A 5' splice-region mutation and a dinucleotide deletion in the lysosomal acid lipase gene in two patients with cholesteryl ester storage disease

Detlev Ameis,[1,*] Gerald Brockmann,[*] Rupert Knoblich,[*] Martin Merkel,[*] Richard E. Ostlund, Jr.,[†] Joseph W. Yang,[†] Paul M. Coates,[**] Jean A. Cortner,[**] S. Victor Feinman,[††] and Heiner Greten[*]

Department of Medicine,[*] University Hospital Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany; Department of Internal Medicine,[†] Washington University School of Medicine, St. Louis, MO 63110; The Children's Hospital of Philadelphia,[**] Philadelphia, PA 19104; and Department of Medicine,[††] University of Toronto, Toronto, Ontario M5G 1X5 Canada

Abstract Cholesteryl ester storage disease (CESD) results from inherited deficiencies of the lysosomal hydrolase, acid lipase (LAL; E.C. 3.1.1.13). To establish the molecular defects in LAL deficiency, two unrelated probands with severely reduced LAL activity were examined. DNA amplification by reverse-transcription polymerase chain reaction and subsequent sequence analysis of LAL cDNA identified two mutant alleles. Patient 1, presenting with hepatosplenomegaly, mildly elevated liver function tests, and hyperlipidemia, was homozygous for a deletion of nucleotides 823 to 894 of the LAL cDNA. This 72-bp deletion maintained the reading frame and resulted in a loss of 24 amino acids from the LAL protein. Analysis of genomic DNA revealed that the 72 bp corresponded to an exon of the LAL gene. A single G to A point mutation at the last exon position was observed in the genomic DNA of patient 1, indicating a splicing defect with consecutive exon skipping underlying the 72-bp deletion. Patient 2 was a compound heterozygote for the 72-bp deletion and a dinucleotide deletion at positions 967 and 968. This deletion resulted in a shifted reading frame carboxy-terminal of codon 296, and 43 random amino acids followed the frame shift. A premature stop at codon 339 truncated the mutant LAL protein by 34 amino acids. Allele-specific hybridization confirmed that patient 1 was homozygous for the 72-bp deletion mutation, and that patient 2 was a compound heterozygote for the 72-bp deletion and the 2-bp deletion.—**Ameis, D., G. Brockmann, R. Knoblich, M. Merkel, R. E. Ostlund, Jr., J. W. Yang, P. M. Coates, J. A. Cortner, S. V. Feinman, and H. Greten.** A 5' splice-region mutation and a dinucleotide deletion in the lysosomal acid lipase gene in two patients with cholesteryl ester storage disease. *J. Lipid Res.* 1995. **36:** 241–250.

Supplementary key words cholesterol metabolism • lysosome • lipase deficiency • exon loss • splice-site mutation

Lysosomal acid lipase (LAL, EC 3.1.1.13) plays a central role in the intracellular degradation of neutral lipids derived from plasma lipoproteins (1). The enzyme hydrolyzes cholesteryl esters and triacylglycerols, releasing cholesterol and fatty acids. Cholesterol liberated is transferred to the cytosol where it participates in cellular sterol homeostasis (2). LAL is synthesized by virtually all cells and tissues of the human body, including liver cells, fibroblasts, macrophages, and lymphocytes (reviewed in ref. 3). Human fibroblast and liver LAL were purified to apparent homogeneity, full-length LAL cDNA clones were isolated, and their DNA sequence was determined (4, 5). The gene for fibroblast LAL is localized on chromosome 10q23.2–23.3 (6). The coding region of liver LAL was found to be virtually identical to fibroblast LAL. However, peptide sequencing of purified liver LAL indicated that the amino terminus of the mature proteins differed significantly (5). cDNA sequence comparisons of hepatic LAL to known lipases revealed significant amino acid similarities with human gastric lipase (7) and rat lingual lipase (8), establishing the three enzymes as members of a gene family of acid lipases. Apart from a region highly conserved in all lipases analyzed (9), no similarity was found with neutral lipases, i.e., lipoprotein lipase, hepatic lipase, and pancreatic lipase, suggesting different gene families of acid and neutral lipases (10). Expression studies have revealed striking differences in LAL mRNA levels among human tissues. Brain, lung, mammary gland, kidney, and adrenal showed high levels of LAL expression, while liver, heart, and skeletal muscle expressed only a small amount of LAL mRNA (5).

Abbreviations: CESD, cholesteryl ester storage disease; LAL, lysosomal acid lipase; RT-PCR, reverse-transcription polymerase chain reaction; bp, base pair; apoB, apolipoprotein B.

[1]To whom correspondence should be addressed.

In humans, the inherited deficiency of LAL activity results in two autosomal recessive disorders of lipid metabolism, cholesteryl ester storage disease (CESD) and Wolman disease (11). In both disorders, the enzyme deficiency leads to a progressive accumulation of cholesteryl esters and triacylglycerols in lysosomes of affected tissues (12–14). Clinically, CESD frequently remains unrecognized until adulthood when hepatomegaly develops. In some cases hepatomegaly evolves to hepatic fibrosis (15) or frank liver cirrhosis (16, 17). Plasma lipoprotein analysis reveals elevated total plasma cholesterol and low density lipoprotein levels, accounting for the premature atherosclerosis observed in some patients with CESD (11). In Wolman disease, a disorder with a brief and severe clinical course, affected infants present with massive hepatosplenomegaly, failure to thrive, adrenal calcifications, and usually succumb to hepatic and adrenal failure at age 3 to 12 months (14). Reduced or absent enzymatic activity of LAL in peripheral blood leukocytes, liver biopsies, or cultured skin fibroblasts establishes the diagnosis of LAL deficiency (12–14).

The availability of LAL-specific cDNA clones has allowed direct investigation of the molecular basis of LAL deficiency. In the present study we sought to identify mutations affecting the gene for LAL in two unrelated individuals with clinically and biochemically diagnosed CESD. We report that a single G to A substitution at position −1 of a 5' splice site leads to aberrant splicing and exon skipping with loss of enzymatic activity in one patient with CESD. This genomic mutation was identical to a recently described mutation in another CESD kindred (18), suggesting either a common ancestry of the afflicted families or a recurrent mutation in unrelated individuals. Another unrelated patient analyzed in this report was a compound heterozygote for the splice-region mutation and a dinucleotide deletion leading to a shifted reading frame with corresponding nonsense amino acids and a truncation of the LAL protein.

