAT cDNA probe, the same membrane was rehybridized with ^{32}P-labeled human β-actin (B). In A and B, all lanes contain poly (A)$^+$ RNA (5 μg) isolated from COS-1 cells. Lane 1, wild-type; lane 2, G^{873} deletion mutant; lane 3, $Gly^{344} \rightarrow$ Ser mutant; lane 4, expression vector that does not contain LCAT cDNA.

PmlI and HphI digestion studies

DNA from case 1's parents including proband was examined by PmlI digestion of the PCR-amplified DNA of the LCAT gene to confirm the carrier status of the G^{873} deletion allele which was characterized by the loss of a PmlI site in exon 6 (**Fig. 5A**). PCR-amplified DNA of the LCAT gene obtained from case 1 subjected to PmlI digestion showed 148 bp and 123 bp bands, indi-

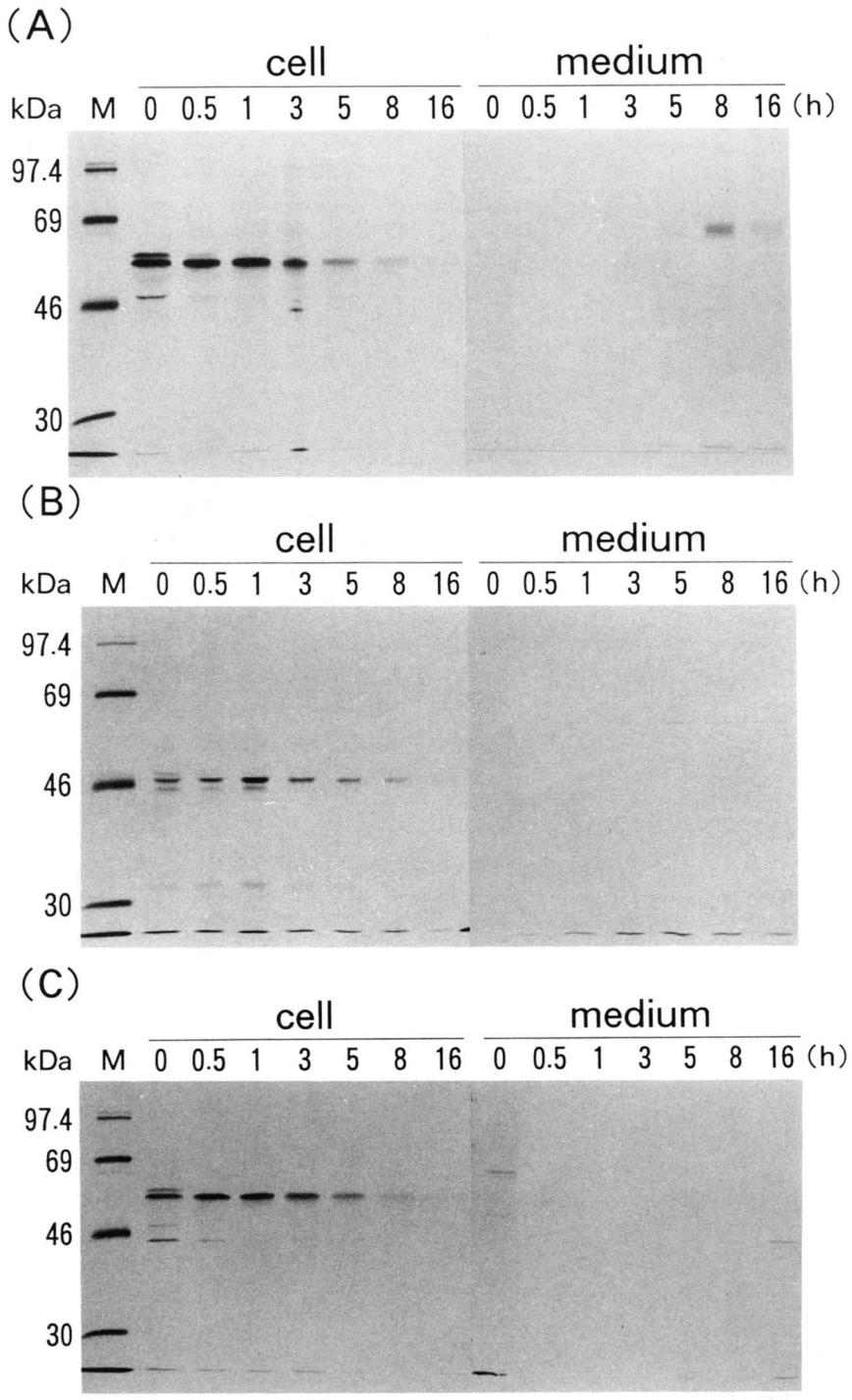

Fig. 8. Pulse-chase analysis of LCATs expressed in the transfected cells. BHK cells were transfected with the wild-type (A), G^{873} deletion mutant (B), and $Gly^{344} \rightarrow Ser$ mutant (C). The cells were labeled with [^{35}S]methionine for 30 min and then chased. At the indicated times of chase, cell lysates were prepared and subjected to immunoprecipitation with rabbit anti-human LCAT serum. The immunoprecipitates were analyzed by SDS-PAGE (9% gels)/fluorography. Lane M, molecular mass marker.

cating loss of the PmlI site (**Fig. 6A**). DNA from a normal control showed 148 bp and 103 bp bands resulting from digestion of PmlI. Case 1's parents exhibited 148 bp, 123 bp, and 103 bp bands. The 148 bp band was produced

by all samples. These data indicated that case 1 was homozygous and his parents were heterozygous for the defect.

