Compound heterozygosity for a Wolman mutation is frequent among patients with cholesteryl ester storage disease

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Abstract Cholesteryl ester storage disease and Wolman disease are rare autosomal recessive lipoprotein-processing disorders caused by mutations in the gene encoding human lysosomal acid lipase. Thus far we have elucidated the genetic defects in 15 unrelated CESD patients. Seven were homozygotes for the prevalent hLAL exon 8 splice junction mutation which results in incomplete exon skipping, while eight probands were compound heterozygotes for E8SJM and a rare mutation on the second chromosome. In this report, we describe the molecular basis of CESD in three compound heterozygous subjects of Czech and Irish origin. RFLP and DNA sequence analysis revealed that they were heteroallelic for the common $G_{934} \rightarrow A$ substitution in exon 8 of the hLAL gene and a mutation which, if inherited on both alleles, would be expected to result in complete loss of enzyme activity and to cause Wolman disease. In patients A. M. and J. J., two nucleotide deletions in exons 7 and 10 were detected, involving a T at position 722, 723, or 724 and a G in a stretch of five guanosines at positions 1064–1068 of the hLAL cDNA. Both mutations result in premature termination of protein translation at residues 219 and 336, respectively, and in the production of truncated, inactive enzymes. Subject D. H., in contrast, is a compound heterozygote for the $Arg_{44} \rightarrow Stop$ mutation previously described in a French CESD proband. Combined with data in the literature, our results demonstrate that compound heterozygosity for a mutation causing Wolman disease is common among cholesteryl ester storage disease patients.—Lohse, P., S. Maas, P. Lohse, M. Elleder, J. M. Kirk, G. T. N. Besley, and D. Seidel. **Compound heterozygosity for a Wolman mutation is frequent among patients with cholesteryl ester storage disease.** *J. Lipid Res.* **2000.** 41: **23–31.**

Supplementary key words lipid metabolism • lysosomal storage disease • enzyme deficiency • genotype • point mutation

Cholesteryl ester storage disease (CESD) and Wolman disease are rare inherited disorders of intracellular neu-

tral lipid metabolism that result from a marked or complete deficiency of human lysosomal acid lipase/cholesteryl ester hydrolase (hLAL; OMIM 278000; for review see refs. 1 and 2). The enzyme is present in lysosomes of all nucleated cells and catalyzes the hydrolysis of highly hydrophobic cholesteryl esters and triglycerides taken up by receptor-mediated endocytosis of plasma lipoproteins, thereby participating in the regulation of the size of the cytoplasmic pool of unesterified cholesterol and free fatty acids and providing substrates for energy production and biosynthetic processes.

Absence of hLAL activity, known as fatal Wolman disease of the infant, gives rise to the intralysosomal accumulation of cholesteryl esters and, to a lesser extent, of triglycerides, which leads to an impairment of normal cell function and finally to organ failure. The disorder becomes clinically evident during the first or second month of life and is characterized by hepato- and splenomegaly, bilateral adrenal calcification, abdominal distension, and failure to thrive. The more benign cholesteryl ester storage disease of the adult, in contrast, is due to very low levels of residual hLAL activity and takes a much more protracted clinical course. It is diagnosed within the first or second decade of life due to the accompanying hepatomegaly, often in combination with hypercholesterolemia. In addition, several CESD patients with no typical clinical symptoms or with very moderate enlargement of the liver even at a relatively advanced age have been reported (3–6).

Elucidation of the hLAL cDNA sequence (7) and of the respective gene structure (8–10), which is composed of ten exons located within a 38.8-kb region on human chro-

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mosome 10 (11), has allowed the identification of mutations responsible for Wolman disease and CESD, thereby providing an explanation for the phenotypic differences between the two allelic disorders. In Wolman disease, 1 bp to 8 bp deletions, nucleotide insertions, splice site and stop codon mutations have been detected which result in the production of catalytically inactive enzymes (8, 12–16). Much rarer are amino acid substitutions which almost completely compromise protein function (8, 17). In all cases, the hydrolytic activity of the mutant enzyme is zero or less than 1% towards cholesteryl esters and triglycerides.

In CESD, on the other hand, the level of residual hLAL activity is slightly higher, the threshold value between the more benign and the fatal form of the disease most likely being around 2–3%. The most common genetic defect associated with this disorder is an exon 8 splice junction mutation (E8SJM) due to a $G_{934} \rightarrow A$ substitution affecting the nucleotide at position -1 relative to the exon/intron border (12, 16, 18–26). This splicing mutant leads to two possible transcripts: one normal (3–5%) and the other aberrantly spliced and lacking the 72 nucleotides of exon 8 encoding amino acids 254–277 (12, 16, 27). Accordingly, seven of our CESD probands are homozygotes and eight are compound heterozygotes for this particular mutation (P. Lohse, M. Elleder, E. Keller, R. Gatti, and Y. S. Shin-Podskarbi, unpublished results).

In this report, we present the molecular basis of CESD in three patients of Czech and Irish origin. In all cases, the lysosomal storage disorder presented with hepatosplenomegaly and mixed hyperlipoproteinemia as early as 4 years of age.

MATERIALS AND METHODS

Study subjects

A. M. is a Caucasian male, whose Irish parents were healthy and not known to be consanguineous. D. H. is the eldest of two females and J. J. the second male child of healthy, non-consanguineous Czech parents. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of the University of Munich. Informed consent was obtained from the participants or, in the case of a minor, from the parents.

Cell culture

Skin fibroblasts of proband A. M. were cultured according to standard protocols in DMEM supplemented with 2 mm l-glutamine, 1 g glucose/l, and 10% fetal calf serum (Sigma-Aldrich) in a humidified incubator (Heraeus Instruments) containing 5% CO_2.

