Lecithin:cholesterol acyltransferase deficiency: identification of two defective alleles in fibroblast cDNA

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Abstract Previous mutations associated with lecithin:cholesterol acyltransferase (LCAT) deficiency have been identified using genomic DNA. To facilitate mutation analysis, we used cDNA from cultured fibroblasts which were shown to express LCAT mRNA. Using reverse-transcriptase PCR, LCAT cDNA was obtained from a 13-year-old boy with complete LCAT deficiency, characterized by low HDL-C (3 mg/dl), nondetectable initial cholesterol esterification rate, LCAT activity, and minimal LCAT mass (0.16 vs. 5–7.5 μg/ml). Sequencing of LCAT cDNA clones identified two mutations. A novel frameshift mutation caused by deletion of cytosine at the third nucleotide position of amino acid 168 (exon 5) predicts a disrupted protein catalytic site by converting Ser_{181}→Ala and creates a *Pvu-II* restriction site prior to premature truncation at amino acid 238. A C→T transition results in a substitution of methionine for threonine at amino acid position 321 and creates an *Nla-III* restriction site on the maternal allele. Expression studies of mutant LCAT cDNA confirmed the virtual absence of LCAT activity in transfected COS-1 cells. The molecular defect in a young male with complete LCAT deficiency has been identified using fibroblast cDNA.—**Miller, M., K. Zeller, P. O. Kwiterovich, J. J. Albers, and G. Feulner.** Lecithin:cholesterol acyltransferase deficiency: identification of two defective alleles in fibroblast cDNA. *J. Lipid Res.* 1995. **36:** 931–938.

Supplementary key words complementary DNA • fibroblasts • LCAT • HDL-C • genetic disease

Lecithin:cholesterol acyltransferase (LCAT) is a glycoprotein that catalyzes the transfer of acyl groups from the *sn*-2 fatty acid of phosphatidylcholine to esterify free cholesterol. It is believed to play a pivotal role in reverse cholesterol transport by facilitating formation of cholesteryl esters in high density lipoprotein cholesterol (HDL-C) for subsequent hepatobiliary excretion (1). Familial deficiency of LCAT is an autosomal recessive disorder associated with very low HDL-C (2). Although there are varying phenotypic expressions of the disorder, most of the affected subjects have been identified after development of clinical manifestations ranging from corneal opacification and mild anemia to severe renal insufficiency. The gene for LCAT, located on chromosome 16q, consists of 6 exons and encodes a protein of 416 amino acids (3). While the primary site of LCAT production is the liver, other tissues, including testes and brain, express the LCAT gene (4–6). To date, mutations within the coding regions of the gene have been identified using genomic DNA (7, 8). The use of cDNA to screen for the presence of splicing defects has been hindered because of the relative inaccessibility of tissues that express LCAT. We now report that cultured skin fibroblasts express the LCAT gene and serve as a useful tool to screen LCAT cDNA for mutations.

MATERIALS AND METHODS

Subjects

The proband is a 13-year-old boy who was identified with a very low level of HDL-C cholesterol after the sudden cardiac death of his 38-year-old non-smoking father. Hematologic studies revealed microcytic anemia and an elevated concentration of unesterified cholesterol and phosphatidylcholine within erythrocyte membranes (9). There was a trace of proteinuria. No corneal opacification was present upon slit-lamp ophthalmologic examination. A skin biopsy was performed on the forearm of the proband after informed consent was obtained from the pro-

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; PCR, polymerase chain reaction; HDL-C, high density lipoprotein-cholesterol; LDL, low density lipoprotein; CAD, coronary artery disease; hypoα, hypoalphalipoproteinemia.

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band's mother. Other family members studied included the sister, mother, maternal uncle, and maternal and paternal grandparents (**Fig. 1**).