PCR-amplified DNA of the LCAT gene obtained from case 2 was subjected to HphI digestion to confirm the missense mutation characterized by the loss of an HphI site in exon 6 (Fig. 5B). Case 2 exhibited a 445 bp band, resulting from the loss of 252 bp and 193 bp derived from digestion of HphI (Fig. 6B).

LCAT activity and LCAT mass of in vitro expressed LCAT

When COS-1 cells were transfected with these mutants and incubated for 72 h in serum-free medium, LCAT activity in the medium was nearly zero and the LCAT mass was undetectable (< 0.01 μg/ml) (**Table 2**). Mutant LCATs showed zero specific activity. In contrast, LCAT activity in the medium of COS-1 cells, transfected with wild-type LCAT, was 1.7 nmol/h per ml and the LCAT mass was 0.09 μg/ml. Specific activity of wild-type LCAT was 20.0 nmol/h per μg. The LCAT mass in the cell lysates of the mutants was less than 12% of control for case 1 and 18% of control for case 2. No detectable LCAT activity or mass was observed in the culture medium or intracellular extract from cells transfected with vector DNA alone.

Northern blot analysis

Northern blot analysis of poly $(A)^+$ RNA isolated from COS-1 cells transfected with mutant LCATs was carried out with ^{32}P-labeled LCAT cDNA. As shown in **Fig. 7A,** both mutants showed mRNA nearly equal to that of wild-type (lanes 1–3), while no detectable amount of LCAT mRNA was detected in the COS-1 cells transfected expression vector that did not contain LCAT cDNA (lane 4). As a control experiment, the same membrane was treated to remove the LCAT cDNA probe and was rehybridized with a ^{32}P-labeled human β-actin cDNA probe. The wild-type (lane 1), two mutants (lanes 2, 3) and expression vector that did not contain LCAT cDNA (lane 4) yielded almost equal amounts of β-actin mRNA (Fig. 7B).

Biosynthesis and localization of mutant LCATs

The plasmid pSG5, containing the normal and mutants cDNA insert, was transfected into BHK cells. First, we investigated the biosynthesis of wild-type LCAT by pulse-chase analysis in transfected COS-1 cell. No mature form of LCAT was observed in cell lysates and media after prolonged chase times (over 5 h) (data not shown). Because Hill et al. (38) have established a BHK cell line that constitutively expresses significant quantities of human recombinant LCAT, we chose BHK cells. After 2 days of culture, the cells were pulse-labeled with [^{35}S]methionine and then chased, followed by immunoprecipitation with rabbit anti-human LCAT serum. SDS-PAGE/fluorography demonstrated that under these conditions wild-type LCAT was synthesized as a high-mannose type of 56 kDa, which was very slowly converted to a mature form to 67 kDa and was secreted into media (**Fig. 8A**). The conversion of the 56 kDa form to the 67 kDa form is due to the processing of its N-linked oligosaccharides, from the high-mannose type to the complex type, as demonstrated by treatment with tunicamycin and Endo-H (**Fig. 9**). In fact, the entire sequence of LCAT includes four potential N-glycosylation sites (Asn-X-Ser/Thr) (39). When LCAT was treated with tunicamycin, molecular weight doubles with an apparent molecular weight of about 47 kDa and 49 kDa were observed. The former was comparable to the mo-

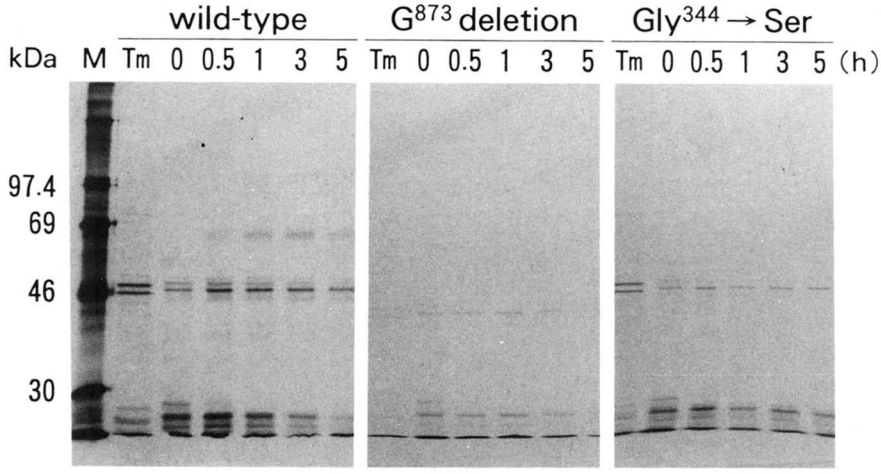

Fig. 9. Endo-H sensitivity of wild-type and mutant LCATs. The same samples shown in Fig. 8 were treated at 37°C with Endo-H (0.2 unit/ml) for 16 h and analyzed by SDS-PAGE (9% gels)/fluorography. Tm, samples obtained from tunicamycin-treated cells.

