# In vitro expression of natural mutants of human lecithin:cholesterol acyltransferase

**Shi-Jing Qu, Hui-Zhen Fan, Francisco Blanco-Vaca, and Henry J. Pownall**[1]

Department of Medicine, Baylor College of Medicine and The Methodist Hospital, 6565 Fannin, MS A-601, Houston, TX 77030

**Abstract** Fish-eye disease (FED) and familial lecithin:cholesterol acyltransferase (LCAT) deficiency (FLD) are rare disorders of lipid metabolism linked to mutations in the LCAT gene. Eleven LCAT cDNA constructs associated with FED and FLD were prepared by site-directed mutagenesis and expressed in COS-6 cells. Analysis of total RNA from wild-type, FED, and FLD transfectants revealed that all contained LCAT-specific mRNA. Western blot analysis demonstrated that all LCAT transfectants synthesized LCAT. Mean LCAT secretion by FED transfectants was slightly lower than secretion by wild-type transfectants, whereas secretion by FLD transfectants was much lower. The specific activities of FED and FLD LCAT against model high density lipoproteins were 6% and 11%, respectively, of wild-type activity. The ratios of the LCAT activities against low density lipoproteins to those against model high density lipoproteins decreased in the order FED mutants > FLD mutants ~ wild type. FED and FLD LCAT mutants are different: the former are more active against low density lipoproteins, and the latter are less secretion-competent. The greater reactivity of FED LCAT against low density lipoproteins may explain the relative mildness of the clinical manifestations of FED compared to those of FLD.—**Qu, S-J., H-Z. Fan, F. Blanco-Vaca, and H. J. Pownall.** In vitro expression of natural mutants of human lecithin:cholesterol acyltransferase. *J. Lipid Res.* 1995. **36:** 967–974.

**Supplementary key words** LCAT deficiency • cholesterol transport • high density lipoproteins

The 416-amino acid glycoprotein lecithin:cholesterol acyltransferase (LCAT) is a key enzyme in the translocation of cholesterol from peripheral tissues to the liver (1–7). Its function is to convert free cholesterol, which transfers freely between lipoprotein surfaces, to an insoluble esterified form. The major substrates for LCAT are high density lipoproteins (HDL), which contain an apolipoprotein (apoA-I) that activates the enzyme. In addition, a lysolecithin:lecithin acyltransferase activity on the surface of low density lipoproteins (LDL) has been assigned to LCAT (8). In the absence of cholesterol, LCAT exhibits a phospholipase $A_2$ activity (9).

Impaired LCAT activity appears to be central to two rare hereditary disorders termed fish-eye disease (FED) and familial LCAT deficiency (FLD). FED and FLD are associated with hypoalphalipoproteinemia and corneal opacities (10–17). Patients with FLD may also exhibit renal insufficiency and anemia. However, despite the strong inverse correlation between plasma level of HDL-cholesterol and incidence of coronary artery disease in the general population (18, 19), premature atherosclerosis has not been described in FED and has been reported only occasionally in FLD (13). To identify the molecular basis for the FED and FLD phenotypes, we expressed LCAT mutants that characterize FED or FLD in COS-6 cells. We then used immunological methods and assays of cholesteryl ester formation to determine *1)* intracellular and secreted levels of LCAT and *2)* specific activities of FED- and FLD-associated LCAT mutants against LDL and model reassembled HDL (R-HDL).

## MATERIALS AND METHODS

### Materials

Restriction enzymes were purchased from Promega (Madison, WI). The Sequenase version 2.0 DNA sequencing kit was from United States Biochemical (Cleveland, OH). Amersham (Arlington Heights, IL) was the source for [$\alpha$-$^{35}$S]dATP (1 Ci/$\mu$mol), [$\alpha$-$^{32}$P]ATP (5 Ci/$\mu$mol), and Na$^{125}$I. Rabbit anti-goat immunoglobulin G (IgG) was obtained from Bio-Rad (Richmond, CA), [$^3$H]cholesterol from NEN-DuPont (Boston, MA), and phospholipids from Avanti Polar Lipids (Birmingham, AL). The LCAT gene is comprised of 6 exons and 5 introns, and its mRNA contains 1550 nucleotides (20). A

Abbreviations: CE, cholesteryl esters; LCAT, lecithin:cholesterol acyltransferase; FED, fish-eye disease; FLD, familial LCAT deficiency; HDL, high density lipoproteins; LDL, low density lipoproteins; R-HDL, model reassembled HDL; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; IgG, immunoglobulin G; apoA-I, apolipoprotein A-I; Tris, tris(hydroxymethyl)-aminomethane.

[1]To whom correspondence should be addressed.

full-length cDNA clone of human LCAT (pUCLCAT.10) spliced into the *Eco*RI/*Bam*HI sites of pUC19 was provided by Richard Lawn of Genentech, Inc. (South San Francisco, CA) (8). The insert contained 102 nucleotides in the 5'-untranslated region, the entire coding region, and 68 nucleotides in the 3'-untranslated region. Both ends were bounded by multiple linkers.