Lipoprotein and apolipoprotein analysis

Total cholesterol and triglyceride concentrations were measured enzymatically on a Hitachi 704 clinical chemistry analyzer (Boehringer-Mannheim, Indianapolis, IN). HDL-C was determined after precipitation of apolipoprotein B lipoproteins with heparin sulfate (1.3 g/l) and manganese chloride (0.092 M) (10). ApoA-I and apoB levels were measured using commercially available immunodiffusion plates (apoA-I, Tago, Inc., Burlingame, CA; apoB, Behring Diagnostics, La Jolla, CA). LDL was estimated using the Friedewald formula (11).

Cholesterol mass measurements initial esterification rate

Fifty μl of whole plasma and the HDL-C fraction were each extracted with hexane–isopropranol 3:2 (12). Stigmasterol (1 mg/ml) was added as an internal standard and the extracts were evaporated under N_2 and redissolved in carbon disulfide for free cholesterol measurement using a Hewlett-Packard 5890 Gas Chromatograph. Separation occurred at 265°C in an HP-17 50% phenyl-methyl silicone cross-linked column after microsaponification (13). The total cholesterol content was measured and esterified cholesterol was calculated as the difference between total and free cholesterol. The initial esterification rate was measured using [^{14}C]cholesterol (sp act: 58 mCi/mmol, New England Nuclear) as the radiolabeled tracer (14).

LCAT mass and activity

LCAT mass was quantified by radioimmunoassay using a polyclonal antibody (15). LCAT activity was determined in vitro (production rate of cholesteryl esters/30 min), using artificial proteoliposomes comprised of apoA-I-[^{14}C]cholesterol-egg phosphatidylcholine (molar ratio, 0.8:12.5:250) (16).

Cloning and sequencing of LCAT cDNA

Fibroblasts were grown and maintained in Eagle's minimal essential media and 10% FCS (Hyclone) as previously outlined (17). For mRNA extraction, fibroblasts were grown to confluency on p60 dishes (Falcon, MA), dissociated with 0.05% trypsin-EDTA, pelleted and resuspended in PBS. mRNA was extracted from approximately 1×10^7 cells using a micro mRNA isolation kit (Pharmacia). A first strand cDNA synthesis kit (Pharmacia) was utilized with 100 ng mRNA as template and an oligo dT primer. Full-length LCAT cDNA was amplified from 1/30th of the first strand reaction with the oligonucleotides: 5' GGGAATTCACCAGGGCTGGAATGGGG 3' and 5' GGAAGCTTCTTTATTCAGGAGGCGGG 3', under the following PCR conditions: 20 pmol of each primer, 100 μM each dNTP, 20 mM Tris-HCl, pH 8.5, and 2 mM $MgCl_2$. The reaction mixtures were denatured at 93°C for 30 sec, annealed at 58°C for 30 sec, and extended at 72°C for 30 sec for a total of 35 cycles using a Hybaid Thermal Reactor (National Labnet). After separation on a 6% polyacrylamide gel and staining with ethidium bromide, PCR products were isolated, blunt-ended with Klenow DNA polymerase, and subcloned into