MATERIALS AND METHODS

Subjects

We studied two unrelated patients with CESD. Patient 1 (T. F.) was from a family of eastern European ancestry, and presented with hepatosplenomegaly, mildly elevated liver function tests, hypercholesterolemia, and hypertriglyceridemia at age 13 (ref. 19). The diagnosis of CESD was confirmed by greatly increased cholesteryl ester content of a liver biopsy and by decreased LAL activity in peripheral leukocytes. The subject had one healthy brother; another brother died at the age of 1 day of unknown reasons. The subject is being treated with the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor lovastatin (19). Patient 2 (P.W.), a 38-year-old white male, presented at the age of 10 months with hepatosplenomegaly. He experienced bouts of right upper quadrant pain from early childhood. Hypercholesterolemia and hypertriglyceridemia were noted at age 21. Liver biopsy at age 23 showed widespread vacuolated hepatocytes. LAL deficiency was diagnosed in liver biopsy and cultured skin fibroblasts. The proband has a 49-year-old brother who was also diagnosed with CESD. Only the proband was available for the present study.

Preparation of RNA, DNA, and oligonucleotide primers

RNA was isolated from fibroblast or Epstein-Barr-virus-transformed lymphoblast cell lines by the guanidinium isothiocyanate method (20). Genomic DNA was extracted either from peripheral blood mononuclear cells or from established cell lines as described (21). Oligonucleotides were synthesized based on sequences of human liver LAL (5) (**Table 1**). In the amplification, one primer was biotinylated at the 5' end (22) to facilitate later purification for solid-state sequencing. The other primer had a 17-base 5' extension containing the bacteriophage M13 universal primer recognition site.

Reverse transcription and PCR amplification of LAL cDNA

LAL cDNAs from control and LAL-deficient subjects were synthesized by incubation of 2 μg of fibroblast or lymphoblast total RNA with 200 units of SuperScript RNase H^- reverse transcriptase (Gibco/BRL) and 20 pmol of primer hLAL1626U (**Table 2**). After incubation at 37°C for 1 h, 1/10 vol of the newly generated cDNA was amplified (23) in a 100-μl reaction volume containing 5 pmol of each oligonucleotide primer; 200 μM each dATP, dCTP, dGTP, and dTTP; 1 × reaction buffer (50 mM KCl, 10 mM Tris • HCl at pH 8.4, 2.5 mM $MgCl_2$, 0.05% polysorbate (Tween) 20, 0.05% Triton X-100, 200 μg/ml gelatin); and 2 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany). A programmable thermocycler (Perkin-Elmer Cetus) was used to perform 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. The final extension time was 10 min at 72°C.

Electrophoresis of amplified products, subcloning and DNA sequence analysis

PCR products were analyzed by electrophoresis on 1% SeaKem agarose gels (FMC Corp.) in Tris-borate buffer containing 0.5 μg/ml ethidium bromide and visualized with UV light (24). For direct solid-phase sequencing of the PCR products, biotinylated DNA strands were purified with steptavidin-coated magnetic beads (22) (Dynabeads M-280 Streptavidin, Dynal, Oslo, Norway). Sequencing was performed with the Taq Cycle Sequencing Kit according to the supplier (Applied Biosystems) with

TABLE 1. Clinical and laboratory data of patients

Variable	Patient 1	Patient 2
Age	21 yr	38 yr
Age at presentation	12 yr	10 months
Hepatomegaly	+	+
Premature coronary artery disease	–	–
Cholesterol (mmol/l)	10.1[a]	7.8
Triglycerides (mmol/l)	2.9[a]	2.7
HDL cholesterol (mmol/l)	0.7	n.d.
ApoB (mg/dl)	242	n.d.
ApoA-I (mg/dl)	76	n.d.
ApoA-II (mg/dl)	38.6	n.d.
ApoE genotype[b]	3/3	3/3
LAL activity (nmol FFA × min^{-1} × mg^{-1} protein)	0.96[c]	0.18[d]
LAL activity (nmol 4-MUB × min^{-1} × mg^{-1} protein)	n.d.	0.45[e]
	n.d.	0.5[f]
	n.d.	0.3[g]

Abbreviations: apoB, apolipoprotein B; apoA, apolipoprotein A; apoE, apolipoprotein E; LAL, lysosomal acid lipase, FFA, free fatty acids; 4-MUB, 4-methylumbelliferone; n.d., not determined.

[a] Mean of three determinations prior to therapy with lovastatin (19).

[b] Determined according to ref. 48.

[c] Normal, 15.65 nmol FFA × min^{-1} × mg^{-1} protein. Determined in peripheral leukocytes according to ref. 49.

[d] Normal, 15.65 nmol FFA × min^{-1} × mg^{-1} protein. Determined in cultured skin fibroblasts as described in ref. 5.

[e] Normal, 7.5 nmol 4-methylumbelliferone × min^{-1} × mg^{-1} protein. Determined in liver biopsy according to ref. 50.

[f] Normal, 5.0–10.0 nmol 4-methylumbelliferone × min^{-1} × mg^{-1} protein. Determined in cultured skin fibroblasts (50).

[g] Normal, 3.0–8.0 nmol 4-methylumbelliferone × min^{-1} × mg^{-1} protein. Determined in isolated lymphocytes (50).

TABLE 2. Synthetic oligonucleotide primers[a] used for amplification, sequence analysis, and allele-specific hybridization

I. Primer for cDNA synthesis	
hLAL1626U	5'-TGAAAACTGCGTACTTTTATACCT-3'
II. Primers for PCR of cDNA and DNA sequence analysis	
hLAL-40D	5'-ACTGCGACTCGAGACAGCGG-3'
hLAL184D	5'-TGCCTTAACCGAATTCC-3'
hLAL389D	5'-AGACACTCTCAGTTTCT-3'
hLAL568D	5'-CTGGCTAAAAGGATTAA-3'
hLAL585D	5'-AATGTTTTTTGCCCTGG-3'
hLAL790D	5'-CTGTGTGGATTTAATGA-3'
hLAL984D	5'-CAATGTGAAGGACATGC-3'
hLAL1092D	5'-GTTCCATGAGAGCATTC-3'
hLAL268U	5'-CCAGCAAGCCATGTTGC-3'
hLAL468U	5'-AATGGAAGCTGGTAGGT-3'
hLAL665U	5'-TGATCTGGTAATCGTCC-3'
hLAL872U	5'-TGCACAGAAGTTCCAGC-3'
hLAL1069U	5'-GTAAGATATTGACGTCGTAGAC-3'
hLAL1244U	5'-TGACATAATCATTGACT-3'
hLAL1481U	5'-ATAATGACCTTTTTACA-3'
II. Primers for PCR of genomic DNA	
hLAL5'In101D	5'-GGCTCTAGTTTTTAGTGCTTTG-3'
hLAL3'In67U	5'-TTTGCATTCCCAGACCTTTCTG-3'
III. Allele-specific oligonucleotides for hybridization	
hLAL872U	5'-TGCACAGAAGTTCCAGC-3'
hLAL815D(Δ823–894)[b]	5'-TAAATATGGCTGTTAAA-3'
hLAL959D[c]	5'-ACAACCAGAGTTATCCT-3'
hLAL958D(Δ967–968)	5'-TACAACCAGTTATCCTC-3'

[a] Nucleotide sequence numbering refers to the 5' nucleotide of respective oligonucleotide and is based on human hepatic LAL (5). D and U denote plus strand and complementary strand orientation of the oligonucleotide, respectively.