### Construction of the mutant clones

The mutagenesis methods are essentially those of Qu et al. (21). The oligonucleotides used as the primers for mutagenesis (**Table 1**) were synthesized on a Cyclone Plus DNA synthesizer and purified using an Oligo-pak oligonucleotide purification column (MilliGen/BioSearch, Burlington, MA). Before use, the oligonucleotides were phosphorylated at the 5' position by $T_4$ polynucleotide kinase. After digestion with *Eco*RI and *Bam*HI, the pUCLCAT.10 was subcloned into M13mp18 and M13mp19. Uracil-containing single-stranded DNA was prepared by using *Escherichia coli* host strain CJ236 ($ung^-$) to culture the M13mp18 or M13mp19 LCAT clones (22). Single-stranded DNA was used as a template and hybridized to oligonucleotides that contained mismatched nucleotides coding for the desired mutant amino acid. After hybridization, the second strand was synthesized using $T_7$ DNA polymerase and $T_4$ ligase. The double-stranded DNA was transformed into an $ung^+$ *Escherichia coli* strain, DH-5$\alpha$'. The plaques of mutant cDNA were identified by the dideoxynucleotide method (23). After digestion with *Eco*RI and *Bam*HI, the wild-type and mutant cDNA were subcloned into the eukaryotic expression vector pSG5 and transformed into *E. coli* AG1 (24). The positive clones were checked by restriction enzyme mapping and direct sequencing. The selected mutant cDNA were prepared in larger quantities by using a kit (Qiagen Midi, Chatsworth, CA) for expression in mammalian cells.

### In vitro expression of LCAT

A modified diethylaminoethyl-dextran method (21, 25) was used to transfect wild-type and mutant LCAT cDNA into COS-6 cells. Cells were grown at 37°C in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum and 1× antibiotic-antimycotic liquid (Gibco BRL, Grand Island, NY) in 75-$cm^2$ flasks. The cells were transfected with 20 $\mu$g of plasmid per flask, incubated with the DNA-diethylaminoethyl stock and chloroquine for 3 h, and shocked with 10% dimethyl sulfoxide for 2 min. Flasks containing COS-6 cells only and COS-6 cells plus pSG5 plasmids were used as controls. After 72 h, the media were immediately screened for LCAT activity using R-HDL as the substrate. The cells of the cultures with LCAT activity (>90% confluence) were then subcultured at a ratio of 1:4 to 1:6 and incubated in serum-free DMEM for 48 h. The medium was collected and centrifuged at 1200 rpm for 10 min, after which 10 $\mu$l was immediately removed to determine LCAT activity. For mass quantification, 4 $\mu$g/ml standard LCAT (10, 20, 40, 80, and 100 $\mu$l) or concentrated sample medium (20, 40, and 80 $\mu$l) was applied to each well of the nitrocellulose membrane. LCAT was detected by reaction with goat anti-human LCAT serum and $^{125}$I-labeled rabbit anti-goat IgG.

TABLE 1. Synthetic oligonucleotides used for LCAT mutants and PCR primers[a]

| | Mutants | Substitution[b] |
|---|---|---|
| FED mutants | | |
| $M_{10}$ | $Pro^{10}$→Leu | 5'-CGT-GGT-GTG-C**A**G-GGG-GAA-GAG-3' |
| $M_{32}$ | $Leu^{32}$→Pro | 5'-CTG-ATT-CCC-C**G**G-GCA-GCC-GGG-3' |
| $M_{123}$ | $Thr^{123}$→Ile | 5'-CTG-CAC-CAG-T**A**T-GTG-CAG-GTA-3' |
| $M_{347}$ | $Thr^{347}$→Met | 5'-GGT-CGC-CAC-C**A**T-GTC-ATC-ACC-3' |
| FLD mutants | | |
| $M_{93}$ | $Ala^{93}$→Thr | 5'-GAC-ACC-AGG-GG**T**-GTT-GGA-CA-3' |
| $M_{135}$ | $Arg^{135}$→Trp | 5'-AGT-CTC-GTC-CC**A**-CAC-GTA-GCC-3' |
| $M_{147}$ | $Arg^{147}$→Trp | 5'-GGG-CTC-CAG-CC**A**-CCA-GTC-ATA-3' |
| $M_{158}$ | $Arg^{158}$→Cys | 5'-TGC-GAG-CTT-GC**A**-GTA-GTA-CTC-3' |
| $M_{209}$ | $Leu^{209}$→Pro | 5'-GGG-AGC-CCC-A**G**G-AGA-GAT-GAA-3' |
| $M_{321}$ | $Thr^{321}$→Ile | 5'-GGT-GCG-GGG-C**A**T-GGG-CAG-GCC-3' |
| PCR primers | | |
| Downstream | | 5'-TTC-TGG-CTC-CTC-AAT-GTG-CTC-3' |
| Upstream | | 5'-CAA-GTG-TAG-ACC-GCC-GAG-GTC-3' |

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; PCR, polymerase chain reaction; FED, fish-eye disease; FLD, familial LCAT deficiency.
[a]Mutagenesis primers are the antisense strand.
[b]Nucleotide substitutions are underlined.

### RNA preparation, cDNA synthesis, and polymerase chain reaction

After collecting media for the LCAT assays, the transfected COS-6 cells were used to prepare total RNA. Extraction of RNA with guanidinium thiocyanate (26) was followed by centrifugation in cesium trifluoroacetate (27) and precipitation in ethanol. The polymerase chain reaction (PCR) was performed on the RNA using the GeneAmp RNA PCR Kit (Perkin-Elmer Cetus, Norwalk, CT) and the primers given in Table 1. For each sample, 1 $\mu$g of total RNA was used. PCR was carried out as follows: 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. These cycles were followed by 10 additional min of PCR at 72°C and an overnight soak at 4°C.