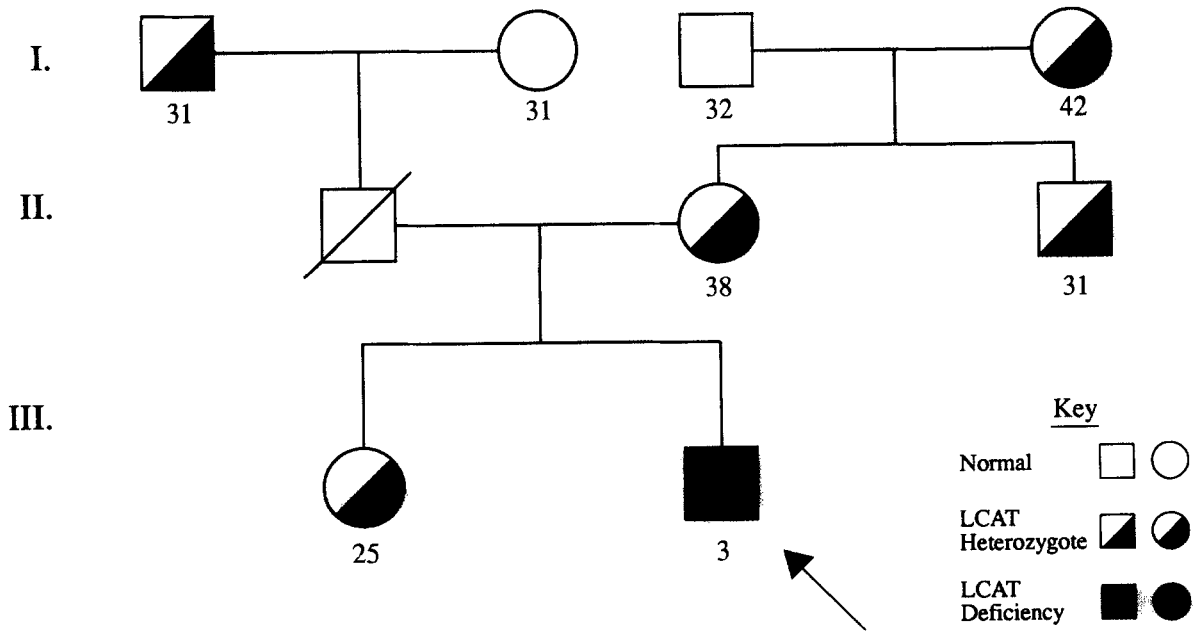


Fig. 1. Pedigree of the family with LCAT deficiency. The proband is indicated by the arrow. HDL-C levels (mg/dl) are listed below the accompanying circle or square.

the *Srf I* restriction enzyme site in the plasmid vector, pBluescript (Stratagene, La Jolla, CA) (18). Full-length clones were isolated and sequenced using cycle sequencing with fluorescent dye terminators and then analyzed on an ABI automated sequencer (19, 20). The universal primers T3 and T7 as well as internal LCAT primers were used to sequence the entire cDNA clones. (see **Table 1** for location of LCAT primers).

Restriction enzyme analysis

To examine polymorphic restriction sites created by identified mutations, genomic DNA spanning exons 5 and 6 of the LCAT gene was amplified from the proband and selected family members. Exon 5 was amplified using the following primers: 5' CTATGACTGGCGGCTGGAG 3' and 5' TCACCTGAGGCGAAGAC 3' under the following PCR conditions: 40 pmol each primer, 100 μM each dNTP, 10% DMSO, 17 mM ammonium sulfate, 67 mM Tris, pH 8.0, 0.67 mM $MgCl_2$, 10 mM DTT, 0.17 mg/ml BSA using 100 ng genomic DNA as template. PCR reactions were cycled 30 times at 94°C for 1 min, 53°C for 45 sec, and 72°C for 1 min. To amplify exon 6, the following primers were used: 5' CTGCAGTCACGTGACCTC 3' and 5' GGCTGTGGCTGGCGGCC 3' with the same conditions noted above except for the primer annealing temperature (58°C). Five hundred nanograms of gel-purified PCR products was digested for 2 h with 5 units of either *Pvu-II* or *Nla-III* (Stratagene). The restriction fragments were separated by polyacrylamide gel (6–8%) electrophoresis and the DNA was visualized after ethidium bromide staining (21).

Expression of LCAT cDNA in COS-1 cells

To evaluate the functional significance of identified mutations associated with LCAT deficiency, mutant and normal full-length LCAT cDNAs were cloned into the expression vector pSVSport (Gibco/BRL). This was accomplished by digestion of the original pBluescript clones with *EcoRI* and *Hind III,* restriction sites that were engineered into the PCR primers used to amplify the LCAT cDNA. After purification from a 0.8% agarose gel, the inserts were ligated into the unique *EcoRI/Hind III* sites of the pSVSport vector. This directional cloning method placed the LCAT cDNA in the correct orientation for expression in Cos-1 cells. For each condition, COS-1 cells (1×10^6) were plated in duplicate and cultured in 10 ml of MEM supplemented with 10% fetal calf serum. The next day, the cells were transfected with 20 μg of each clone or carrier DNA by the calcium phosphate coprecipitation method (22). After an overnight incubation, media were changed to 10 ml DMEM (Gibco, Gaithersburg, MD) without FCS. At 72 h post-transfection, media were removed and stored at −70°C. LCAT activity was assessed using a proteoliposome substrate and 10 μl of thawed media (16). Using a micro RNA isolation kit (Stratagene) total RNA was extracted from the transfected cells for Northern blot analysis using a radiolabeled full-length human LCAT cDNA probe.