Isolation of genomic DNA and total RNA

High molecular weight chromosomal DNA and total RNA were isolated from fibroblasts of subject A. M. with genomic tips and the RNeasy total RNA purification system (QIAGEN Inc.). In the case of patients J. J. and D. H., DNA was extracted from white blood cells using the QIAamp blood kit (QIAGEN Inc.).

Oligonucleotides

Synthetic oligonucleotide primers (17) were prepared by the phosphoramidite method on a DNA synthesizer (Perkin-Elmer Applied Biosystems, Inc., model 381A), desalted on NAP-5 col-umns (Amersham Pharmacia Biotech), and used for reverse transcription and PCR amplification without further purification.

Reverse transcription of total RNA

A partial hLAL cDNA of patient A. M. was synthesized by incubation of 3 μg of total fibroblast RNA in a 10-μl reaction volume with Superscript II RNase H⁻ reverse transcriptase (GIBCO BRL Life Technologies) and oligonucleotide AL-11 (5′-TTTGCACAG*GAA*TTCCAGCAGGAG-3′), located in exon 8 of the hLAL gene and containing an artificial EcoR I restriction site (altered nucleotides in italics and in bold face). After incubation at 37°C for 1.5 h, the newly generated cDNA was further amplified by the PCR technique.

cDNA and DNA amplification by the polymerase chain reaction

The 50 μl or 100 μl reactions contained 10 mm Tris-HCl, pH 9.0, 50 mm KCl, 1.5 mm MgCl₂, 200 μm of each dNTP, 20 μm each of two hLAL-specific primers, the newly generated cDNA or approximately 1 μg of high molecular weight DNA, and 1.25 units Taq DNA Polymerase (Amersham Pharmacia Biotech). After denaturation at 95°C for 3 min, DNA was amplified for 40 cycles at 95°C for 30 sec, 52°C or 55°C for 30 sec, and 72°C for 30 sec.

Single-strand conformation polymorphism (SSCP) analysis

Heat-denatured PCR products of proband A. M. were loaded on precast 15% Clean Gels (Amersham Pharmacia Biotech) for mutation screening (28, 29).

Direct automated sequencing of PCR products

PCR fragments were gel-purified with the Geneclean kit (Bio 101) and sequenced with 3′ dye-labeled dideoxynucleotide triphosphates (dye terminators) and AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Inc.) on an Applied Biosystems 377A DNA sequencer.

Expression of thioredoxin/hLAL fusion proteins in *E. coli*

To demonstrate the formation of truncated hLAL enzymes, we used the pBAD/TOPO ThioFusion expression system (Invitrogen Corp.). The stop codon mutation and the two deletions were first introduced into the normal hLAL cDNA by the overlap extension PCR method (30). The resulting mutant cDNAs were then amplified by PCR, cloned into the pBAD/Thio-TOPO vector, and the vector was transformed into TOP10 *E. coli*. Cells containing the normal and the three mutant pBAD/Thio-hLAL constructs were induced with 0.2% arabinose for 4 h and lysed by sonication. Aliquots of the soluble fraction were size-separated on a 10%/12% SDS-polyacrylamide gel, blotted on a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp.), and fusion proteins were detected with an Anti-Thio antibody (Invitrogen).

Restriction fragment length polymorphism analysis

The common hLAL exon 8 splice junction mutation (E8SJM) was detected with oligonucleotide AL-84 (5′-TATCAATGC*AA G*CTTAATGCTGTT-3′), located in intron 7 and containing an artificial site for the restriction endonuclease Hind III (altered nucleotides in italics and in bold face), and mutation detection primer AL-111 (5′-GATTTTACATGAACCCCAAATGCACTCC TGGAATG*AG*TAC-3′), placed at the exon 8/intron 8 border and harboring an incomplete Sca I site.

As the three other genetic defects identified also do not create or destroy an endonuclease restriction site, additional mutation detection primers were designed for simplified genotyping of the patients and their immediate relatives. Amplification of exon 3 was performed with primer pair AL-70 (5′-GCTTTCTTAAAGC*T*

TABLE 1. Serum lipid and apolipoprotein concentrations as well as hLAL enzymic activities of CESD probands J. J. and D. H. and their immediate relatives

Subject	Born	Cholesterol	VLDL-Chol	LDL-Chol	HDL-Chol	Triglycerides	ApoB	ApoA-I	hLAL Activity
		mmol/l					*g/l*		
J. J.	1971	8.71	0.87	6.87	0.96	2.79–4.50	2.67	1.19	27.3
F. J.	1942	6.27	0.33	4.73	1.21	0.74	1.09	1.79	337
M. J.	1943	6.65	0.48	3.93	1.11	1.64	1.16	1.62	207
I. J.	1969	6.09	0.42	4.06	1.61	0.94	1.28	2.32	242
D. H.	1965	8.87	0.98	7.24	0.65	2.16	2.11	0.89	12
K. H.	1986	3.84	0.28	1.71	1.85	0.62	0.60	1.59	146
L. H.	1994	5.32	0.75	3.18	1.39	1.66	0.95	1.44	192
Controls		3.83–5.80	0.50–1.00	1.50–3.50	1.10–1.60	0.10–2.10	0.60–1.30	0.90–1.90	194–706

Cholesterol and triglyceride values are mmol/l and apoA-I and apoB concentrations are g/l. Enzyme assays were performed with extracts of peripheral leukocytes and the results are expressed as nanomoles of 4-methylumbelliferyl-palmitate hydrolyzed per hour per mg of cell protein.