### Substrate preparation and LCAT assays

R-HDL was prepared according to the techniques of Matz and Jonas (28). Cholesterol (2 mol%), [$^3$H]cholesterol (7,500 disintegrations/min/pmol), and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) were mixed, and the solvent was removed under a stream of nitrogen. The lipids were suspended in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (pH 7.4) and combined with human apoA-I at a lipid to protein ratio of 100:1 (M/M). Sodium cholate (10%) was added until the turbidity of the solution disappeared, and the solution was exhaustively dialyzed against the same buffer. Assays were conducted with R-HDL in which the final concentrations of POPC, cholesterol, and apoA-I were 600 $\mu$M, 12 $\mu$M, and 6 $\mu$M, respectively. Kinetic constants for LCAT activity were determined by measuring the activity against R-HDL as a function of substrate concentration. The constants were calculated using a standard curve-fitting routine for enzyme kinetics.

LCAT activity was also measured using human LDL (0.65 mg/ml apoB-100; 0.64 $\mu$mol/ml free cholesterol) as a substrate. LDL was labeled by injection of [$^3$H]cholesterol (50 $\mu$Ci; 50 $\mu$l ethanolic solution) into a solution of LDL, 10 mM Tris (pH 7.4), and 150 mM NaCl to give a final volume of 4 ml. All LCAT assays were performed as previously described (29). Each assay contained 0.5% human serum albumin, 2 mM dithiothreitol, and 25 $\mu$l medium in a final volume of 160 $\mu$l.

### Quantification of LCAT and Western blot analysis

Western blot analysis of lysates and media of transfected COS-6 cells was performed by using goat anti-sera raised against human plasma LCAT according to the technique of Qu et al. (21). A polyclonal antibody was raised in a goat by injection of purified human plasma LCAT. A standard solid-phase enzyme-linked immunoassay was used to determine the mass of secreted LCAT in the medium; purified human plasma LCAT was the standard. Isolated human LCAT and media from the transfected COS-6 cells and the controls were concentrated tenfold with a Centriprep-30 (Amicon, Inc., Beverly, MA) and applied to a nitrocellulose membrane in a dot-blot apparatus. The nitrocellulose was incubated first with goat antiserum to human LCAT, and then with rabbit anti-goat $^{125}$I-labeled IgG. After extensive washing to ensure adequate signal/background ratio, autoradiography was performed. The dots were cut and the radioactivity was measured by gamma counting. Electrophoresis was conducted using a 12% sodium dodecyl sulfate-polyacrylamide gel, after which the proteins were transferred to a nitrocellulose membrane and reacted with goat antiserum to human LCAT. The bands were visualized using a rabbit anti-goat IgG (heavy and light chains) horseradish peroxidase-conjugated IgG kit (Bio-Rad).

## RESULTS

### Mutagenesis strategy

Eleven LCAT mutants associated with FED and FLD were prepared in vitro. The primers used in site-directed mutagenesis and the resulting amino acid substitutions are listed in Table 1. These mutants include single as well as compound mutations found in some FED and FLD subjects. Five of the mutations are found in FLD patients; of these, four are point mutations and the other is a compound of two point mutations. They are $M_{135}$, Arg$^{135}\rightarrow$Trp; $M_{147}$, Arg$^{147}\rightarrow$Trp; $M_{93,158}$, Ala$^{93}\rightarrow$Thr, Arg$^{158}\rightarrow$Cys; $M_{209}$, Leu$^{209}\rightarrow$Pro; and $M_{321}$, Thr$^{321}\rightarrow$Ile (13). Mutations that characterize FED include $M_{10}$, Pro$^{10}\rightarrow$Leu (30); $M_{32}$, Leu$^{32}\rightarrow$Pro (15); $M_{123}$, Thr$^{123}\rightarrow$Ile (16); and $M_{347}$, Thr$^{347}\rightarrow$Met (31). For cases in which a compound mutation is associated with FLD or FED, single point mutants corresponding to each of the two mutation sites were prepared to determine their separate contributions to the impaired secretion and activity. After mutagenesis using M13 vectors, the mutant cDNA were subcloned into the mammalian cell expression vector pSG5 (21). Before transfection of the cells with the mutant cDNA for expression, the changes in the codons were verified by direct sequencing and autoradiography.

Total RNA from COS-6 cells transfected by pSG5 (with or without LCAT cDNA) were analyzed as previously described (21). After PCR, a 561-bp fragment confirmed the presence of LCAT-specific mRNA in the mutant and wild-type transfectants (data not shown). As expected, no specific band was found for cells transfected by the pSG5 control lacking the insert.