TABLE 1. Primers used for sequencing LCAT cDNA

Primer Sequence	Nucleotide Position
5' CTATGACTGGCGGCTGGAG 3'	511–529
5' CAGCAGGAAATAGAGCAAG 3'	641–659
5' ATAACCACCACCTCCCCCT 3'	816–834
5' CCTGTGTAGTTGAAGCTGG 3'	889–907
5' TACATCTACGACCACGG 3'	1056–1072
5' GGCTGTGGCTGGCGGCC 3'	1164–1180

Nucleotide position in the cDNA of each primer is indicated: nucleotide 1 is equal to the first position of the initiator methionine.

TABLE 2. Lipids, lipoproteins, and apolipoprotein A-I levels of the proband and family members

Subject	Age	TC	TG	HDL-C	A-I
	yr		*mg/dl*		
Proband (IIIb)	**13**	**96**	**136**	**3**	**7**
Sister (IIIa)	16	175	62	25	87
Mother (IIa)	41	213	78	38	126
(m) Uncle (IIb)	39	175	136	31	119
(p) gf (Ia)	73	184	232	31	104
(p) gm (Ib)	70	214	181	31	134
(m) gf (Ic)	73	186	168	32	120
(m) gm (Id)	69	294	159	42	148

Abbreviations: m, maternal; p, paternal; gf, grandfather; gm, grandmother.

RESULTS

Lipoprotein and apolipoprotein analysis

The members of the pedigree (Fig. 1) are nonconsanguineous 5th generation Americans of German (paternal) and Irish-Scottish (maternal) descent. Lipid, lipoprotein, and apolipoprotein levels of the proband (shown in bold) and family members are shown in **Table 2.** Maternal and paternal family members exhibited low levels of HDL-C, although extremely low levels were only detected in the proband.

Cholesterol mass, esterification rate, LCAT mass and activity

The results of cholesterol mass, esterification rate, and LCAT mass and activity are displayed in **Table 3.** Compared with controls, the proband (shown in bold) demonstrated reduced mass of cholesteryl esters in plasma and HDL-C. An initial cholesterol esterification rate was not detectable and the mass and activity of LCAT were both negligible, consistent with complete LCAT deficiency. A

TABLE 3. Cholesterol mass[a], initial cholesterol esterification rate (CER), LCAT mass and activity of proband and family members

Subject	Cholesterol Mass		CER	LCAT	
	Plasma	HDL-C		Mass	Activity
	% CE		nmol/ml/h	μg/ml	nmol/ml/h
Proband	**11**	**20**	**0**	**0.16**	**0**
Sister	75	80	83	3.8	78
Mother	78	82	78	3.7	48
(m) Uncle	77	75	70	4.0	47
(p) GF	78	79	32	3.0	34
(p) GM	78	73	86	7.2	92
(m) GF	80	80	90	6.7	127
(m) GM	75	75	79	4.2	58
Controls (n = 5)	80	80	60–100	5–7.5	100–130

Abbreviations: m, maternal; p, paternal; gf, grandfather; gm, grandmother.

[a]Values given as percent of total cholesterol in cholesteryl esters (CE).

reduction in mass of LCAT of approximately 50% was found in certain family members, consistent with heterozygosity for LCAT deficiency.

Expression of LCAT in cultured skin fibroblasts

To evaluate LCAT expression in fibroblasts, confluent cells from the proband and a normolipidemic control (passages 3–10), as well as HepG2 cells (positive control) and leukocytes (negative control) (4) were collected for mRNA extraction. The isolated RNA was subjected to reverse transcription and PCR amplification. LCAT expression was evident in fibroblasts but not in leukocytes (**Fig. 2**). However, the quantity expressed in fibroblasts was extremely low and not detectable by Northern blot analysis of 1 μg of mRNA (data not shown).