lecular weight (46917) of the mature protein moiety (21). The reason for the presence of a molecular weight doublet under treatment with tunicamycin remains to be determined. In transfected cells with two mutants, a newly synthesized protein was also immunoprecipitated with rabbit anti-human LCAT serum and was shown to have a different molecular mass (43 kDa and 46 kDa in the presence and absence of tunicamycin) for the G^{873} deletion mutant and the same molecular mass (47 kDa and 56 kDa) for the $Gly^{344} \rightarrow Ser$ mutant as the wild-type precursor (Fig. 8B, 8C). In contrast to the wild-type LCAT, however, the mutant precursors were not processed into the mature form but slowly disappeared along with chase times. The finding that no radioactive form was detectable in the medium indicated that no mutant LCATs would be secreted into the medium. Treatment with Endo-H confirmed that the mutant molecules contain only the high-mannose-type oligosaccharides (Fig. 9). We also performed the steady labeling experiments ([^{35}S]methionine; 300 μCi/dish, labeled for 8 h). As shown in **Fig. 10,** in the case of wild-type LCAT, the mature 67 kDa form was observed in both the cell lysate and media, whereas no mature form was detected in the cell lysate and media which were transfected mutant LCATs. These results suggest that the mutant LCATs are actually synthesized in an amount comparable to that of the wild-type, but they are slowly degraded without being processed into the mature form. Moreover, in contrast to the wild-type LCAT, no maturation of their oligosaccharides into the complex type occurs, suggesting that the mutant LCATs are not able to be transported to the Golgi apparatus and retained in the endoplasmic reticulum (ER).

We then examined the intracellular localization of mutant LCATs expressed in the transfected cells. In control BHK cells without transfection, no significant reaction with rhodamine-labeled goat anti-rabbit IgG was observed (**Fig. 11A**). In contrast, cells transfected with the wild-type and mutant LCAT cDNAs yield a reticular pattern of immunofluorescence throughout the cytoplasm (Fig. 11C, D, E). The staining profiles of these cells are different from those stained for WGA binding (Fig. 11B). The lectin WGA specifically binds to oligosaccharides of proteins concentrated in the Golgi complex (40, 41). Thus, the immunocytological observations suggest that expressed wild-type and mutant LCATs are localized mainly in ER rather than the Golgi complex. These observations support the biochemical data obtained in this study.

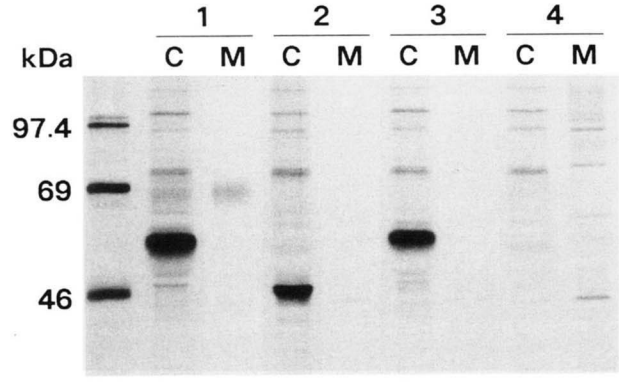

Fig. 10. Immunoprecipitation of wild-type and mutant LCATs on steady label experiments. Transfected BHK cells were labeled for 8 h with [^{35}S]methionine (300 μCi/dish). After 8 h label, cell lysates and media were prepared and subjected to immunoprecipitation of LCAT and analyzed by SDS-PAGE (9% gels)/fluorography. 1, wild-type; 2, G^{873} deletion mutant; 3, $Gly^{344} \rightarrow Ser$ mutant; lane 4, mock. C and M represent the cell lysates and medium, respectively.

DISCUSSION

We identified two different mutations in the LCAT gene in patients with familial LCAT deficiency. Both cases had low levels of HDL cholesterol and apoA-I and high ratios of free cholesterol to total cholesterol. Clinical findings included corneal opacities, anemia, and glomerulosclerosis, which are characteristic of familial LCAT deficiency. Patients with familial LCAT deficiency show heterogeneity in their degree of renal involvement. Case 1 was diagnosed as early onset nephrotic syndrome, and a renal biopsy showed progressive glomerulonephritis. Initially, his renal dysfunction was slightly decreased but proteinuria rapidly progressed. In case 2, the renal function was markedly reduced (creatinine clearance: 6 ml/min) and more impaired than case 1. Allelic mutations differed in our patients. The clinical phenotype appears to be determined by other factors in addition to the genetic defect of the LCAT gene, such as environmental factors and/or other genetic defects. The level of triglycerides was higher in case 1 than in case 2. Thus, the progression of renal involvement might be related to the plasma triglyceride level.