### Western blot analysis

By Western blot analysis, LCAT-specific bands were detected in native human plasma LCAT and in cell lysates

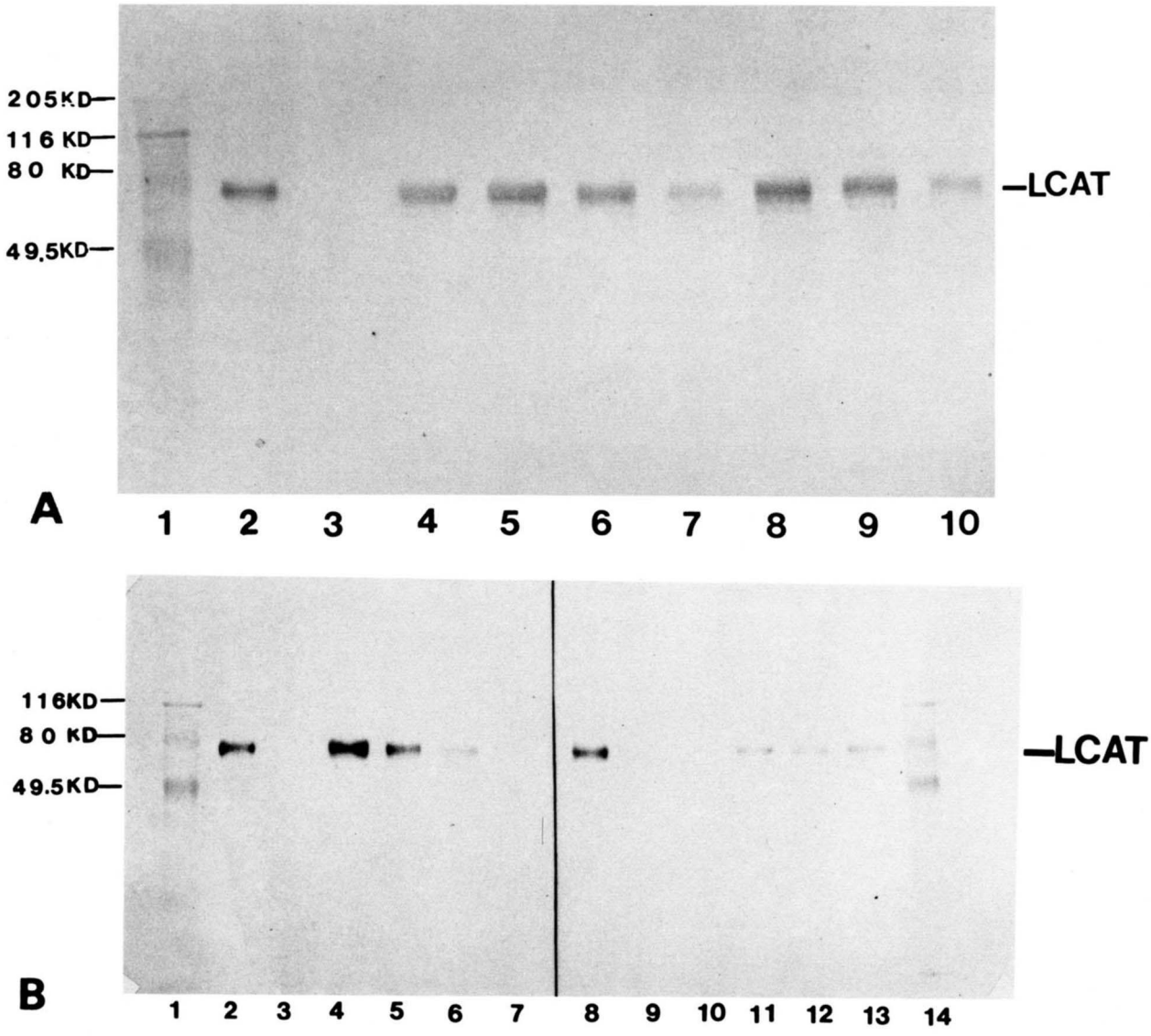


**Fig. 1.** Western blot analysis of isolated human plasma LCAT and wild-type or mutant transfected COS-6 cell culture media. The media from COS-6 cells were concentrated 10-fold and 5 μl was applied to each lane. Lane assignments were: A (FED mutants): 1, marker; 2, human plasma LCAT; 3, cells transfected by pSG5; 4, wild-type LCAT; 5, $M_{123}$; 6, $M_{347}$; 7, $M_{10}$; 8, $M_{32}$; 9, human plasma LCAT. B (FLD mutants): 1, 14, marker, 2, human plasma LCAT; 3, cells transfected by pSG5; 4, wild-type LCAT; 5, $M_{93}$; 6, $M_{135}$; 7, $M_{147}$; 8, $M_{93,158}$; 9, $M_{209}$; 10, $M_{321}$; 11, $M_{158}$.

of the wild-type, FED, and FLD transfectants, but not in lysates from cells transfected by pSG5 lacking the LCAT insert (data not shown). Western blot analysis of media from the transfected cells revealed major differences in intensity (**Fig. 1**). Tests performed on the medium from the transfectant lacking the LCAT insert were negative. All samples of media from the cells transfected with the wild-type and FED cDNA were positive (Fig. 1A). Similar analyses of media from FLD transfectants were consistent with secretion rates that were much lower than that of the wild-type transfectant (Fig. 1B). Generally, band intensities were either not detectable ($M_{147}$, $M_{209}$, $M_{321}$) or extremely faint ($M_{135}$). However, Western blot results for $M_{93,158}$ were similar to results for the wild type. Band intensities from the media of transfectants secreting the model mutants $M_{93}$ and $M_{347}$ were comparable to the band intensity of the wild type, whereas those for $M_{158}$, $M_{193}$, and $M_{201}$ were less intense.