[b] Oligonucleotide hLAL815D(Δ823–894) comprises nucleotides 815 to 822 and 895 to 903.

[c] The AG dinucleotide of the normal allele which is deleted in the mutant allele is underlined.

fluorochrome-labeled M13 universal primers and analyzed on an Applied Biosystems 373A DNA sequencer. For subcloning of PCR products from patient 2, RT-PCR was performed with oligonucleotide primer hLAL-40D and hLAL1244U. The resulting PCR products were gel-purified, and the two alleles were separately subjected to blunt end ligation in puC18 linearized with *Sma* I. As a control, the cDNA clone hLAL4/1 containing the full-length human liver LAL cDNA (5) was amplified and sequenced in the same manner.

Partial genomic cloning of LAL gene

A human cosmid library constructed in pWE15 (Stratagene) (25) was screened with a full-length human liver LAL cDNA (5) as described (26). Of approximately 1×10^6 recombinants screened, four clones hybridized with a ^{32}P-labeled (27) human LAL cDNA probe. Positive clones were purified by two rounds of colony hybridization (24). Cosmid DNA was digested with several restriction enzymes, fractionated on 1% agarose gels, and analyzed by blot hybridization. Fragments containing human LAL-specific fragments were gel-purified and subcloned in pBS-KS (Stratagene). DNA sequencing of double-stranded DNA was performed as described above. Sequence analysis was performed by manual assignment of exon–intron junctions or by sequence comparison using the program Tetra (SoftGene, Berlin, FRG).

Allele-specific oligonucleotide hybridization

One-tenth of the PCR products (10 μl) generated with primers hLAL568D and hLAL1244U was immobilized on nitrocellulose membranes (Schleicher and Schüll). The filters were prehybridized in hybridization buffer (0.3 M NaCl, 0.03 M sodium citrate, 0.12 M sodium phosphate, 4 × Denhardt's solution, 0.1% $NaDodSO_4$, 1% N-lauryl sarcosine, 1 mM EDTA, 100 μg/ml denatured salmon sperm DNA) at 41°C for 4 h. They were then hybridized with the respective allele-specific oligonucleotides labeled with [γ-^{32}P]ATP by polynucleotide kinase (24). Oligonucleotides for the normal alleles were hLAL872U and hLAL959D; for the mutant alleles, hLAL815D(Δ823–894) and hLAL958D(Δ967–968), respectively (Table 1). Hybridization was at 41°C for all used oligonucleotides for 16 h. The filters were washed in 0.3 M NaCl, 0.03 M sodium citrate at 22°C for 30 min. Subsequently, the filters were washed in 0.3 M NaCl, 0.03 M sodium citrate at 41°C. Filters were air-dried and exposed to Kodak XAR film for 2 h.

RESULTS

To determine the molecular basis of familial CESD, we assessed the structure of the LAL gene in two unrelated subjects with primary LAL deficiency. The enzyme deficiency was diagnosed by a reduced intracellular acid lipase activity in fibroblast or lymphoblast cell lines from the two patients. To examine the LAL gene in detail, mRNA was extracted from lymphoblast or fibroblast cell lines of the two patients and relatives, and reverse-transcription with an LAL-specific oligonucleotide primer in conjunction with PCR amplification (RT-PCR) was performed. Oligonucleotides (Table 2) were synthesized for PCR amplification covering the complete coding region of the LAL cDNA (**Fig. 1A**). The length of the fragments obtained from the patient's total RNA was compared with that of the corresponding control fragment. Agarose gel electrophoresis of RT-PCR products revealed a reduced fragment size in patient 1, suggesting a deletion in the LAL cDNA (Fig. 1B). Both parents showed two products of normal and reduced fragment size, respectively, indicating heterozygosity for the observed deletion.

Analysis of RT-PCR products of patient 2 revealed two fragments, suggesting that he was heterozygous for the same or a similar deletion as patient 1 (Fig. 1B). RT-PCR using various oligonucleotide primers in combination with primer hLAL1244U was performed to localize the deletion to the region between nucleotides 790 and 984 (not shown). For subsequent DNA sequence analysis, each pair of oligonucleotides consisted of one member with a 5' biotin modification to facilitate purification on Streptavidin-coated Dynabeads. The other oligonucleotide was synthesized with a 5' extension of 17 bases complementary to the universal M13 primer recognition site. Using this strategy, the complete LAL cDNA sequence was determined on both DNA strands using fluorochrome-labeled M13 primers. When compared to a control subject or to the cloned LAL cDNA sequence (5), a 72-bp deletion mutation was observed in patient 1 (**Fig. 2A**). The deletion encompassing nucleotides 823 through 894, referred to as the Δ823–894 mutation, preserved the reading frame of the LAL cDNA. It resulted in a loss of 24 amino acids at codons 248 to 271 of the mature LAL protein.

To investigate the genomic alterations leading to the 72-bp deletion, the genomic structure in the vicinity of the deletion was established. Exon–intron junctions were determined by DNA sequencing and revealed that the 72-bp region encompassing the deletion in patient 1 precisely corresponded to an exon of the LAL gene. Intron-specific oligonucleotide primers were derived from 5' and 3' flanking sequences and utilized to specifically amplify genomic DNA of patient 1. DNA sequence analysis revealed the presence of the 72-bp exon in the patient's genomic DNA, excluding a deletion in the LAL gene responsible for the cDNA deletion. Comparison of the patient's exon and flanking 5' and 3' intron sequences with a normal control identified a single nucleotide substitution at the last exon position, changing the wild-type G residue to A. This transition occurred in the third base of codon 271, and