In both cases, a small amount of LCAT protein was detected in their plasma. The gene mutations described here offer a possible explanation of the biochemical profiles of the two patients. The one base deletion of G in the LCAT gene of case 1 resulted in the loss of N-glycosylation sites Asn^{272} and Asn^{384} and the creation of N-glycosylation site Asn^{279}. The one base deletion also resulted in the loss of one disulfide bridge between Cys^{313} and Cys^{356} (39). Loss of an N-glycosylation site reduces its enzyme activity (21). Loss of one disulfide bridge creates structural flexibility that may be disadvantageous in the maintenance of higher protein structures. Thus, the deletion of G may cause a significant alteration in the enzyme conformation that could destabilize the LCAT molecule. The glycine to serine change in case 2 is

a substitution between neutral amino acids. This mutation occurs in a possibly important amino acid that is conserved among species (15, 42–44).

Previously identified defects in the LCAT gene in patients with classic LCAT deficiency include missense mutations in codons 93, 147, 158, 209, 228, 293, and 321 (22, 23, 24, 26), a single nucleotide insertion in exon 1 (27) and a frame GGC (Gly) insertion between codons 140 and 141 (23), nonsense mutation in codon 83 (26), and two compound heterozygotes (25, 26). The mutations in codons 147, 228, and 293 are associated with very little or no LCAT activity and 40–60% of normal mass.

In order to establish the molecular basis of these substitutions, the two mutant LCATs were expressed in the mammalian expression system. Analysis of in vitro expressed LCATs (G^{873} deletion, $Gly^{344} \rightarrow Ser$) revealed that LCAT activity in the medium was nearly zero and the LCAT mass was undetectable (< 0.01 μg/ml). In contrast, LCAT activity in the medium of COS-1 cells transfected with wild-type LCAT was 1.7 nmol/h per ml and the LCAT mass was 0.09 μg/ml. After concentrating the culture media about 10 times, wild-type LCAT mass increased up to 1.4 μg/ml but mutant LCAT masses were undetectable (< 0.02 μg/ml). One of the explanations for this is their instability in the culture media. LCAT mass in the cell lysates of the mutants was not detectable for the G^{873} deletion mutant and 18% of control for the $Gly^{344} \rightarrow Ser$ mutant. These data suggested that these two mutations may influence the transcriptional, translational, and/or posttranslational level. Although it is important to know the physiological functions of these two mutants, no LCAT activities were detected in cell lysates for wild-type LCAT as well as the two mutants in our experiments.

Takagi et al. (45) reported that one base deletion (G^{916}) in exon 5 of lipoprotein lipase (LPL) gene caused no detectable LPL protein due to the absence of LPL mRNA transcript. Therefore it is necessary to investigate the LCAT mRNA transcript of these two mutants. Northern blot analysis of the mRNA of COS-1 cells transfected with the mutants showed no difference compared to wild-type mRNA.

The biosynthesis of wild-type and two mutant LCATs that were expressed in transfected BHK cells was then analyzed by pulse-chase experiments. Although the primary sequence of LCAT has characteristic features of a secretory protein (39), processing of the oligosaccharides is relatively inefficient (Fig. 8A). The same behavior of wild-type LCAT, which was expressed in transfected COS-1 cells, was observed (data not shown). Recently, it has been reported that immunoprecipitation of the cell lysate of transfected COS-1 cells reveals a specific protein band with a molecular mass lower than the mature LCAT (about 52 kDa). No mature form of