### Quantification of secreted LCAT mass and activity

Secreted LCAT mass was quantified by solid-phase enzyme-linked immunoassay (**Table 2**). Mean secretion of the FED mutants was 75% of that of the wild type, with a range of 60–83%. Secretion of the FLD mutants was much lower than that of the wild type; the mean value was 15%, and the range was 0–56%. However, the mean value

TABLE 2. Mass, activity, and specific activity of media from mutant and wild-type LCAT transfectants

| Transfectant | Mass | Activity | Specific Activity | $K_m$ × $10^5$ M |
|---|---|---|---|---|
| | *μg/ml* | *nmol CE/ml media/h* | *nmol CE/μg LCAT/h* | |
| Wild type[a] | 1.53 ± 0.40 (1.00) | 6.35 ± 0.39 (1.0) | 4.15 ± 0.25 (1.00) | 8. |
| FED mutants | | | | |
| $M_{10}$ | 1.23 ± 0.27 (0.78) | 0.47 ± 0.03 (0.07) | 0.38 ± 0.02 (0.09) | 20. |
| $M_{32}$ | 1.01 ± 0.26 (0.64) | 0.03 ± 0.01 (0.005) | 0.03 ± 0.01 (0.01) | |
| $M_{123}$ | 1.30 ± 0.34 (0.83) | 0.42 ± 0.03 (0.06) | 0.32 ± 0.02 (0.08) | 13. |
| $M_{347}$ | 0.94 ± 0.09 (0.60) | 0.43 ± 0.06 (0.06) | 0.46 ± 0.06 (0.11) | 6. |
| Mean (FED) | (0.75) | (0.045) | (0.06) | |
| FLD mutants | | | | |
| $M_{135}$ | 0.30 ± 0.09 (0.20) | 0.02 ± 0.01 (0.003) | 0.07 ± 0.01 (0.02) | |
| $M_{147}$ | None detected | None detected | | |
| $M_{93,158}$ | 0.86 ± 0.20 (0.56) | 0.70 ± 0.11 (0.11) | 0.81 ± 0.13 (0.20) | 6. |
| $M_{209}$ | None detected | None detected | | |
| $M_{321}$ | None detected | None detected | | |
| Mean (FLD) | (0.15) | (0.023) | (0.11) | |
| $M_{158}$ | 0.38 ± 0.01 (0.25) | 0.85 ± 0.02 (0.14) | 2.24 ± 0.05 (0.55) | 4. |
| $M_{93}$ | 1.15 ± 0.26 (0.75) | 1.23 ± 0.04 (0.20) | 1.07 ± 0.04 (0.26) | 6. |

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; CE, cholesteryl esters; FED, fish-eye disease; FLD, familial LCAT deficiency.

[a]Values in parentheses are normalized to wild-type LCAT = 1.0.

for the secretion of the FLD mutants is misleading because of a single high value for $M_{93,158}$. If this mutant is not included, the mean is reduced to 5% of that of the wild type. The secretion of $M_{93}$ was greater than that of $M_{93,158}$, but that of $M_{158}$ was unexpectedly low.

The LCAT activity against R-HDL for the media from the FED transfectants was lower than that of the wild type (mean, 4.5% of that of the wild type; range, 0.5–7%); activity for FLD transfectants was even lower (mean, 2.3% of that of the wild type; range, 0.3–11%). If the value for $M_{93,158}$ is disregarded, the mean activity for FLD transfectants decreases to almost nothing. Mean specific activity against R-HDL was only 6% of that of the wild type for FED mutants (range, 1–11%) and only 11% of that of the wild type for FLD mutants, although the latter value may not be representative as specific activity could be calculated only for the two FLD mutants for which there was measurable mass. Although the specific activities of the mutants varied greatly, the range of values for $K_m$ was small ($4 \times 10^{-5}$–$20 \times 10^{-5}$ M; Table 2).

When LCAT activity against R-HDL (Table 2) and LDL (**Table 3**) was compared (**Fig. 2**), a clear pattern distinguishing FED from FLD mutants emerged. The ratio of cholesterol esterification in LDL to that in R-HDL was higher in the FED mutants than in wild-type LCAT. The highest esterification ratio was observed for $M_{123}$; the ratios for $M_{10}$ and $M_{32}$ were lower but still higher than the ratio for wild-type LCAT. Only two of the FLD mutants, $M_{135}$ and $M_{93,158}$, were sufficiently active for similar comparisons. The esterification ratios of both of these mutants were slightly higher than that of the wild-type LCAT. Separate mutation of the sites corresponding to the compound mutant had opposite effects. The esterification ratio for $M_{93}$ was lower than that of $M_{93,158}$, while that of $M_{158}$ was higher than in the compound mutant.

## DISCUSSION

FED and FLD are rare disorders of lipid metabolism that have been attributed to mutations in the LCAT gene. FLD is characterized by the presence of little or no plasma LCAT mass or activity; in FED, the concentration of plasma LCAT concentration is less than normal but