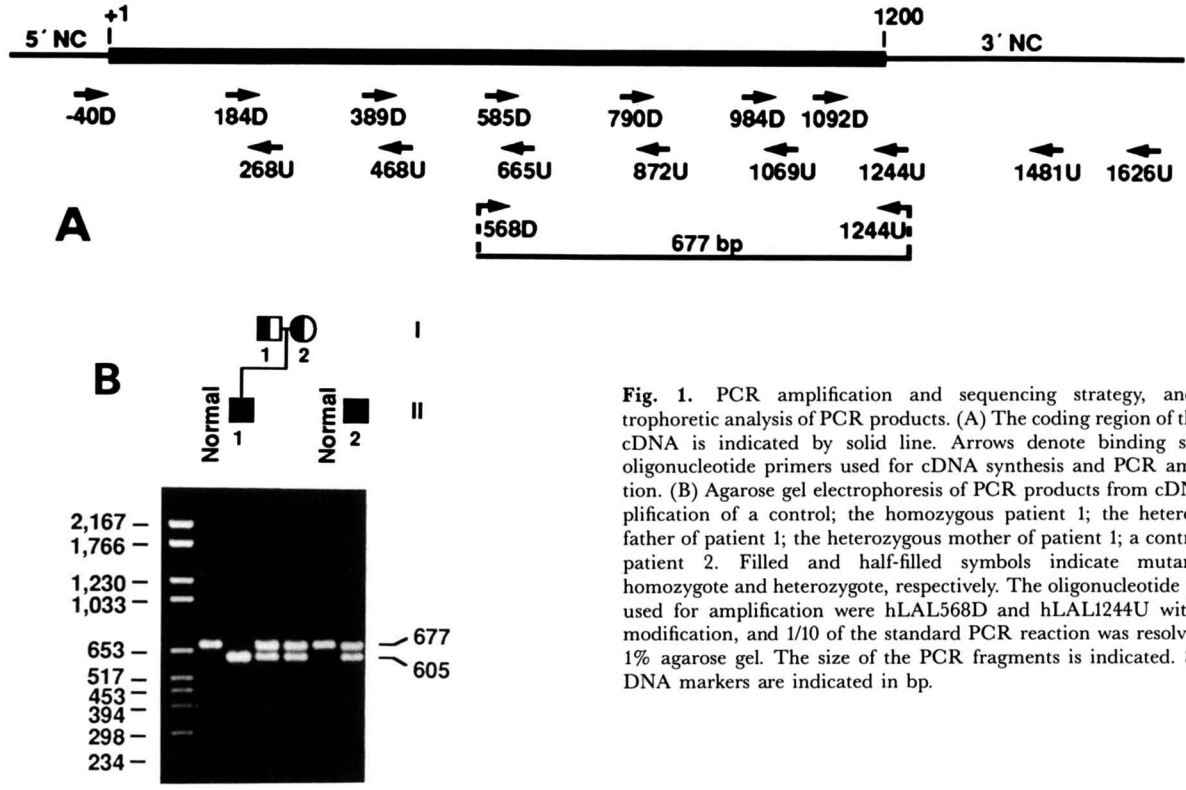

Fig. 1. PCR amplification and sequencing strategy, and electrophoretic analysis of PCR products. (A) The coding region of the LAL cDNA is indicated by solid line. Arrows denote binding sites for oligonucleotide primers used for cDNA synthesis and PCR amplification. (B) Agarose gel electrophoresis of PCR products from cDNA amplification of a control; the homozygous patient 1; the heterozygous father of patient 1; the heterozygous mother of patient 1; a control; and patient 2. Filled and half-filled symbols indicate mutant-allele homozygote and heterozygote, respectively. The oligonucleotide primers used for amplification were hLAL568D and hLAL1244U without 5' modification, and 1/10 of the standard PCR reaction was resolved on a 1% agarose gel. The size of the PCR fragments is indicated. Sizes of DNA markers are indicated in bp.

resulted in a translationally silent change from CAG to CAA (Fig. 2B). The location of the G to A substitution at the −1 position of the 5' splice donor site strongly suggested a splicing defect with a consecutive skipping of the respective exon leading to the 72-bp deletion in the LAL cDNA of patient 1.

Mutant alleles responsible for the LAL deficiency in patient 2 were assessed after RT-PCR with oligonucleotides hLAL-40D and hLAL1244U and subcloning of PCR products in puC18. Two clones for each allele were sequenced individually. Allele 1 showed the Δ823–894 mutation as detected in patient 1 (Fig. 2A). Allele 2 carried a deletion of residues A and G at positions 967 and 968, referred to as Δ967–968 mutation (**Fig. 3**). All mutations were confirmed by determining the DNA sequences for the complementary DNA strands.

Dot blot hybridization with allele-specific oligonucleotides was used to verify the deletion mutations and to identify heterozygous carriers of the Δ823–894 mutation (**Fig. 4**). Hybridization with wild-type oligonucleotide hLAL872U or mutation-specific oligonucleotide hLAL815D (Δ823–894) confirmed that patient 1 was homozygous for the Δ823–894 mutation. Both parents were heterozygous for the mutant allele. Allele-specific hybridization demonstrated that patient 2 was heterozygous for the Δ823–894 mutation. Hybridization with wild-type oligonucleotide hLAL959D or the deletion-specific oligonucleotide hLAL958D(Δ967–968) showed that allele 2 of patient 2 carried the Δ967–968 dinucleotide deletion.

The effects of both mutations on the LAL coding sequence are depicted in **Fig. 5.** In allele 1, the in-frame deletion of 72 bp leads to a loss of 24 amino acids and thus reduces the mature lipase protein to 348 amino acids. In allele 2, the Δ967–968 mutation results in a shifted reading frame starting at codon 296. The predicted LAL protein contains 43 random amino acids at the carboxy terminus. At codon 339, a premature stop codon occurs and truncates the lipase by 34 amino acids.

DISCUSSION

We have analyzed the complete coding region of the LAL gene in two unrelated subjects with CESD. Two deletion mutations were identified as responsible for the LAL deficiency. One deletion, Δ823–894, for which patient 1 was homozygous, resulted in the loss of 24 amino acids at codons 248 to 271. DNA sequence analysis of genomic DNA revealed that nucleotides 823 to 894 precisely represented an exon of the LAL gene. Comparison of normal and patient DNA sequences of exon and flanking intron sequences detected a single G to A transition mutation at the last position of the exon. This substitution