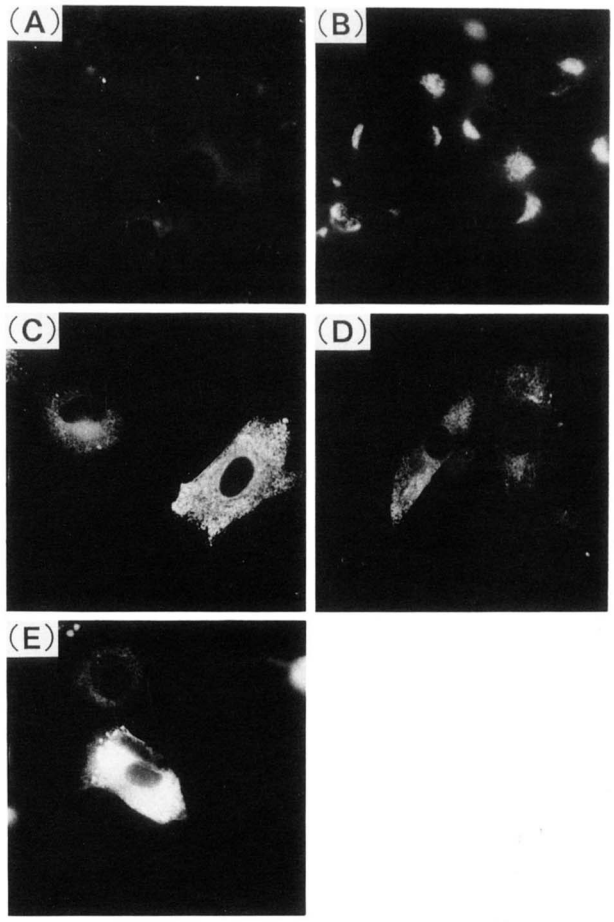

Fig. 11. Intracellular localization of LCATs expressed in BHK cells. BHK cells before (panels A and B) or after transfection with the wild-type LCAT cDNA (panel C) or G^{873} deletion mutant cDNA (panel D) or $Gly^{344} \rightarrow Ser$ mutant cDNA (panel E) were cultured for 2 days. The cells grown on coverslips were fixed in 2% paraformaldehyde, permeabilized, and subjected to immunochemical (rhodamine-coupled IgG) or cytochemical (FITC-WGA) reactions as described in Materials and Methods. Panels A, C, D, and E, rabbit anti-human LCAT serum; panel B, WGA.

LCAT was observed in cell lysate after a 4-h chase (46). These observations indicate that the inefficient processing of LCAT in BHK cells cannot be simply explained by differences in secretory pathways. However, a complex type of wild-type LCAT was observed in the media, after prolonged chase times (over 5 h) (Fig. 8A). Thus, these results indicate that LCAT transport out of the ER into the Golgi apparatus is very slow, but once LCAT reaches the Golgi apparatus, it is secreted into the media immediately. These observations were identical for the result of the immunocytochemistry which the wild-type LCAT expressed in BHK cells that are localized mainly in the ER. Inefficient secretion may be an intrinsic property of LCAT protein. Alternatively, overexpression might result in the formation of insoluble aggregates or cause

some of the protein to fold inappropriately through failure of correct glycosylation.

In contrast to the wild-type LCAT, the mutant precursors were not processed into the mature form but slowly disappeared during the chase experiments. Finally, no complex type of mutant LCATs were observed in the media after a 16-h chase (Fig. 8B, 8C). Treatment with Endo-H confirmed that the mutant molecules contained only the high-mannose-type oligosaccharides (Fig. 9). The intracellular amount of two mutants was considered to be comparable with that of wild-type LCAT on steady label experiments (Fig. 10). However, only immature forms of mutant LCATs were observed in cell lysate, whereas the mature form of wild-type LCAT was observed. In addition, no immunoprecipitable band was detected in the media for two mutants. These results suggest that the mutant LCATs are actually synthesized in an amount comparable to that of the wild-type, but they are gradually degraded without being processed into the mature form. No maturation of their oligosaccharides into the complex type also suggests that the degradation occurs within the ER and/or early Golgi compartment. The immunocytological observations are also favorable to our conclusion that the mutant LCATs are retained and degraded in the ER.

Similar findings have been reported for mutant α2-plasminogen inhibitor (α2-PI Okinawa) which causes α2-PI deficiency (47). Immunoprecipitation analysis and enzyme-linked immunosorbent assay revealed that the mutant α2-PI (deletion of Glu^{137}) synthesized is mostly retained within the cells as an endo H-sensitive form, and only a small portion of it is secreted into the medium as a neuraminidase-sensitive form. However, how the cell is able to distinguish between properly folded and assembled proteins and incomplete proteins is only partially understood. Proper folding and oligomerization of secretory and membrane proteins take place in the ER prior to their transport to appropriate cellular sites (48). Misfolded polypeptides or incompletely assembled oligomeric proteins are often retained or degraded in the ER (49, 50). It is thought that molecular chaperones, a class of functionally defined proteins, may facilitate folding and oligomerization of polypeptides that are transported across the ER membrane (51, 52). BiP/GRP78, the HSP70 analogue in the ER lumen, binds to hydrophobic sequences exposed on the surface of partially folded or unassembled proteins (53–56), and calnexin (a membrane-bound chaperone also called p88 and IP90) (57–59) binds to glycoproteins that have undergone partial trimming of glucose residues in their N-linked oligosaccharide side chains (60, 61). Both of these chaperones associate transiently with folding and assembly intermediates, and more permanently with misfolded proteins. Therefore, it is interesting to know the mechanism by which mutant LCATs are retained and degraded in the ER.

In this paper we report two novel molecular defects of LCAT. In conclusion, our data suggest that these two mutations may disrupt the mutant LCATs transport from the ER into Golgi apparatus, resulting in LCAT deficiency. The characterization of the molecular defect in these two patients will provide a better understanding of the structure–biosynthesis relationships of LCAT and the clinical course of a patient with familial LCAT deficiency.

This work was supported in part by grant HL30086 from the National Institutes of Health, USA to S.M.M. and J.J.A. We thank Dr. Y. Misumi (Fukuoka University) for providing human liver cDNA library and suggestions. We also thank Drs. T. Fujiwara and K. Shibata (Fukuoka University) for technical advice and discussions, K. Nishi for excellent technical assistance, and J. Urakawa and Y. Hashimoto for manuscript preparation.