TGGAGAACATAG-3′), positioned at the 3′-end of intron 2, and AL-165 (5′-CTTTGTCAGAATGGTTCTTCCTCCCATGAGGAA*C*TC-3′) in exon 3 which contains three nucleotides of an artificial, partial Dde I site (5′-C/TGAG-3′). hLAL exon 7 PCR products were obtained with oligonucleotides AL-130 (5′-GTTCTTGGGTTTCTTTTTATTTTGTAGGAC*CA*ATT-3′), located at the intron 6/exon 7 border and harboring an incomplete Mfe I site (5′-C/AATTG-3′), and AL-117 (5′-CAGCAGGTGG*AT*CCGTATTCTAGG-3′) in intron 7. Exon 10 was amplified from genomic DNA with the primer pair AL-138 (5′-GAAGGACATGCTTGTGCCGACTGCAGTCTGG*GA*CG -3′) in exon 10, containing a partial Tth111 I site (5′-GACG/GGGTC-3′), and AL-1 (5′-GACAT*A*AT*C*ATTGAATTCGTGGTACAC-3′) at the 5′-end of the 3′-nontranslated region. After an overnight incubation, restriction digests were loaded on 2–3% low melting point agarose (Gibco BRL Life Technologies) gels and the DNA fragments were visualized by ethidiumbromide staining and UV transillumination.

RESULTS

Characterization of subject A. M.

The case history, histopathological and laboratory findings as well as lipid analyses of liver and fibroblast extracts of patient A. M. have been described in detail elsewhere (31). At the time of diagnosis (1980), he was a 37-year-old Caucasian male of Irish descent who had suffered from re-
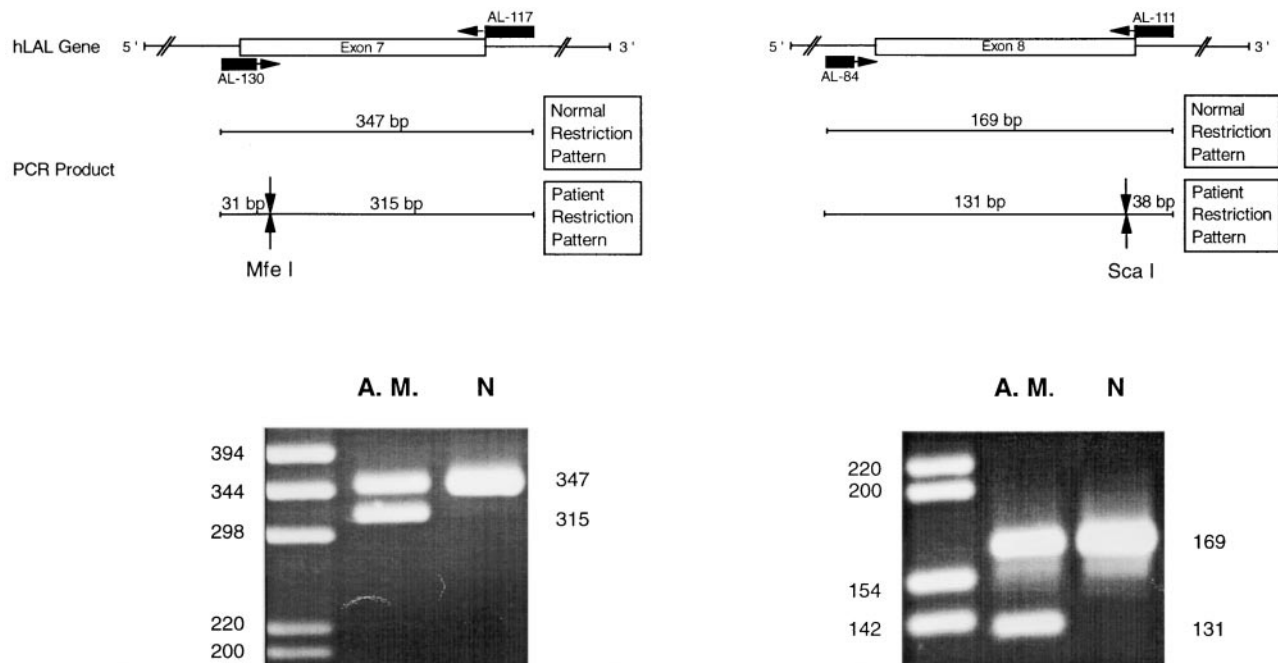