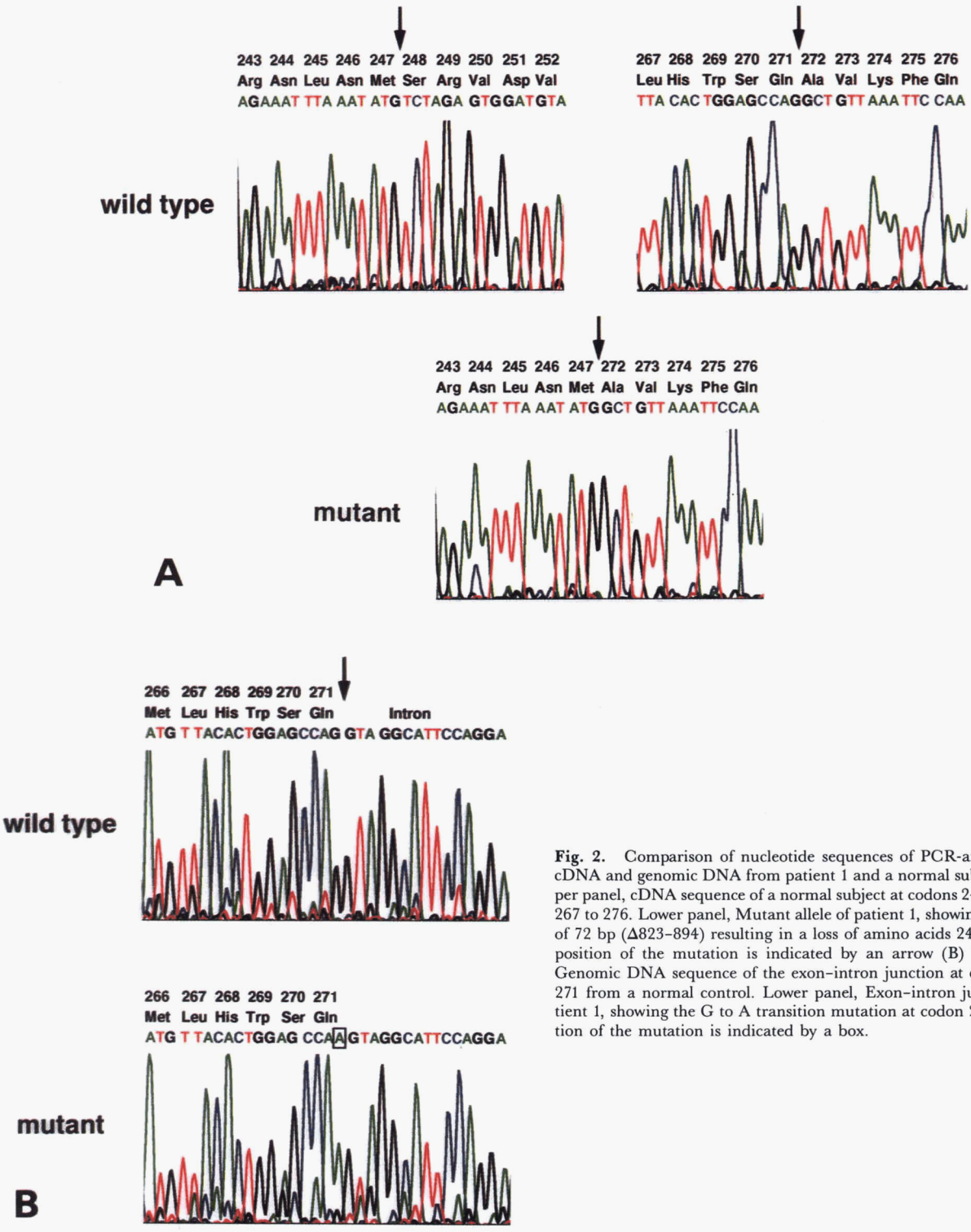

Fig. 2. Comparison of nucleotide sequences of PCR-amplified LAL cDNA and genomic DNA from patient 1 and a normal subject. (A) Upper panel, cDNA sequence of a normal subject at codons 243 to 252, and 267 to 276. Lower panel, Mutant allele of patient 1, showing the deletion of 72 bp (Δ823–894) resulting in a loss of amino acids 248 to 271. The position of the mutation is indicated by an arrow (B) Upper panel, Genomic DNA sequence of the exon–intron junction at codons 266 to 271 from a normal control. Lower panel, Exon–intron junction of patient 1, showing the G to A transition mutation at codon 271. The position of the mutation is indicated by a box.

was not found in four control individuals. It strongly suggested aberrant splicing of the patient's genomic DNA with exon skipping and a consecutive loss of the respective exon from the mRNA. Interestingly, a recent study of a family of Polish–German ancestry with CESD revealed an identical splice-site mutation in the LAL gene (18). As patient 1 of the present study was also of eastern European ancestry, a founder effect might be responsible for this exon loss mutation. Another possibility is that a recurrent mutation in the LAL gene lead to the splice-site defect in unrelated individuals.

Mutations in comparable exon positions and with simi-

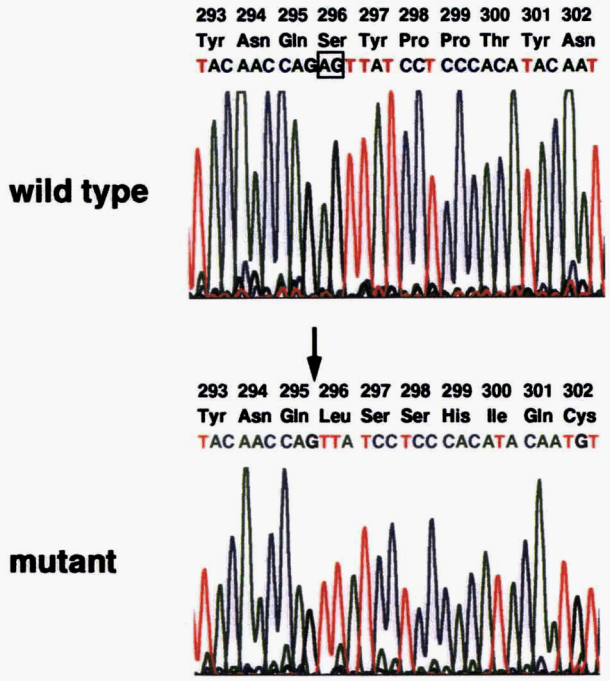

Fig. 3. Comparison of nucleotide sequences of the normal and mutant LAL cDNA from patient 2. Upper panel, DNA sequence of a normal subject at codons 293 to 302. The wild type AG dinucleotide is indicated by a box. Lower panel, DNA sequence of patient 2 at codons 293 to 302. An AG dinucleotide deletion (Δ967–968) leads to a shifted reading frame at codon 296. The position of the frame shift is indicated by an arrow.

lar effects have been defined in cerebrotendinous xanthomatosis (28), protein C deficiency (29), Tay-Sachs disease (30), Ehlers-Danlos syndrome type VII (31), acute intermittent porphyria (32), and β^+-thalassemia (33), emphasizing the frequency and importance of this type of splice-site defects. Further studies are required to show whether the deleted LAL mRNA is transcribed in normal amounts or is prematurely degraded due to mRNA instability.

To confirm the observed mutations and to detect heterozygote carriers of the altered alleles, allele-specific oligonucleotide hybridization was used. In accordance with an autosomal recessive mode of inheritance for LAL deficiency, both parents of patient 1 were heterozygous for the Δ823–894 deletion. The second subject studied was also found to carry the Δ823–894 deletion on one allele. Sequence analysis of allele 2 of this patient revealed a 2-bp deletion, Δ967–968. This dinucleotide deletion resulted in a shifted reading frame starting at amino acid 296 followed by 43 random amino acids. A premature stop codon was predicted at position 339, truncating the enzyme by 34 amino acids. Thus, patient 2 was a compound heterozygote for two deletion mutations. As for patient 1, the observed mutations were confirmed in patient 2 by hybridization with allele-specific oligonucleotides. Both deletion mutations are likely to have major adverse effects on the tertiary structure of the LAL molecule, resulting in a catalytically incompetent protein.