Manuscript received 14 March 1995 and in revised form 17 August 1995.

REFERENCES

1. Glomset, J. A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* **9:** 155–167.
2. Doblasova, M. 1983. Lecithin:cholesterol acyltransferase and the regulation of endogenous cholesterol transport. *Adv. Lipid Res.* **20:** 107–194.
3. Norum, K. R., E. Gjone, and J. A. Glomset. 1989. Familial lecithin:cholesterol acyltransferase deficiency, including fish eye disease. *In* The Metabolic Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill Information Services, New York. 1181–1194.
4. Norum, K. R., and E. Gjone. 1967. Familial plasma lecithin:cholesterol acyltransferase deficiency. Biochemical study of a new inborn error of metabolism. *Scand. J. Clin. Lab. Invest.* **20:** 231–243.
5. Gjone, E., and K. R. Norum. 1968. Familial serum cholesterol ester deficiency. Clinical study of a patient with a new syndrome. *Acta Med. Scand.* **183:** 107–112.
6. Utermann, G., W. Schoenborn, K. H. Langer, and P. Dicker. 1972. Lipoproteins in LCAT deficiency. *Hum. Genet.* **16:** 295–306.
7. Bron, A. J., J. K. Lloyd, A. S. Fosbrooke, A. F. Winder, and R. C. Tripathi. 1975. Primary LCAT deficiency disease. *Lancet.* **1:** 928–929.
8. Chevet, D., M. P. Ramee, P. L. Pogamp, R. Thomas, M. Garre, and L. D. Alcindor. 1976. Hereditary lecithin:cholesterol acyl-transferase deficiency: report of a new family with two afflicted sisters. *Kidney Int.* **10:** 185–188.
9. Frohlich, J., W. J. Godolphin, C. E. Reeve, and K. A. Evelyn. 1978. Familial lecithin:cholesterol acyltransferase deficiency. Report of two patients from a Canadian family of Italian and Swedish descent. *Scand. J. Clin. Lab. Invest.* **Suppl. 150. 38:** 156–161.
10. Iwamoto, A., C. Naito, T. Teramoto, H. Kato, M. Kato, T. Kariya, T. Shimizu, H. Oka, and T. Oda. 1978. Familial lecithin:cholesterol acyltransferase deficiency compli-