TABLE 3. LCAT activity of FED and FLD mutants against LDL

| | Activity | Specific Activity[a] |
|---|---|---|
| | *nmol CE/ml media/h* | *nmol CE/mg protein* |
| Wild type | 2.7 ± 0.2 (1.00)[b] | 1.7 ± 0.1 (1.00) |
| FED mutants | | |
| $M_{10}$ | 1.4 ± 0.03 (0.55) | 1.17 ± 0.03 (0.69) |
| $M_{32}$ | 0.034 ± 0.00 (0.01) | 0.034 ± 0.005 (0.02) |
| $M_{123}$ | 3.2 ± 0.06 (1.22) | 2.4 ± 0.050 (1.43) |
| $M_{347}$ | 0.20 ± 0.039 (0.08) | 0.21 ± 0.04 (0.02) |
| Mean (FED) | (0.66) | (0.71) |
| FLD mutants | | |
| $M_{135}$ | 0.025 ± 0.005 (0.01) | 0.082 ± 0.02 (0.05) |
| $M_{93,158}$ | 0.082 ± 0.02 (0.03) | 0.094 ± 0.023 (0.06) |
| Mean (FLD) | (0.02) | (0.06) |
| $M_{158}$ | 0.76 ± 0.05 (0.29) | 2.0 ± 0.1 (1.17) |
| $M_{93}$ | 0.076 ± 0.04 (0.03) | 0.066 ± 0.04 (0.04) |

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; FED, fish-eye disease; FLD, familial LCAT deficiency; LDL, low density lipoproteins; CE, cholesteryl esters.

[a]Calculated using the mass data from Table 2.

[b]Values in parentheses are normalized to wild-type LCAT = 1.0.

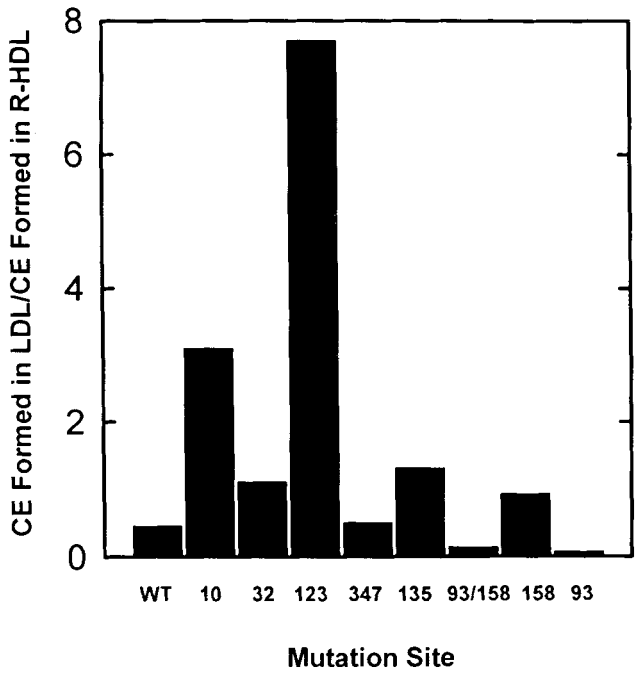


**Fig. 2.** Comparison of the ratios of cholesterol esterification of LDL and R-HDL by wild-type and mutant LCAT. Data are from Tables 1 and 2.

greater than in FLD. To improve understanding of these two diseases and the role of LCAT in normal lipid metabolism, we have used COS-6 cells as a model system for the study of the secretion, activity, and specificity of LCAT mutants associated with FED or FLD.

Wild-type LCAT, four LCAT mutants associated with FED, and five LCAT mutants associated with FLD were transfected into COS-6 cells and compared on the basis of transcription, translation, secretion, specific activity, and macromolecular specificity. According to PCR amplification of cDNA derived from total RNA, all transfectants produced LCAT-specific mRNA. Western blot analysis of cell lysates demonstrated that each transfectant synthesized LCAT. Mean LCAT secretion by FED transfectants was slightly lower (−25%) than that of the wild-type enzyme. In contrast, mean secretion by the FLD transfectants was much lower (−85%) and was not detectable for $M_{147}$, $M_{209}$, and $M_{321}$. For both FED and FLD mutants, the total activity as measured by esterification of R-HDL was much lower than that of the wild-type enzyme. Activity was typically higher for the FED mutants than for the FLD mutants. The exception was the FLD mutant $M_{93,158}$, which exceeded all four FED mutants in activity.

R-HDL was used as a substrate to compare the specific activities because it is more reactive than native HDL and is believed to emulate putative LCAT precursors found in LCAT deficiencies (29, 32, 33). Moreover, plasma HDL is rich in cholesteryl esters (CE) and is likely to be the mature product of LCAT action rather than its primary substrate (34). The specific activities of FED and FLD mutants were low; none was greater than 20% of that of the wild type. Although average specific activity was higher for the FLD mutants than for the FED ones, the difference may not be meaningful as only two FLD transfectants were secretion-competent and one ($M_{93,158}$) was much more active than the other. Furthermore, of all the FED and FLD mutants, $M_{93,158}$ exhibited the highest specific activity against R-HDL.

Although there were small differences between the $K_m$ values (Table 2) of the mutants and the wild-type enzyme, the differences are much smaller than those observed for $V_{max}$. Moreover, the plasma concentration of HDL-phospholipid (~1 mM) is greater than the $V_{max}$ measured for LCAT and the FED and FLD mutants. Therefore, the physiologic effects associated with FLD and FED are likely to be due to differences in $V_{max}$ rather than $K_m$.