Fig. 1. Restriction enzyme digestion of PCR-amplified hLAL exon 7 and exon 8 products from proband A. M. and from a control subject. The seventh and eighth exon of the hLAL gene were amplified with the polymerase chain reaction using primer pairs AL-130 + AL-117 and AL-84 + AL-111, respectively, as shown in the upper panel. Artificial Mfe I and Sca I restriction enzyme sites created by the T-deletion in exon 7 and by the $G_{934} \rightarrow A$ substitution in exon 8 are indicated below. The lower panel contains two electropherograms of digested PCR products from a control subject (N; right lane) and from the patient (middle lane) which demonstrate that A. M. is a heterozygous carrier of both mutations. A nucleic acid size standard was loaded in the left lane and the fragment sizes are given on the left side. The sizes of the uncut and digested products are indicated on the right side.
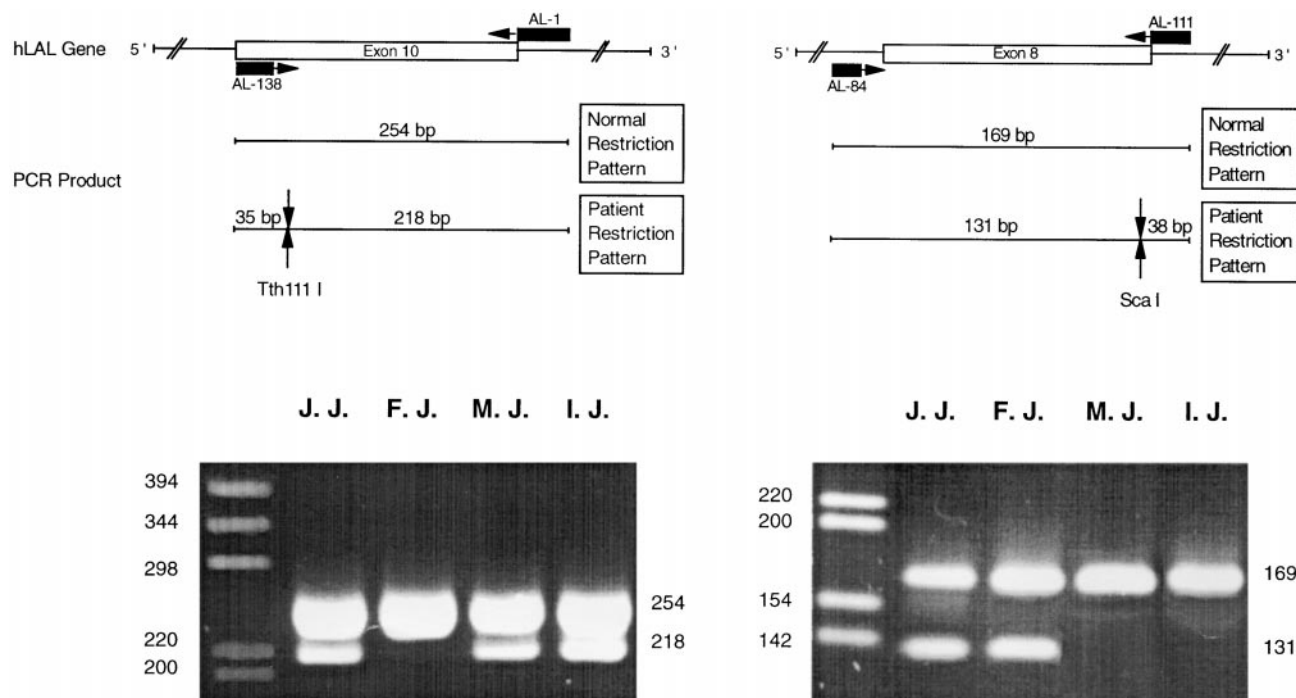
Fig. 2. Analysis of the segregation of the hLAL exon 8 and exon 10 mutations in family J. by restriction enzyme digestion. Part of the coding region of exon 10 and exon 8 were amplified from DNA of the proband (J. J.), his father (F. J.), his mother (M. J.), and his sister (I. J.) by PCR with oligonucleotide combinations AL-138 + AL-1 and AL-84 + AL-111 (upper panel). The products were then digested with Tth111 I and Sca I, respectively, and the fragments were separated on low melting point agarose gels together with a nucleic acid size standard (lower panel, left lane). In the presence of the mutations, the enzymes are expected to cleave the amplification products once, leading to the production of two smaller fragments. The results demonstrate that both the proband and his sister inherited the G-deletion in exon 10 from their mother, while the exon 8 splice junction mutation is located on one of the two paternal alleles.

curring episodes of non-specific malaise and intermittent diarrhoea since age 21. A mild hepatomegaly was diagnosed at that time. Five years later, a liver biopsy was taken which revealed the presence of foamy macrophages, granulomas, and piecemeal necroses. The proband was reassessed at age 37 because he was icteric. Liver function tests showed raised serum bilirubin (56 μmol/l) and SGPT (70 IU/l) levels. Total cholesterol (9 mmol/l) and triglycerides (2.45 mmol/l) were also elevated. A new liver biopsy demonstrated an accumulation of ceroid-loaded macrophages in the portal tract. The hepatocytes were swollen and some of them showed vacuolization and necrosis. A bone marrow aspirate also revealed increased numbers of foamy macrophages as well as of sea-blue histiocytes. Thin-layer chromatography of lipid extracts prepared from the liver sample showed a 70-fold increase in cholesteryl esters which accounted for 88% of total liver cholesterol and nearly 10% of wet weight. Consistent with the diagnosis of CESD, hLAL hydrolytic activity towards glycerol-tri-oleate and 4-methylumbelliferyl palmitate was 4.5% and 6% of control values. The patient was treated with phenobarbitone which resulted in the disappearance of jaundice, but had no influence on acid lipase activity and neutral lipid hydrolysis.

Characterization of subject J. J.

The Czech proband was born in 1971. At 4 years of age, he was discovered to have a grossly enlarged liver during a routine medical examination for a common respiratory infection. Laboratory tests revealed a slightly abnormal liver function in combination with hypercholesterolemia. Histological examination of a liver biopsy and subsequent HPTLC chromatography of extracted lipids disclosed a pathological storage of cholesteryl esters within hepatocytes as well as a considerable number of lipid- and ceroid-loaded intralobular and portal macrophages. In 1981, the diagnosis of CESD was confirmed by a 93% reduction of hLAL-catalyzed 4-methylumbelliferyl palmitate hydrolysis. At the present time, the patient's clinical course is stable. Total and LDL-cholesterol as well as triglycerides are elevated, while HDL-cholesterol is reduced (**Table 1**). The liver is palpable 3–4 cm below the costal margin, the spleen size is also slightly increased, and the differential blood count shows vacuoles in peripheral lymphocytes.

Both parents and his sister are healthy, but have approximately 50% of normal levels of esterase activity (Table 1).

Characterization of subject D. H.