cated with unconjugated hyperbilirubinemia and peripheral neuropathy. *Acta Med. Scand.* **204:** 219–227.
11. Hamnstrom, B., E. Gjone, and K. R. Norum. 1979. Familial lecithin:cholesterol acyltransferase deficiency. *Br. Med. J.* **2:** 283–286.
12. Vergani, C., A. L. Catapano, P. Roma, and G. Giudici. 1983. A new case of familial LCAT deficiency. *Acta Med. Scand.* **214:** 173–176.
13. Gjone, E., K. R. Norum, and J. A. Glomset. 1983. Familial lecithin:cholesterol acyltransferase deficiency. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. S. Goldstein, and M. S. Brown, editors. McGraw-Hill, Information Services, New York. 643–654.
14. Frohlich, J., and R. Mcleod. 1986. Lecithin:cholesterol acyltransferase deficiency syndromes. *In* Hypoalphalipoproteinemia Syndromes. P. Angel and J. Frohlich, editors. Plenum Press, New York. 181–184.
15. McLean, J., C. Fielding, and D. Drayna. 1986. Cloning and expression of human lecithin:cholesterol acyltransferase cDNA. *Proc. Natl. Acad. Sci. USA.* **83:** 2335–2339.
16. McLean, J., K. Wion, D. Drayna, C. Fielding, and R. Lawn. 1986. Human lecithin:cholesterol acyltransferase gene: complete gene sequence and sites of expression. *Nucleic Acids Res.* **14:** 9397–9406.
17. Albers, J. J., C. H. Chen, and A. G. Lacko. Isolation, characterization, and assay of lecithin:cholesterol acyltransferase. *Methods Enzymol.* **129:** 763–783.
18. Chung, J., D. A. Abano, G. M. Fless, and A. M. Scanu. 1979. Isolation, properties and mechanism of in vitro action of lecithin:cholesterol acyltransferase from human plasma. *J. Biol. Chem.* **254:** 7456–7464.
19. Sinder, M. D. 1984. Biosynthesis of glycoproteins: formation of N-linked oligosaccharides. *In* Biology of Carbohydrates. V. Ginsburg, and P. W. Robbins, editors. Vol. 2. Wiley-Interscience, New York. 163–198.
20. Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54:** 631–664.
21. Collet, X., and C. J. Fielding. 1991. Effects of inhibitors of N-linked oligosaccharide processing on the secretion, stability, and activity of lecithin:cholesterol acyltransferase. *Biochemistry.* **30:** 3228–3234.
22. Taramelli, R., M. Pontoglio, G. Candiani, S. Ottolenghi, H. Dieplinger, A. Catapano, J. Albers, C. Vergani, and J. McLean. 1990. Lecithin:cholesterol acyltransferase deficiency: molecular analysis of a mutated allele. *Hum. Genet.* **85:** 195–199.
23. Gotoda, T., N. Yamada, T. Murase, M. Sakuma, N. Murayama, H. Shimano, K. Kozaki, J. J. Albers, Y. Yazaki, and Y. Akanuma. 1991. Differential phenotypic expression by three mutant alleles in familial lecithin:cholesterol acyltransferase deficiency. *Lancet.* **338:** 778–781.
24. Maeda, E., Y. Naka, T. Matozaki, M. Sakuma, Y. Akanuma, G. Yoshino, and M. Kasuga. 1991. Lecithin:cholesterol acyltransferase (LCAT) deficiency with a missense mutation in exon 6 of the LCAT gene. *Biochem. Biophys. Res. Commun.* **178:** 460–466.
25. Klein, H. G., P. Lohse, N. Duverger, J. J. Albers, D. J. Rader, L. A. Zech, S. Santamarina-Fojo, and H. B. Brewer, Jr. 1993. Two different allelic mutations in the lecithin:cholesterol acyltransferase (LCAT) gene resulting in classic LCAT deficiency: LCAT ($tyr^{83} \rightarrow$ stop) and LCAT ($tyr^{156} \rightarrow$ asn). *J. Lipid Res.* **34:** 49–58.
26. Funke, H., A. von Eckardstein, P. H. Pritchard, A. E. Hornby, H. Wiebusch, C. Motti, M. R. Hayden, C. Dachet, B. Jacotot, U. Gerdes, O. Faergeman, J. J. Albers, N. Colleoni, A. Catapano, J. Frohlich, and G. Assmann. 1993. Genetic and phenotypic heterogeneity in familial lecithin:cholesterol acyltransferase (LCAT) deficiency. *J. Clin. Invest.* **91:** 677–683.
27. Bujo, H., J. Kusunoki, M. Ogasawara, T. Yamamoto, Y. Ohta, T. Shimada, Y. Saito, and S. Yoshida. 1991. Molecular defect in familial lecithin:cholesterol acyltransferase (LCAT) deficiency: a single nucleotide insertion in LCAT gene causes a complete deficient type of the disease. *Biochem. Biophys. Res. Commun.* **181:** 933–940.
28. Midorikawa, K., K. Satoh, K. Abe, E. Kinoshita, G. Nomura, H. Toshima, M. Morimatsu, and S. Nanbu. 1983. Familial LCAT deficiency. *J. Jpn. Atheroscler. Soc.* **11:** 1215–1221 (in Japanese)
29. Sasaki, J., and K. Arakawa. 1987. Effect of nifedipine on serum lipids, lipoproteins, and apolipoproteins in patients with essential hypertension. *Curr. Ther. Res.* **41:** 845–851.
30. Goto, Y., Y. Akanuma, and Y. Harano. 1986. Determination by the SRID method of normal values of serum apolipoproteins (A-I, A-II, B, C-II, C-III, and E) in normolipidemic healthy Japanese subjects. *J. Clin. Biochem. Nutr.* **1:** 73–88.
31. Albers, J. J., J. L. Adolphson, and C. H. Chen. 1981. Radioimmunoassay of human plasma lecithin:cholesterol acyltransferase. *J. Clin. Invest.* **67:** 141–148.
32. Takada, Y., J. Sasaki, M. Seki, S. Ogata, Y. Teranishi, and K. Arakawa. 1991. Characterization of a new human apolipoprotein A-I Yame by direct sequencing of polymerase chain reaction-amplified DNA. *J. Lipid Res.* **32:** 1275–1280.
33. Gyllensten, U. B., and H. A. Erlich. 1988. Generation of single-stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQA locus. *Proc. Natl. Acad. Sci. USA.* **85:** 7652–7656.
34. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463–5467.
35. Misumi, Y., Y. Hayashi, F. Arakawa, and Y. Ikehara. 1992. Molecular cloning and sequence analysis of human dipeptidyl peptidase IV, a serine proteinase on the cell surface. *Biochim. Biophys. Acta.* **1131:** 333–336.
36. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning; a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 368–369.
37. Green, S., I. Issemann, and E. Sheer. 1988. A versatile in vivo and in vitro eukaryotic expression vector for protein engineering. *Nucleic Acids Res.* **16:** 369.
38. Hill, J. S., K. O. X. Wang, S. Paranjape, D. Dimitrijevich, A. G. Lacko, and H. Pritchard. 1993. Expression and characterization of recombinant human lecithin:cholesterol acyltransferase. *J. Lipid Res.* **34:** 1245–1251.
39. Yang, C. Y., D. Manoogian, Q. Pao, F. S. Lee, R. D. Knapp, A. M. Gotto, Jr., and H. J. Pownall. 1987. Lecithin:cholesterol acyltransferase. Functional regions and a structural model of the enzyme. *J. Biol. Chem.* **262:** 3086–3091.
40. Tartakoff, A. M., and P. Vassalli. 1983. Lectin-binding sites as markers of Golgi subcompartments: proximal-to-distal maturation of oligosaccharides. *J. Cell Biol.* **97:** 1243–1248.
41. Virtanen, I., P. Ekblom, and P. Laurila. 1980. Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. *J. Cell Biol.* **85:** 429–434.
42. Warden, C. H., C. A. Langner, J. I. Gordon, B. A. Taylor, J. W. McLean, and A. J. Lusis. 1989. Tissue-specific expression, developmental regulation, and chromosomal mapping of the lecithin:cholesterol acyltransferase gene. Evi-