CESD and Wolman disease have both been attributed to deficiencies of LAL enzymatic activity (12–14). However, the precise molecular mechanisms responsible for the striking differences in clinical phenotype are poorly defined. Residual enzymatic activity in the range of 1 to 10 percent of normal LAL activity in the two patients described in this study and previous reports (12, 34, 35) suggests that remaining LAL activity may prevent CESD patients from expressing the more severe phenotype of Wolman disease. Currently, it is unclear whether residual enzymatic activity is due to LAL or an as yet uncharacterized second lipase with similar properties. Partially reduced LAL activity may also arise from mutations resulting in minor changes of enzyme structure that affect various properties, such as enzyme half-life, substrate affinity, or substrate preference. Analysis of a patient with Wolman disease has recently revealed compound heterozygosity for a single-base insertion and a transition mutation in the LAL gene (36). Molecular investigation of the genetic defects in other CESD and Wolman disease patients should help to better understand the allelic variants responsible for these disorders. In vitro expression studies of mutant LAL alleles from CESD and Wolman disease patients will then reveal which types of mutations result in residual enzymatic activity or complete loss of catalytic function.

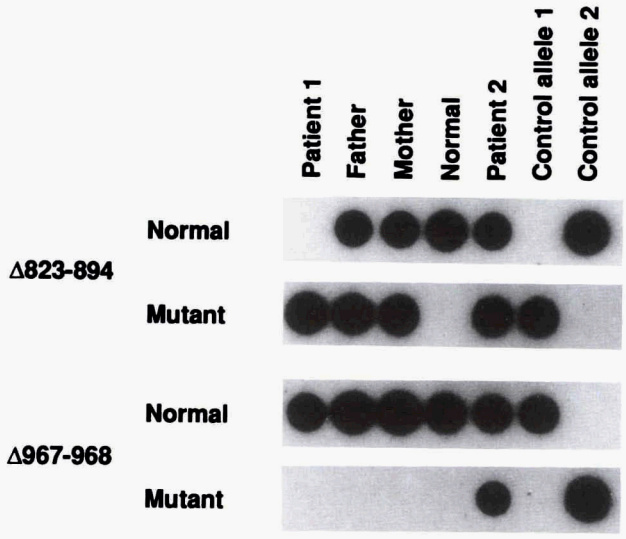

Fig. 4. Analysis of the Δ823–894 and Δ967–968 mutations by allele-specific oligonucleotide hybridization. The cDNA region spanning the deletion mutations was amplified with primers hLAL568D and hLAL1244U as indicated in Fig. 1A, and the products were applied onto nitrocellulose membrane by a dot-blot apparatus. The filters were hybridized to normal oligonucleotide hLAL856U; to mutant oligonucleotide hLAL815D(Δ823–894); to normal oligonucleotide hLAL959D or to mutant oligonucleotide hLAL958D(Δ967–968) as described under Materials and Methods. Control alleles 1 and 2 denote the cloned LAL cDNAs of patients 1 and 2, respectively.

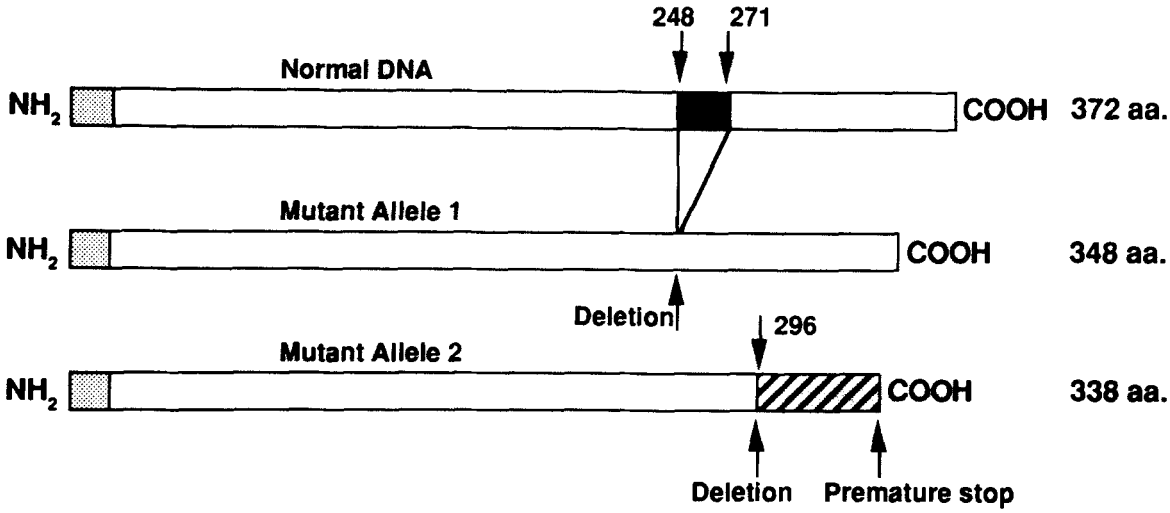

Fig. 5. Schematic representation of the LAL cDNA mutations in two subjects with CESD. The coding sequence of LAL is given by an open box. The signal peptide is indicated by a dotted box. In the mutant allele 1, for which patient 1 is homozygous and patient 2 is heterozygous, an in-frame deletion of 72 bp leads to a loss of codons 248 to 271, reducing the size of the mature lipase to 348 amino acids. In the mutant allele 2, for which patient 2 is heterozygous, a deletion of nucleotides 967 and 968 results in a shifted reading frame at codon 296. The resulting lipase protein contains 43 amino acids of random sequence and is truncated prematurely at amino acid 338.

At present, no specific therapy is available to replace defective LAL protein in CESD or Wolman disease. Symptomatic therapy of hypercholesterolemia and hepatomegaly has been attempted in CESD patients, using the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, lovastatin. The clinical response to treatment with lovastatin has been variable, however. Patient 1 of the present study (19) as well as CESD patients in three independent studies responded favorably to a therapy with lovastatin (34, 37, 38). Kinetic studies of lipoproteins in a 9-year-old subject with CESD indicated an elevated production rate of apoB (34). Lovastatin treatment decreased both the plasma LDL cholesterol and the VLDL and LDL apoB production rates, thereby reducing the secretion of apoB-containing lipoproteins (34). In a study of three unrelated CESD patients, no clinical benefit from the lovastatin treatment could be demonstrated (15). Splenomegaly with a splenic abscess resulted in subtotal splenectomy in one patient (39). The patient later developed progressive cirrhosis and portal hypertension and was one of two patients subjected to orthotopic liver transplantation (17, 40). Gaucher's disease, a lysosomal storage disorder caused by a deficiency in glucocerebrosidase, has recently been treated by bone marrow transplantation (41). This therapeutic strategy should also be considered in the severely affected patients with Wolman disease. Recent attempts of bone marrow transplantation in Wolman disease patients have been successful in some of the transplant recipients (42). Whether bone marrow transplantation will be beneficial for some severely affected CESD patients will have to await further studies.