An early hypothesis about FED proposed that normal subjects had an α-LCAT that preferentially esterified the free cholesterol in HDL, but those with FED had a β-LCAT that preferentially used the cholesterol in LDL (10, 11). The failure to find more than one gene for LCAT dispelled the notion that there were separate genes for α- and β-LCAT (8). However, in one investigation it was reported that a recombinant LCAT corresponding to $M_{123}$ esterified cholesterol in LDL but not in HDL (35). This finding supports the hypothesis that FED is due to one or more mutations in the LCAT gene that alter the activity and specificity of the enzyme. However, our data are not totally consistent with this model. Our observed specific activities against R-HDL and LDL are higher and lower, respectively, than those reported by O et al. (35). As a consequence, our ratios of the reactivity of this mutant against LDL and R-HDL are much lower.

The ratios of the rates of esterification of LDL-cholesterol to those of R-HDL-cholesterol are higher for the FED mutants (Fig. 2), but this result is misleading. The higher ratio is due not to increased activity against LDL but to greatly depressed activity against R-HDL (Tables 2 and 3). However, because HDL has a much lower reactivity than R-HDL (29), a much higher ratio would have been found if HDL had been substituted for R-HDL. On the other hand, it is possible that the greater reactivity of HDL precursors relative to mature HDL is the main reason for the high levels of plasma HDL in FED subjects.

Four other mutants that are not known to be associated with FED or FLD were also prepared and tested. Two of these, $M_{93}$ and $M_{158}$, contain single point mutations corresponding to each of the sites of the compound FLD mutant $M_{93,158}$. $M_{93,158}$, $M_{93}$, and $M_{158}$ were similar in secretion and specific activity. The ratios of the activities of $M_{93,158}$ and $M_{93}$ were also similar but lower than that of $M_{158}$. This result largely reflects the much lower activities of $M_{93,158}$ and $M_{93}$ against LDL. Thus,

most of the biochemical effects that distinguish $M_{93,158}$ from the wild-type LCAT are associated with $M_{93}$ and not with $M_{158}$. This conclusion is consistent with the report of Hill et al. (35), who also studied this mutant using COS-1 cells.

Clinically, FED appears to be a mild form of FLD in which corneal opacities in late life are the primary symptoms. It is tempting to think of FED as a mild form of FLD and to look among the in vitro FED and FLD mutants for biochemical characteristics that would differentiate between the two diseases. As measured using R-HDL as the substrate, the total cholesterol-esterifying activity secreted by either FED or FLD transfectants was not more than 11% of that of wild-type LCAT. With only one exception, very low secreted activity due to a combination of poor secretion and low specific activity distinguishes FLD from FED. Also, the relative activity against LDL was higher among the FED mutants than among the FLD mutants. None of the FED mutants was characterized by greatly impaired secretion.

It is possible that FED and FLD are the same disease at different levels of LCAT impairment. There are a number of explanations for the absence of a distinct correlation between the biochemistry of FED and FLD LCAT and the severity of the attendant diseases. First, the effects of FED and FLD mutations may be modulated through interactions with other genes or with diet. As pointed out by Klein et al. (36), there is a great deal of heterogeneity in the expression of clinical manifestations of LCAT deficiency. Second, COS-6 cells may not be a good physiological model for the secretion of a hepatically derived enzyme. Third, the number of reported FED and FLD cases is too small to support a statistically significant correlation. Finally, the levels of plasma LCAT found in FED and FLD are a function of both secretion and turnover rates, and an in vitro expression system cannot be used to address questions relating to differences in the rates of plasma LCAT catabolism. More definitive answers to mechanistic questions will require the expression and isolation of larger amounts of normal and mutant LCAT that may be compared in vivo and by physicochemical methods.

This work was supported by a grant-in-aid from the National Institutes of Health (HL-30914) and the Welch Foundation (Q906). FBV was partially supported by a fellowship from de Fondo de Investigación Sanitaria (Spain), FIS 88/2876.