In 1977, D. H., a 12-year-old Czech girl, was diagnosed with gross hepatomegaly (16 cm below the costal margin) while recovering from parotitis. During a hospital stay in 1979, a bone marrow aspirate demonstrated vacuolized plasmocytes, monocytes, and granulocytes as well as some foamy histiocytes. Plasma triglycerides were increased, while total cholesterol was in the normal range. A liver biopsy showed changes typical of CESD and the tentative
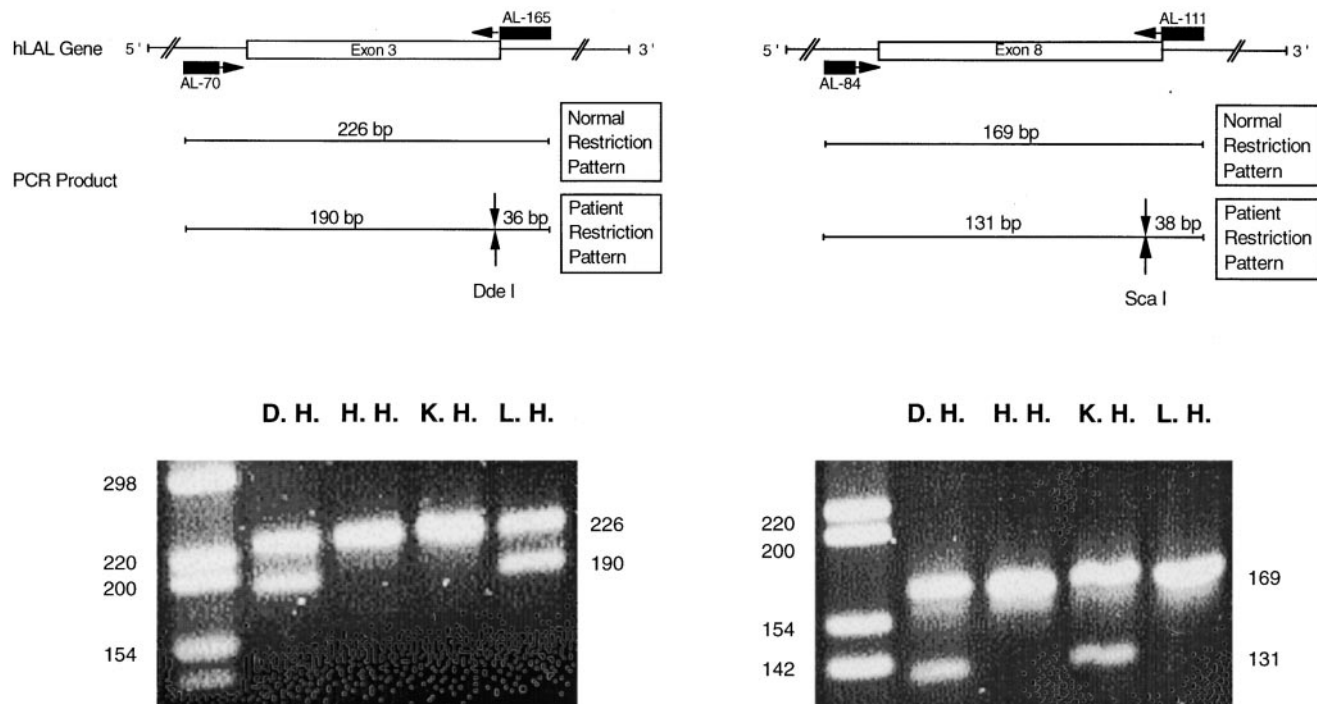
Fig. 3. Analysis of the segregation of the hLAL exon 3 and exon 8 mutations in family H. by restriction enzyme digestion. PCR products were generated by amplification of DNA from the proband (D. H.), her two sons (K. H. and L. H.), and her husband (H. H.) with oligonucleotides AL-70 + AL-165 and AL-84 + AL-111 and digested with Dde I and Sca I as illustrated in the upper panel. The respective agarose gels are shown in the lower panel. From DNA sequencing, the proband is known to carry a stop codon mutation in exon 3 and a splice site mutation in exon 8. RFLP analysis of her two sons demonstrates that K. H. has inherited the E8SJ mutation, while L. H. is a heterozygous carrier of the $C_{233} \rightarrow T$ substitution in exon 3, thereby also providing evidence that their mother is a compound heterozygote. The unaffected husband H. H. has a normal band pattern.

diagnosis was verified biochemically in two independent laboratories. In 1986 and 1994, she gave birth to two sons and both pregnancies were uneventful. In 1998, the patient was without health problems, but had increased plasma total cholesterol, LDL-cholesterol, and triglyceride as well as decreased HDL-cholesterol levels (Table 1). The spleen was enlarged to the costal margin, the liver protruded 3 to 4 cm below.

The grandmother on her mother's side (born 1926) suffers from coronary heart disease, while her parents and her younger sister are healthy.

Screening for the hLAL exon 8 splice junction mutation

As a first step in the genetic analysis of the hLAL gene locus, all three probands were studied for the presence or absence of the most common mutation causing CESD, which accounts for approximately 70% of all mutant chromosomes in the Central European population (17) and corresponds to the replacement of an A for a G at nucleotide position 934 (12, 16, 18–26; numbering according to Anderson and Sando, 7). Restriction fragment length polymorphism (RFLP) analysis, utilizing a mutation detection primer with an incomplete Sca I site, revealed that all three CESD patient carried the E8SJ mutation on only one of their two chromosomes (**Fig. 1, Fig. 2, Fig. 3**), suggesting that the remaining mutation on the second CESD chromosome is less frequent and heterogeneous in nature.

Single-strand conformation polymorphism (SSCP) analysis

hLAL exons 1–7, 9, and 10 of subject A. M. were subsequently characterized by mobility shift analysis of heat-denatured PCR amplification products on 15% neutral polyacrylamide gels in order to detect the second causal mutation. Only the exon 7 fragment exhibited a number of additional bands and this result was reproducible. The other single-stranded DNA products, in contrast, had a band pattern indistinguishable from that of a normal control (data not shown).