dence for expression in brain and testes as well as liver. *J. Biol. Chem.* **264:** 21573–21581.
43. Meroni, G., N. Malgaretti, P. Magnaghi, and R. Taramelli. 1990. Nucleotide sequence of the cDNA for lecithin:cholesterol acyltransferase (LCAT) from the rat. *Nucleic Acids Res.* **18:** 5308.
44. Hizson, J. E., D. M. Driscoll, S. Birnbaum, and M. L. Britten. 1993. Baboon lecithin:cholesterol acyltransferase (LCAT): cDNA sequences of two alleles, evolution, and gene expression. *Gene.* **128:** 295–299.
45. Takagi, A., Y. Ikeda, Z. Tsutsumi, T. Shoji, and A. Yamamoto. 1992. Molecular studies on primary lipoprotein lipase (LPL) deficiency. One base deletion (G916) in Exon 5 of LPL gene causes no detectable LPL protein due to the absence of LPL mRNA transcript. J. *Clin. Invest.* **89:** 581–591.
46. O, K., J. S. Hill, X. Wang, and P. H. Pritchard. 1993. Recombinant lecithin:cholesterol acyltransferase containing a $Thr_{123} \rightarrow Ile$ mutation esterifies cholesterol in low density lipoprotein but not in high density lipoprotein. *J. Lipid Res.* **34:** 81–88.
47. Miura, O., Y. Sugahara, and N. Aoki. 1989. Hereditary a2-plasmin inhibitor deficiency caused by a transport-deficient mutation (a2-PI-Okinawa). *J. Biol. Chem.* **264:** 18213–18219.
48. Hurtly, S. M., and A. Helenius. 1989. Protein oligomerization in the endoplasmic reticulum. *Annu. Rev. Cell Biol.* **5:** 277–307.
49. Chen, C., J. S. Bonifacino, L. C. Yuan, and R. D. Klausner. 1988. Selective degradation of T cell antigen receptor chains retained in a pre-Golgi compartment. *J. Cell Biol.* **107:** 2149–2161.
50. Lippincott-Schwartz, J. S. Bonifacino, L. C. Yuan, and R. D. Klausner. 1988. Degradation from the endoplasmic r(line through square) (line through circle(line through square) (line through circleeticulum: disposing of newly synthesized proteins. *Cell.* **54:** 209–220.
51. Ellis, R. J., and S. M. van der Vries. 1991. Molecular chaperones. *Annu. Rev. Biochem.* **60:** 321–347.
52. Wickner, W., A. J. M. Driessen, and F. U. Hartl. 1991. The enzymology of protein translocation across the *Escherichia coli* plasma membrane. *Annu. Rev. Biochem.* **60:** 101–124.
53. Hass, I. G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature.* **306:** 387–389.
54. Bole, D. G., L. M. Hendershot, and J. F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* **102:** 1558–1566.
55. Flynn, G. C., J. Pohl, M. T. Flocco, and J. E. Rothman. 1991. Peptide-binding specificity of the molecular chaperone BiP. *Nature.* **353:** 726–730.
56. Blond-Elguindi, S., S. E. Cwirla, W. J. Dower, R. J. Lipshutz, S. R. Spang, J. F. Sambrook, and M-J. H. Gething. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell.* **75:** 717–728.
57. Degen, E., and D. B. Williams. 1991. Participation of a novel 88-kD protein in the biogenesis of murine class I histocompatibility molecules. *J. Cell Biol.* **112:** 1099–1115.
58. Wada, I., D. Rindress, P. H. Cameron, W-J. Ou, J. J. Doherty, D. Louvard, A. W. Bell, D. Dignard, D. Y. Thomas, and J. J. M. Bergeron. 1991. SSRα and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. *J. Biol. Chem.* **266:** 19599–19610.
59. Hochstenbach, F., V. David, S. Watkins, and M. B. Brenner. 1992. Endoplasmic reticulum resident protein of 90 kilodaltons associates with the T- and B-cell antigen receptors and major histocompatibility complex antigens during their assembly. *Proc. Natl. Acad. Sci. USA.* **89:** 4737–4738.
60. Ou, W-J., P. H. Cameron, D. Y. Thomas, and J. J. M. Bergeron. 1993. Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature.* **364:** 771–776.
61. Hammond, C., I. Braakman, and A. Helenius. 1994. Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. *Proc. Natl. Acad. Sci. USA.* **91:** 913–917.