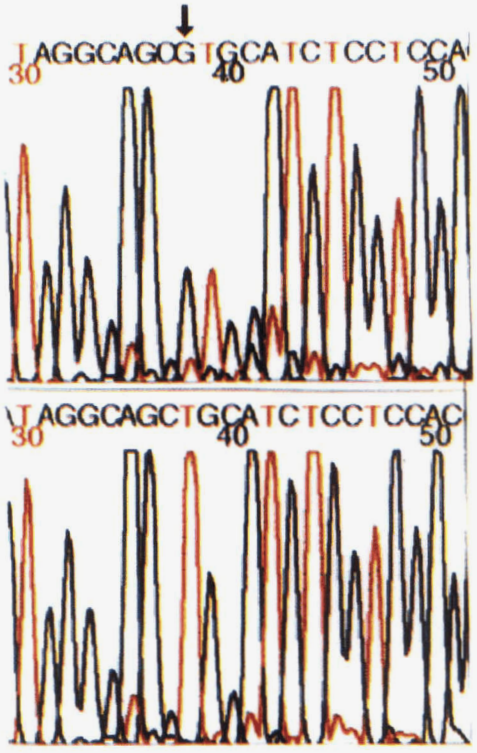


Fig. 3. Single base-pair deletion in amino acid 168. Top panel: normal LCAT sequence. Bottom panel: sequence of mutant LCAT cDNA. Arrow indicates the base-pair that is deleted in the mutant (sequence is of the antisense strand).

DNA sequence analysis and restriction digestion

Full-length LCAT cDNA was amplified and cloned from skin fibroblasts of the proband and a normal control. Sequence analysis revealed two mutations on two different cDNA clones from the proband. The first mutation

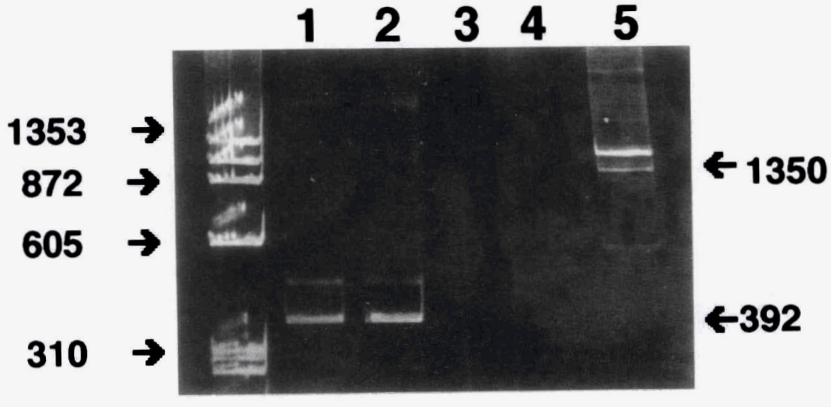


Fig. 2. Expression of LCAT mRNA in liver and fibroblasts. Markers on the left refer to molecular weight standards (Φx Hae III). Lane 1: 392 base-pair PCR fragment of exons 4 and 5 from HepG2 cells; lane 2: 392 base-pair fragment from fibroblasts of the proband; lane 3: PCR amplification of mRNA from leukocytes; lane 4: no sample applied; lane 5: full-length LCAT cDNA amplified from normal human liver cDNA.

was a deletion of a cytosine residue at the third nucleotide position of amino acid 168 in exon 5 (**Fig. 3**). This deletion creates a novel *Pvu-II* restriction site (**Fig. 4**) and a frameshift that produces a stop codon at amino acid 239 and truncates the LCAT protein by 178 amino acids. A 334 base-pair genomic DNA fragment containing this mutation was amplified from several family members and used for restriction mapping. This region contains a fixed *Pvu-II* site as well as the variable restriction site. When digested with the appropriate restriction enzyme, the normal allele is cleaved into two fragments, 256 and 78 base-pairs in length while the mutant allele is cut twice to produce three fragments, 174, 82 and 78 base-pairs in length. A heterozygote for this mutation (e.g., the proband) will exhibit four fragments of sizes 256, 174, 82, and 78 base-pairs (Fig. 4). As evident from this restriction digest, the mutant allele was inherited from the paternal grandfather.