DNA sequence analysis

Determination of the nucleotide sequences of the coding and non-coding exons of the hLAL gene revealed that subject A. M. was a heterozygote for the deletion of a T residue at nucleotide position 722, 723, or 724 in exon 7 (**Fig. 4**), thereby confirming the results of the SSCP analysis, while proband J. J. was a heterozygous carrier of a G-deletion in exon 10 which affects one of five Gs at cDNA positions 1064–1068 (**Fig. 5**). These mutations lead to a reading frame shift and to premature termination of protein translation at amino acid positions 219 and 336 of the mature enzyme, respectively. The second Czech patient, D. H., in contrast, had inherited a single $C_{233} \rightarrow T$ substitution in hLAL exon 3 (**Fig. 6**), replacing arginine (CGA) at position 44 with an Opal termination signal (TGA).
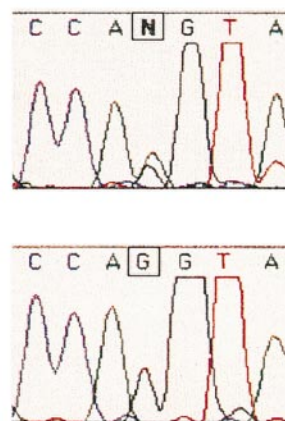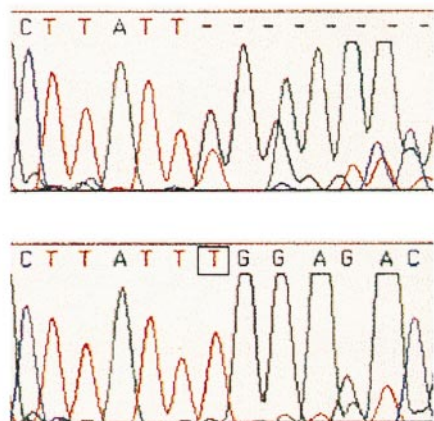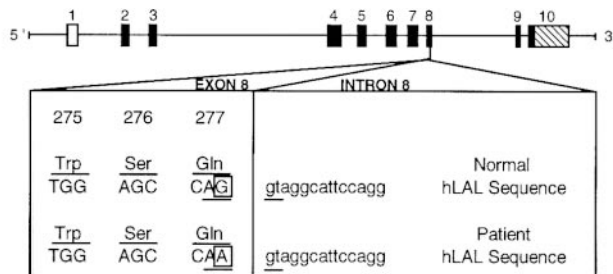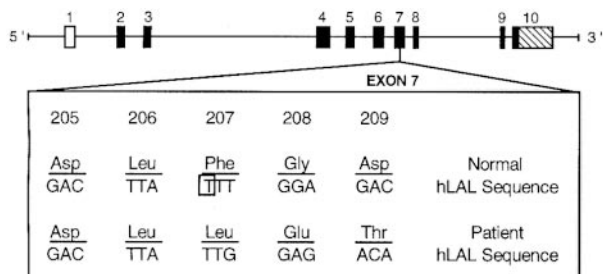
Fig. 4. Sequence analysis of hLAL exons 7 and 8 in CESD patient A. M. The upper panel illustrates the genomic structure and the location of the T-deletion and of the exon 8 splice junction mutation within the seventh and eighth exon of the proband's hLAL gene, respectively. The lower panel contains the corresponding DNA sequence electropherograms from the patient (top) and from a normal subject (bottom). The substituted and deleted nucleotides are boxed.

Expression of thioredoxin/hLAL fusion proteins in *E. coli*

In order to document directly that the mutations result in truncated proteins, we introduced the nucleotide substitution and the two deletions into the wild-type hLAL cDNA by oligonucleotide-mediated site-directed mutagenesis and synthesized the respective mutant proteins as well as the normal protein product in vitro in *E. coli* with thioredoxin (13 kDa) as the N-terminal fusion partner. As predicted from the cDNA sequence, the reactions yielded single bands of calculated molecular mass (7.2 kDa for D. H.; 26.7 kDa for A. M.; 40.1 kDa for J. J.; 45.4 kDa for normal hLAL; 21-amino-acid signal peptide included) on SDS-polyacrylamide gels (**Fig. 7**).

Allele-specific cDNA amplification

In the case of subject A. M., no immediate relatives were available for genetic analysis. In order to determine whether the two mutations were located on different chromosomes, we reverse transcribed total RNA from the patient with 3′-oligonucleotide AL-11 in exon 8 and PCR-amplified the product with primer pair AL-11 and AL-20 (5′-TAGGTTTTA*A*AGC*T*TTTTCACAGA-3′), located at the exon 5/exon 6 border. The reasoning behind this strategy was that in about 95–97% of the hLAL mRNA transcripts generated from the chromosome with the splice junction mutation, exon 8 would be missing (16, 20, 31). Using an anti-sense primer located in this exon, we should be able to amplify a 339 bp fragment specific for the second allele

which should contain only the T-deletion in exon 7. Sequence analysis of the amplification product confirmed our assumption, as it demonstrated homozygosity for the mutation (data not shown).

Restriction fragment length polymorphism analysis

The two deletions and the base substitution did not result in the formation or loss of a restriction enzyme site, as revealed by software (PC/GENE; IntelliGenetics, Mountain View, CA)-assisted computer analysis. We therefore synthesized three mutation detection primers in order to confirm the results of the DNA sequence analysis and to genotype the subject's immediate relatives. AL-130 at the intron 6/exon 7 border contained an incorporated partial Mfe I site (5′-C/AATTG-3′) which is completed by G_{725} of the allele with the T-deletion. AL-138 introduced a partial, artificial Tth111 I site (5′-GACG/GGGTC-3′) into the exon 10 PCR amplification product which is cleaved only in the presence of four (mutated allele), but not five G's (normal allele). AL-165 in exon 3 harbored an incomplete Dde I site (5′-C/TGAG-3′) which is cut in case the $C_{233} \rightarrow T$ mutation is present.