*Manuscript received 23 September 1993 and in revised form 31 January 1995.*

## REFERENCES

1. Fielding, C. J. 1990. Lecithin:cholesterol acyltransferase. *In* Advances in Cholesterol Research. M. Esfahani and J. B. Swaney, editors. The Telford Press, Caldwell, NJ 271–314.
2. Jonas, A. 1991. Lecithin:cholesterol acyltransferase in the metabolism of high-density lipoproteins. *Biochim. Biophys. Acta.* **1084:** 205–220.
3. 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.
4. Subbaiah, P. V. 1986. Lysolecithin acyltransferase of human plasma: assay and characterization of enzyme activity. *Methods Enzymol.* **129:** 790–797.
5. 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.
6. Chong, K. S., M. Janani, S. Hara, and A. G. Lacko. 1983. Characterization of lecithin:cholesterol acyltransferase from human plasma. 3. Chemical properties of the enzyme. *Can. J. Biochem. Cell Biol.* **61:** 875–881.
7. Collet, X., and C. J. Fielding. 1991. Effects of inhibitors of N-linked oligosaccharide processing of the secretion, stability, and activity of lecithin:cholesterol acyltransferase. *Biochemistry.* **30:** 3228–3234.
8. McLean, J., C. Fielding, D. Drayna, H. Dieplinger, B. Baer, W. Kohr, W. Henzel, and R. Lawn. 1986. Cloning and expression of human lecithin:cholesterol acyltransferase cDNA. *Proc. Natl. Acad. Sci. USA.* **83:** 2335–2339.
9. Aron, L., S. Jones, and C. J. Fielding. 1978. Human plasma lecithin:cholesterol acyltransferase. Characterization of cofactor-dependent phospholipase activity. *J. Biol. Chem.* **253:** 7220–7226.
10. Carlson, L. A., and L. Holmquist. 1985. Evidence for the presence in human plasma of lecithin:cholesterol acyltransferase activity ($\beta$-LCAT) specifically esterifying free cholesterol of combined pre-$\beta$- and $\beta$-lipoproteins. Studies of fish eye disease patients and control subjects. *Acta Med. Scand.* **218:** 197–205.
11. Carlson, L. A., and L. Holmquist. 1985. Evidence for deficiency of high density lipoprotein lecithin:cholesterol acyltransferase activity ($\alpha$-LCAT) in fish eye disease. *Acta Med. Scand.* **218:** 189–196.
12. Frohlich, J., and R. McLeod. 1986. Lecithin:cholesterol acyltransferase (LCAT) deficiency syndromes. *In* Lipoprotein Deficiency Syndromes. A. Angel and J. Frohlich, editors. Plenum Press, New York, NY. 181–194.
13. Assmann, G., A. von Eckardstein, and H. Funke. 1991. Lecithin:cholesterol acyltransferase deficiency and fish-eye disease. *Curr. Opin. Lipidol.* **2:** 110–117.
14. Norum, K. R., E. Gjone, and J. A. Glomset. 1989. Familial lecithin:cholesterol acyltransferase, 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 Inc., New York, NY. 1181–1194.
15. McLean, J. W. 1992. Molecular defects in the lecithin:cholesterol acyltransferase gene. *In* High Density Lipoproteins and Atherosclerosis III. N. E. Miller and A. R. Tall, editors. Elsevier Science Publishers, Amsterdam, The Netherlands, 59–65.
16. Funke, H., A. von Eckardstein, P. H. Pritchard, J. J. Albers, J. J. Kastelein, C. Droste, and G. Assmann. 1991. A molecular defect causing fish eye disease: an amino acid exchange in lecithin:cholesterol acyltransferase (LCAT) leads to the selective loss of $\alpha$-LCAT activity. *Proc. Natl. Acad. Sci. USA.* **88:** 4855–4859.
17. Frohlich, J., R. McLeod, P. H. Pritchard, J. Fesmire, and W. McConathy. 1988. Plasma lipoprotein abnormalities in heterozygotes for familial lecithin:cholesterol acyltransfer-

ase deficiency. *Metabolism.* **37:** 3-8.
18. Gordon, D. J., and B. M. Rifkind. 1989. High-density lipoprotein-the clinical implications of recent studies. *N. Engl. J. Med.* **321:** 1311-1316.
19. Tall, A. R. 1990. Plasma high density lipoproteins. Metabolism and relationship to atherogenesis. *J. Clin. Invest.* **86:** 379-384.
20. 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.
21. Qu, S-J., H-Z. Fan, F. Blanco-Vaca, and H. J. Pownall. 1993. Roles of cysteines on human lecithin:cholesterol acyltransferase. *Biochemistry.* **32:** 3089-3094.
22. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* **82:** 488-492.
23. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* **74:** 5463-5467.
24. 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.
25. Selden, R. F., K. B. Howie, M. E. Rowe, H. M. Goodman, and D. D. Moore. 1986. Human growth hormone as a reporter gene in regulation studies employing transient gene expression. *Mol. Cell Biol.* **6:** 3173-3179.
26. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* **18:** 5294-5299.
27. Okayama, H., M. Kawaichi, M. Brownstein, F. Lee, T. Yokota, and K. Arai. 1987. High-efficiency cloning of full-length cDNA; construction and screening of cDNA expression libraries for mammalian cells. *Methods Enzymol.* **154:** 3-28.
28. Matz, C. E., and A. Jonas. 1982. Miscellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. *J. Biol. Chem.* **257:** 4535-4540.
29. Pownall, H. J., W. B. Van Winkle, Q. Pao, M. Rohde, and A. M. Gotto, Jr. 1982. Action of lecithin:cholesterol acyltransferase on model lipoproteins: preparation and characterization of model nascent high density lipoprotein. *Biochim. Biophys. Acta.* **713:** 494-503.
30. Skretting, G., and H. Prydz. 1992. An amino acid exchange in exon I of the human lecithin:cholesterol acyltransferase (LCAT) gene is associated with fish eye disease. *Biochem. Biophys. Res. Commun.* **182:** 583-587.
31. Glomset, J. A., K. R. Norum, and W. King. 1970. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: lipid composition and reactivity in vitro. *J. Clin. Invest.* **49:** 1827-1837.
32. Francone, O., and C. J. Fielding. 1991. Effects of site-directed mutagenesis at residues cysteine-31 and cysteine-184 on lecithin:cholesterol acyltransferase activity. *Proc. Natl. Acad. Sci. USA.* **88:** 1716-1720.
33. Agnani, G., and Y. L. Marcel. 1993. Cholesterol efflux from fibroblasts to discoidal lipoproteins with apolipoprotein A-I (LpA-I) increases with particle size but cholesterol transfer from LpA-I to lipoproteins decreases with size. *Biochemistry.* **32:** 2643-2649.
34. O, K., J. S. Hill, X. Wang, and P. H. Prichard. 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.
35. Hill, J. S., K. O., X. Wang, and P. H. Pritchard. 1993. Lecithin:cholesterol acyltransferase deficiency: identification of a causative gene mutation and a co-inherited protein polymorphism. *Biochim. Biophys. Acta.* **1181:** 321-323.
36. 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.