Sequence analysis of two independent LCAT clones from the proband's fibroblasts (in both 5'→3' and 3'→5' direction), revealed a single base-pair substitution in exon 6 of the maternal allele (**Fig. 5**). A C→T transition in the second position of amino acid 321 results in the substitution of methionine for threonine and creates an *Nla-III* restriction site. The normal allele in the amplified genomic region does not contain any *Nla-III* sites and migrates as a single 214 base-pair band. The *Nla-III* restriction site detected in the proband and several maternal relatives cleaves the fragment into two smaller fragments of 133 and 81 base-pairs (**Fig. 6**).

In vitro expression studies

Northern blot analysis of total RNA from transfected COS-1 cells demonstrated that the normal and both mutant forms of LCAT cDNA were expressed (data not shown). Using a proteoliposome substrate, LCAT activity was assessed in the culture media of transfected COS-1 cells. Expression of either mutation resulted in virtual absence of LCAT activity (**Table 4**), suggesting that instability of the altered proteins was a likely cause of LCAT deficiency.

DISCUSSION

An important observation in this study was the demonstration that LCAT mRNA is expressed in cultured fibroblasts. The proband, in whom the molecular defects causing LCAT deficiency were detected after analysis of fibroblast cDNA, is the youngest subject identified to date with this abnormality. The genetic compound is the result of a novel frameshift mutation in exon 5 and a previously reported missense mutation, Thr_{321}→Met, in exon 6 (2). Although nucleotide insertions resulting in a frameshift mutation have been reported previously (23, 24), this is the first case, to our knowledge, of a nucleotide deletion associated with LCAT deficiency. The significance of this mutation is the loss of a cytosine at the third nucleotide of amino acid 168 which predicts a disrupted putative enzyme catalytic center by converting Ser_{181}→Ala. Recently, Francone and Fielding (25) showed that Ser_{181} is

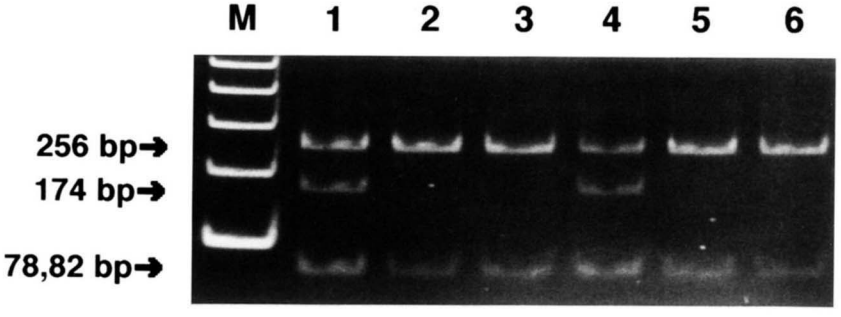
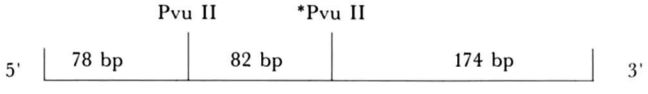


Fig. 4. Novel *Pvu-II* restriction site created by the base-pair deletion in exon 5. Restriction map of 334 base-pair PCR fragment illustrates position of the fixed and variable (indicated by the asterisk) *Pvu-II* sites. The PCR fragments amplified from the genomic DNA of maternal and paternal family members were restricted with *Pvu-II* and analyzed by polyacrylamide gel electrophoresis. Lane 1: proband; lanes 2 and 3: proband's sister and mother, respectively; lanes 4 and 5: paternal grandfather and grandmother, respectively; lane 6: maternal grandfather. Lane M represents a 100 base-pair DNA ladder (Gibco/BRL).