Agarose gel electrophoresis of Mfe I- and Sca I-digested exon 7 and exon 8 amplification products demonstrated that subject A. M. is a heterozygote for both the E8SJ mutation and the T-deletion in exon 7 (Fig. 1). Analysis of family J. revealed that the proband J. J. had inherited the splice junction mutation in exon 8 from his father and
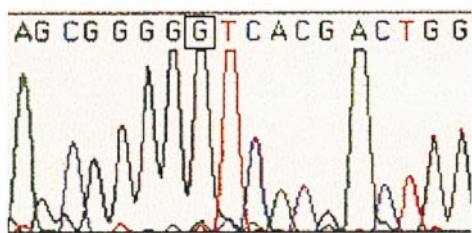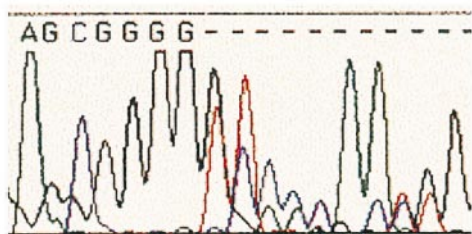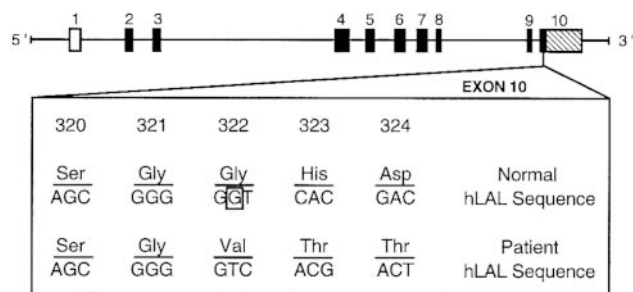
Fig. 5. Sequence analysis of hLAL exon 10 in CESD patient J. J. A schematic diagram of the hLAL genomic structure (upper panel) shows the position of the G-deletion in exon 10 (indicated by a box) which results in premature termination of protein translation at amino acid position 336. The lower panel presents the corresponding sequence electropherograms of hLAL exon 10 amplification products from the proband (top) and from a normal control (bottom).

Fig. 6. Sequence analysis of hLAL exon 3 in CESD patient D. H. The schematic diagram (upper panel) illustrates the location of the mutation (boxed) within the third exon of the hLAL gene which replaces arginine (CGA), amino acid 44 of the mature hLAL, with an Opal termination signal (TGA). The electropherograms of sequencing gels of exon 3 fragments from the proband (top) and from a normal control (bottom) are shown in the lower panel and the position of the mutation is indicated by a box.

the G-deletion in exon 10 from his mother, while his sister carried only the deletion mutation (Fig. 2). Patient D. H. is also a compound heterozygote for E8SJM and the $C_{233}{\rightarrow}T$ substitution in exon 3, as shown by restriction enzyme digest of exon 3 and exon 8 PCR fragments of her and her offspring with Sca I and Dde I (Fig. 3). One of her sons has inherited the splice defect, the other the stop codon mutation, while her husband served as normal control.

DISCUSSION

As of 1995, more than 40 patients with CESD and more than 50 cases of Wolman disease had been recorded in the literature (1). Of our 26 probands, three have been described earlier. In conjunction with case reports published recently by others, our estimate of the number of patients with partial and complete hLAL deficiency known today is about 130 to 140, suggesting that both diseases are extremely and similarly rare. However, as Wolman already pointed out (32), the true incidence of both
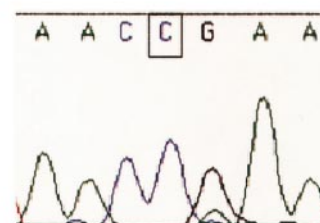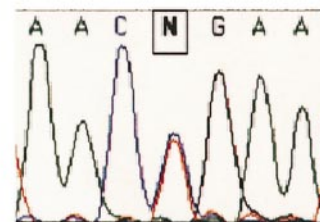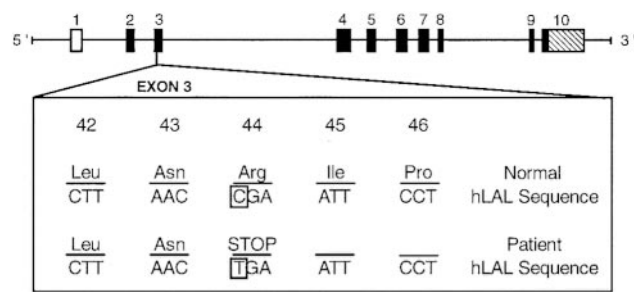
disorders cannot be determined with certainty because *1*) CESD is sometimes very difficult to diagnose as illustrated by the clinically asymptomatic and purely accidentally discovered cases (3–6), *2*) in certain areas of the world, the medical profession is not aware of these diseases, and *3*) clinical cases are no longer published. With the ability to detect CESD and Wolman mutations at the DNA level and to perform genetic testing, additional data on the heterogeneity and on the frequency of different mutations in various populations will accumulate in the near future, hopefully also providing a better estimate of disease incidence.