Gene replacement strategies using retroviral-mediated delivery of genes that have been used in vitro or in animal models of lysosomal storage disorders (43, 44) may also be pursued for CESD and Wolman disease. A recently described strain of rats with absent acid lipase activity and striking hepatic accumulation of cholesteryl esters, free cholesterol, and triacylglycerol (45) provides a valuable animal model of LAL deficiency. It should help to establish ex vivo or in vivo gene transfer protocols aimed at correcting the metabolic alterations due to LAL deficiency. This may also contribute to defining the pathogenesis of premature atherosclerosis that has been associated with reduced LAL activity (46, 47).

We gratefully acknowledge the expert technical assistance of M. Schulz and A. Pauer. The help of Dr. R. A. Levy and C. Miller-Owen in obtaining patient specimen is greatly appreciated. This work was supported in part by the Deutsche Forschungsgemeinschaft (Am 65/3-1).

Manuscript received 16 May 1994 and in revised form 31 August 1994.

REFERENCES

1. Goldstein, J. L., S. E. Dana, J. R. Faust, A. L. Beaudet, and M. S. Brown. 1975. Role of lysosomal acid lipase in the metabolism of plasma low density lipoproteins. *J. Biol. Chem.* **250:** 8487–8795.
2. Brown, M. S., P. T. Kovanen, and J. L. Goldstein. 1981. Regulation of plasma cholesterol by lipoprotein receptors. *Science.* **212:** 628–635.
3. Fowler, S. D., and W. J. Brown. 1984. Lysosomal acid lipase. *In* Lipases. B. Borgström and H. L. Brockman, editors. Elsevier, New York. 329–364.

4. Anderson, R. A., and G. N. Sando. 1991. Cloning and expression of cDNA encoding human lysosomal acid lipase/cholesteryl ester hydrolase. *J. Biol. Chem.* **266:** 22479–22484.
5. Ameis, D., M. Merkel, C. Eckerskorn, and H. Greten. 1994. Purification, characterization and molecular cloning of human hepatic lysosomal acid lipase. *Eur. J. Biochem.* **219:** 905–914.
6. Anderson, R. A., N. Rao, R. S. Byrum, C. B. Rothschild, D. W. Bowden, R. Hayworth, and M. Pettenati. 1993. In situ localization of the genetic locus encoding the lysosomal acid lipase/cholesteryl esterase (LIPA) deficient in Wolman disease to chromosome 10q23.2-q23.3. *Genomics.* **15:** 245–247.
7. Bodmer, M. W., S. Angal, G. T. Yarranton, T. J. R. Harris, A. Lyons, D. J. King, G. Pieroni, C. Riviere, R. Verger, and P. A. Lowe. 1987. Molecular cloning of a human gastric lipase and expression of the enzyme in yeast. *Biochim. Biophys. Acta.* **909:** 237–244.
8. Docherty, A. J. P., M. W. Bodmer, S. Angal, R. Verger, C. Riviere, P. A. Lowe, A. Lyons, J. S. Emtage, and T. J. R. Harris. 1985. Molecular cloning and nucleotide sequence of rat lingual lipase cDNA. *Nucleic Acids. Res.* **13:** 1891–1903.
9. Hide, W. A., L. Chan, and W. H. Li. 1992. Structure and evolution of the lipase superfamily. *J. Lipid Res.* **33:** 167–178.
10. Ameis, D., H. Greten, and M. C. Schotz. 1992. Hepatic and plasma lipases. *Semin. Liver Dis.* **12:** 397–402.
11. Schmitz, G., and G. Assmann. 1989. Acid lipase deficiency: Wolman disease and cholesteryl ester storage disease. *In* The Metabolic Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 1623–1644.
12. Sloan, H. R., and D. S. Fredrickson. 1972. Enzyme deficiency in cholesteryl ester storage disease. *J. Clin. Invest.* **51:** 1923–1926.
13. Burke, J. A., and W. K. Schubert. 1972. Deficient activity of hepatic acid lipase in cholesterol ester storage disease. *Science.* **176:** 309–310.
14. Patrick, A. D., and B. D. Lake. 1969. Deficiency of an acid lipase in Wolman's disease. *Nature.* **222:** 1067–1068.
15. Di Bisceglie, A. M., K. G. Ishak, L. Rabin, and J. M. Hoeg. 1990. Cholesteryl ester storage disease: hepatopathology and effects of therapy with lovastatin. *Hepatology.* **11:** 764–772.
16. Beaudet, A. L., G. D. Ferry, B. D. Nichols, Jr., and H. S. Rosenberg. 1977. Cholesterol ester storage disease: clinical, biochemical, and pathological studies. *J. Pediatr.* **90:** 910–914.
17. Ferry, G. D., H. H. Whisennand, M. J. Finegold, E. Alpert, and A. Glombicki. 1991. Liver transplantation for cholesteryl ester storage disease. *J. Pediatr. Gastroenterol. Nutr.* **12:** 376–378.
18. Klima, H., K. Ullrich, C. Aslanidis, P. Fehringer, K. J. Lackner, and G. Schmitz. 1993. A splice junction mutation causes deletion of a 72-base exon from the mRNA of lysosomal acid lipase in a patient with cholesteryl ester storage disease. *J. Clin. Invest.* **92:** 2713–2718.
19. Levy, R., R. E. Ostlund, G. Schonfeld, P. Wong, and C. F. Semenkovich. 1992. Cholesteryl ester storage disease: complex molecular effects of chronic lovastatin therapy. *J. Lipid Res.* **33:** 1005–1015.
20. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
21. Ameis, D., J. Kobayashi, R. C. Davis, O. Ben-Zeev, M. J. Malloy, J. P. Kane, H. Wong, R. J. Havel, and M. C. Schotz. 1991. Familial chylomicronemia (type I hyperlipoproteinemia) due to a single missense mutation in the lipoprotein lipase gene. *J. Clin. Invest.* **87:** 1165–1170.
22. Hultman, T., S. Stahl, E. Hornes, and M. Uhlen. 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucleic Acids. Res.* **17:** 4937–4946.
23. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science.* **239:** 487–491.
24. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. Wahl, G. M., K. A. Lewis, J. C. Ruiz, B. Rothenberg, J. Zhao, and G. A. Evans. 1987. Cosmid vectors for rapid genomic walking, restriction mapping, and gene transfer. *Proc. Natl. Acad. Sci. USA.* **84:** 2160–2164.
26. Ameis, D., G. Stahnke, J. Kobayashi, M. Bücher, G. Lee, J. McLean, M. C. Schotz, and H. Will. 1990. Isolation and characterization of the human hepatic lipase gene. *J. Biol. Chem.* **265:** 6552–6555.
27. Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132:** 6–13.
28. Leitersdorf, E., A. Reshef, V. Meiner, R. Levitzki, S. P. Schwartz, E. J. Dann, N. Berkman, J. J. Cali, L. Klapholz, and V. M. Berginer. 1993. Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous xanthomatosis in Jews of Moroccan origin. *J. Clin. Invest.* **91:** 2488–2496.
29. Soria, J. M., J. Fontcuberta, M. Chillon, M. Borrell, X. Estivill, and N. Sala. 1993. Acceptor splice site mutation in the invariant AG of intron 5 of the protein C gene, causing type I protein C deficiency. *Hum. Genet.* **92:** 506–508.
30. Akli, S., J. Chelly, C. Mezard, S. Gandy, A. Kahn, and L. Poenaru. 1990. A "G" to "A" mutation at position −1 of a 5' splice site in a late infantile form of Tay-Sachs disease. *J. Biol. Chem.* **265:** 7324–7330.
31. Weil, D., M. D'Alessio, F. Ramirez, B. Steinmann, M. K. Wirtz, R. W. Glanville, and D. W. Hollister. 1990. Temperature-dependent expression of a collagen splicing defect in the fibroblasts of a patient with Ehlers-Danlos syndrome type VII. *J. Biol. Chem.* **264:** 16804–16809.
32. Grandchamp, B., C. Picat, F. de Rooij, C. Beaumont, P. Wilson, J. C. Deybach, and Y. Nordmann. 1989. A point mutation G → A in exon 12 of the porphobilinogen deaminase gene results in exon skipping and is responsible for acute intermittent porphyria. *Nucleic Acids. Res.* **17:** 6637–6649.
33. Vidaud, M., R. Gattoni, J. Stevenin, D. Vidaud, S. Amselem, J. Chibani, J. Rosa, and M. Goossens. 1989. A 5' splice-region G → C mutation in exon 1 of the human β-globin gene inhibits pre-mRNA splicing: a mechanism for β^+-thalassemia. *Proc. Natl. Acad. Sci. USA.* **86:** 1041–1045.
34. Ginsberg, H. N., N-A. Le, M. P. Short, R. Ramakrishnan, and R. J. Desnick. 1987. Suppression of apolipoprotein B production during treatment of cholesteryl ester storage disease with lovastatin. *J. Clin. Invest.* **80:** 1692–1697.
35. Desai, P. K., K. H. Astrin, S. N. Thung, R. E. Gordon, M. P. Short, P. M. Coates, and R. J. Desnick. 1987. Cholesteryl ester storage disease: pathologic changes in an affected fetus. *Am. J. Hum. Genet.* **26:** 689–698.