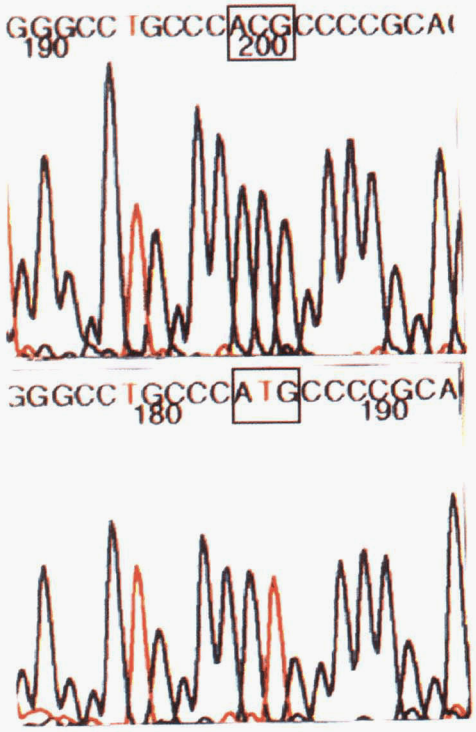


Fig. 5. The C→T transition in exon 6. Top panel: normal LCAT sequence. Bottom panel: mutant sequence from proband.

the active site for transfer of acyl intermediates. Using site-directed mutagenesis, conversion of Ser_{181}→Ala resulted in the inhibition of LCAT activity (25). Based on the predicted 1° and 2° amino acid sequence, the mutation would be expected to produce a protein that contains a greater predominance of serine and threonine residues (11 and 5, respectively) resulting in a more polar and hydrophilic polypeptide. Another consequence of the nucleotide deletion is premature truncation which results in a protein of 238 amino acids.

TABLE 4. LCAT activity in the culture media of in vitro expressed normal and mutant LCAT

LCAT	Activity
	nmol/h
Normal	84 ± 22
Mutant	7 ± 5
$LCAT_{168}$ (frameshift)	
Thr_{321}→Met	ND

Total amount of cholesteryl esters formed per hour in the cell culture media using proteoliposomes as substrate (see Methods). Values represent mean ± SD of triplicate measurements from two separate dishes and were subtracted from the background values (e.g., carrier DNA and cultured cells only); ND, no detectable activity noted.

Although the second mutation, Thr_{321}→Met, was recently reported in an individual from Sardinia who was homozygous for this change (2), the phenotypic effect of this alteration was not studied. When COS-1 cells were transfected with LCAT cDNA containing the Thr_{321}→Met mutation, LCAT mRNA was expressed but there was complete absence of enzymatic activity (Table 4). As anticipated by the computer-predicted secondary structure, the allelic substitution increases the hydrophobicity of the

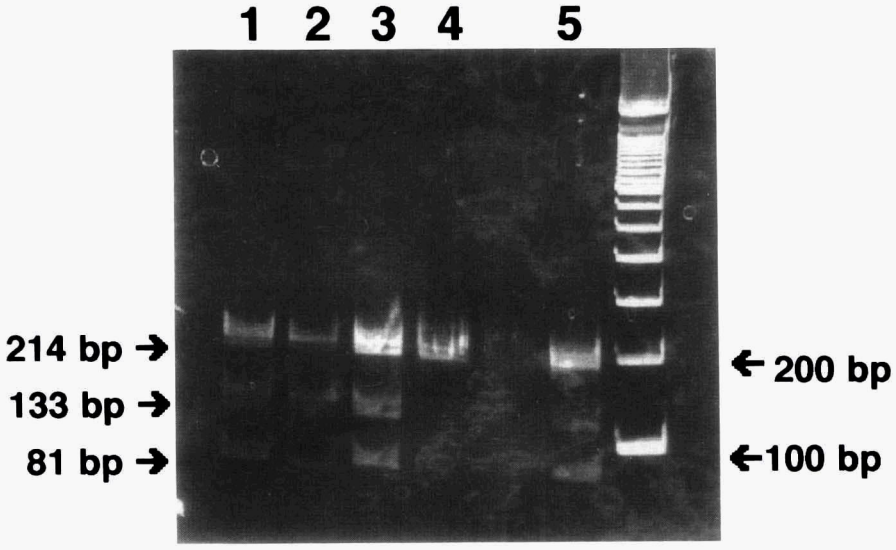
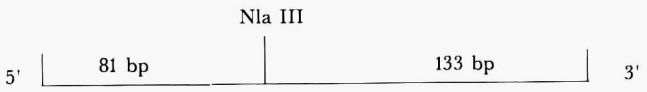