In the present study, we investigated the genetic defects leading to CESD in three probands of Czech (J. J. and D. H.) and Irish (A. M.) origin. DNA sequence and restriction fragment length polymorphism analysis as well as allele-specific amplification provided evidence that all three subjects were compound heterozygotes for the prevalent hLAL exon 8 splice junction mutation. A. M. was, in addition, a heterozygous carrier of a T-deletion in exon 7,
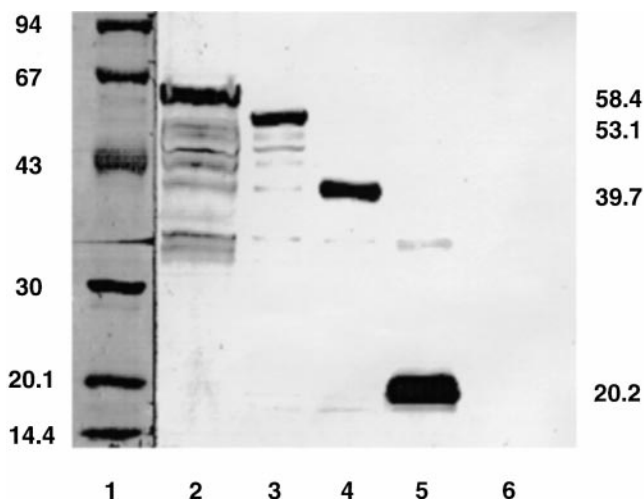
Fig. 7. Expression of thioredoxin-hLAL fusion proteins in *E. coli.* The Western blot shows the three truncated hLAL protein products of CESD probands J. J. (40.1 kDa; lane 3), A. M. (26.7 kDa; lane 4), and D. H. (7.2 kDa; lane 5) in comparison with the wild-type enzyme (45.4 kDa; lane 2) after PCR amplification of the respective hLAL cDNAs, regulated in vitro expression in *E. coli*, and one-dimensional SDS-polyacrylamide gel electrophoresis. Lane 1 contains a molecular mass marker with protein standards of 94, 67, 43, 30, 20.1, and 14.4 kDa (shown on the left side), lane 6 a negative control. Note that the fusion with the N-terminal thioredoxin peptide increases the size of the normal and mutant hLAL proteins (illustrated on the right side) by 13 kDa.

affecting one of three Ts at cDNA positions 722–724 and resulting in the production of a truncated enzyme of 219 instead of 378 amino acids. J. J. and his sister had inherited a G-deletion at nucleotide positions 1064–1068 in exon 10 on their maternal chromosome. This mutation results in premature termination of protein translation at residue 336 and in complete loss of enzymic activity due to the absence of the catalytic triad residue histidine at position 353 (30). D. H., in contrast, was heterozygous for an in-frame stop codon mutation at amino acid position 44. This defect has already been described once in an adult male CESD patient from France who presented with hepatomegaly, mildly elevated liver function tests, hypercholesterolemia, and 7% hLAL hydrolytic activity towards triglycerides (24, 25). The same mutation has also been found, in homozygous form, in the only Wolman case from the Czech Republic (M. Elleder and P. Lohse, unpublished results).

Our analyses add two new mutations to the growing list of genetic defects of the Wolman type in CESD patients which is already fairly long and includes an $Arg_{44} \rightarrow$ stop mutation in exon 3 (24, 25; also this manuscript), a $Leu_{179} \rightarrow$ Pro substitution in exon 6 (20), the replacement of glycine at position 245 in exon 7 by a termination signal (12), an $A \rightarrow G$ 3′-splice site mutation resulting in the loss of exon 7 from the hLAL mRNA transcript (22), a dinucleotide deletion affecting the first two bases of exon 10 (19), and a partial deletion of the hLAL gene (26). Only the replacement of leucine at position 179 by proline has also been described as one of two causal mutations in a compound heterozygous Wolman patient (8).

With the notable exception of the hLAL exon 8 splice junction mutation, the genetic defects of the CESD type, in contrast, are all amino acid substitutions, which allow for some residual hLAL activity to be maintained: $His_{108} \rightarrow$ Arg (26), $Pro_{181} \rightarrow$ Leu (22), $Thr_{267} \rightarrow$ Ile (16), $His_{274} \rightarrow$ Tyr (24, 33), and $Leu_{336} \rightarrow$ Pro (34). At present, it is unclear whether the replacement of Gln_{64} by Arg (16), of Gly_{66} by Val (22), of His_{108} by Pro (6, 26), and of Leu_{273} by Ser (22) would lead to CESD or Wolman disease, if inherited on both chromosomes. Recent in vitro expression studies of Pagani et al. (16) strongly suggest, however, that mutant enzymes with the respective amino acid substitutions at positions 64, 66, and 273 are partially active, their esterase activities ranging from 1.9 to 3.9%.

Our previous genetic analyses had also shown that, in almost all cases, infants with Wolman disease are born to consanguineous parents (17), again lending support to the notion that it is a very rare autosomal recessive disorder and that the likelihood of two heterozygous individuals with different Wolman mutations marrying each other is extremely low. It is therefore noteworthy that, among our compound heterozygous CESD patients, four out of eight are carriers of a Wolman mutation. Our observation is in complete agreement with CESD case reports in the literature. Of the 11 compound heterozygotes published thus far, six had also inherited a defect of the Wolman type (deletion, nonsense or splice site mutation) on one of their two chromosomes (12, 16, 19, 20, 22, 24–26). In four patients, the Wolman mutation was associated with the prevalent $G_{934} \rightarrow A$ transition in exon 8 (12, 19, 20, 24, 25), in one proband with a $Pro_{181} \rightarrow$ Leu substitution (22), and in another subject with the replacement of histidine, amino acid 108, by arginine (26).

In summary, we have elucidated the molecular basis of CESD in three probands of Czech and Irish origin using multiple, confirmatory approaches. All patients were compound heterozygotes for the common hLAL exon 8 splice junction mutation (E8SJM) and for a genetic defect which results in complete loss of enzymic activity. These Wolman mutations included the substitution of arginine at position 44 by a stop codon, a T-deletion in exon 7 affecting nucleotide 722, 723, or 724 of the hLAL cDNA, and the deletion of a guanosine within a stretch of five Gs at cDNA position 1064–1068 in exon 10. Both frameshift mutations result in premature termination of protein translation at amino acid residues 219 and 336, respectively.

Based on our genetic analyses of a total of 15 CESD patients, we conclude that *1)* the hLAL exon 8 splice defect is the most frequent mutation causing CESD and accounts for approximately 70% of all mutant chromosomes, *2)* the remaining CESD chromosomes are heterogeneous, probably consisting of a large number of infrequent mutant alleles, and *3)* compound heterozygosity for a Wolman mutation is a common cause of CESD.

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