36. Anderson, R. A., R. S. Byrum, P. M. Coates, and G. N. Sando. 1994. Mutations at the lysosomal acid cholesteryl ester hydrolase gene locus in Wolman disease. *Proc. Natl. Acad. Sci. USA.* **91:** 2718–2722.
37. Tarantino, M. D., D. J. McNamara, P. Granstrom, R. D. Ellefson, E. C. Unger, and J. N. Udall, Jr. 1991. Lovastatin therapy for cholesterol ester storage disease in two sisters. *J. Pediatr.* **118:** 131–135.
38. Glueck, C. J., P. Lichtenstein, T. Tracy, and J. Speirs. 1992. Safety and efficacy of treatment of pediatric cholesteryl ester storage disease with lovastatin. *Pediatr. Res.* **32:** 559–565.
39. Edelstein, R. A., M. R. Filiing-Katz, P. Pentchev, A. Gal, R. Chandra, T. Shawker, P. Guzzetta, M. Comly, C. Kaneski, R. O. Brady, and N. Barton. 1988. Cholesteryl ester storage disease: a patient with massive splenomegaly and splenic abscess. *Am. J. Gastroenterol.* **83:** 687–692.
40. Arterburn, J. N., W. M. Lee, R. P. Wood, B. W. Shaw, and R. S. Markin. 1991. Orthotopic liver transplantation for cholesteryl ester storage disease. *J. Clin. Gastroenterol.* **13:** 482–485.
41. Beutler, E. 1991. Gaucher's disease. *N. Engl. J. Med.* **325:** 1354–1360.
42. Krivit, W., D. Freese, K. W. Chan, and R. Kulkarni. 1992. Wolman's disease: a review of treatment with bone marrow transplantation and considerations for the futures. *Bone Marrow Transpl.* **10 (Suppl 1):** 97–101.
43. Suchi, M., T. Dinur, R. J. Desnick, S. Gatt, L. Pereira, E. Gilboa, and E. H. Schuchman. 1992. Retroviral-mediated transfer of the human acid sphingomyelinase cDNA: correction of the metabolic defect in cultured Niemann-Pick disease cells. *Proc. Natl. Acad. Sci. USA.* **89:** 3227–3231.
44. Kyle, J. W., E. H. Birkenmeier, B. Gwynn, C. Vogler, P. C. Hoppe, J. W. Hoffmann, and W. S. Sly. 1990. Correction of murine mucopolysaccharidosis VII by a human beta-glucuronidase transgene. *Proc. Natl. Acad. Sci. USA.* **87:** 3914–3918.
45. Kuriyama, M., H. Yoshida, M. Suzuki, J. Fujiyama, and A. Igata. 1990. Lysosomal acid lipase deficiency in rats: lipid analyses and lipase activities in liver and spleen. *J. Lipid Res.* **31:** 1605–1612.
46. Yatsu, F. M., F. C. Hagemenas, L. C. Manaugh, and T. Galambos. 1980. Cholesteryl ester hydrolase activity in human symptomatic atherosclerosis. *Lipids.* **15:** 1019–1022.
47. Coates, P. M., T. Langer, and J. A. Cortner. 1986. Genetic variation of human mononuclear leukocyte lysosomal acid lipase activity. Relationship to atherosclerosis. *Atherosclerosis.* **62:** 11–20.
48. Wenham, P. R., C. R. Newton, and W. H. Price. 1991. Analysis of apolipoprotein E genotypes by the amplification refractory mutation system. *Clin. Chem.* **37:** 241–244.
49. Koster, J. F., H. Vaandrager, and T. J. C. Van Berkel. 1980. Study of the hydrolysis of 4-methylumbelliferyl oleate by acid lipase and cholesteryl oleate by acid cholesteryl esterase in human leukocytes, fibroblasts, and liver. *Biochim. Biophys. Acta.* **618:** 98–105.
50. Coates, P. M., J. A. Cortner, G. M. Hoffman, and S. A. Brown. 1979. Acid lipase activity of human lymphocytes. *Biochim. Biophys. Acta.* **572:** 225–234.