Fig. 6. Novel *Nla-III* site created by the C→T transition in exon 6. Restriction map illustrates position of *Nla-III* site in the mutant allele. The gel represents an *Nla-III* digestion of amplified genomic DNA from proband and family members. Lane 1: proband; lanes 2 and 3: proband's sister and mother, respectively; lane 4: paternal grandfather; lane 5: maternal grandfather. Lane on right represents 100 base-pair DNA ladder (Gibco/BRL).



protein segment. Therefore, the markedly reduced LCAT mass found in both the subject homozygous for the mutation (0.3 μg/ml) (2) and in our subject most likely represents irregular folding of the protein leading to enhanced degradation.

Despite the severity of the genetic abnormalities present in the proband, the devastating sequelae often attributed to complete LCAT deficiency (e.g., renal insufficiency) have not yet occurred. This may in part reflect the phenotypic heterogeneity of the disorder, or delayed expression of clinical renal complications. Unfortunately, the homozygote subject with the $Thr_{321} \rightarrow Met$ substitution developed renal insufficiency during his fourth decade (2). The predicted abolishment of the catalytic site owing to a frameshift mutation on the paternal allele further suggests that the compound heterozygote reported herein is likely to develop the serious manifestations of this disorder. At present, microcytic anemia and trace proteinuria are the sole clinical findings. However, these premonitory findings suggest that close observation and a vigorous search for potential therapeutic strategies, including liver transplantation and/or gene therapy, may be warranted in the future.

Previous studies using fibroblast cDNA (26) can now be extended to include LCAT deficiency states. Although liver, brain, and testes all express LCAT in appreciable quantities (4–6), routine biopsies of these tissues for mRNA isolation are not feasible. Therefore, it is fortuitous that fibroblasts express LCAT mRNA. Although the quantity of LCAT mRNA was below the threshold for detection by Northern blot analysis, fibroblasts may be particularly useful in facilitating the identification of splicing defects. For example, the inability to find the second mutation in an LCAT-deficient compound heterozygote using genomic DNA (27) raises the possibility of abnormal splicing. Thus, examination of fibroblast cDNA may help to elucidate the molecular defect in such subjects.

Although most family members studied were heterozygotes for LCAT deficiency (Fig. 1), the paternal grandmother (pgm) and maternal grandfather (mgf) displayed normal LCAT mass and activity (Table 3). That HDL-C levels in both subjects were low (31 mg/dl and 32 mg/dl, respectively) suggests the coexistence of familial hypoalphalipoproteinemia (hypoα), a polygenic disorder characterized by low HDL-C and premature coronary artery disease (CAD) (28). Consistent with this possibility was a fatal myocardial infarction in the proband's 38-year-old non-smoking, normolipidemic, and normotensive father. Levels of HDL-C had not been determined in the deceased father although postmortem examination revealed corneal clouding, suggestive of very low HDL-C (1). Nonetheless, the absence of CAD in other family members with low HDL-C who were not heterozygotes for LCAT deficiency (e.g., paternal grandmother) makes familial hypoα less likely and raises the possibility of an unidentified abnormal genetic and/or metabolic condition that may have predisposed the proband's father to sudden cardiac death.

In summary, at least 16 different mutations have now been reported from among 30 families with LCAT deficiency. The widespread screening measures recently advocated for identifying subjects with low HDL-C (29) increase the likelihood that additional mutations will be described and characterized in the near future.

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