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Genetics

Mutations at the lysosomal acid cholesteryl ester hydrolase gene locus in Wolman disease

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ABSTRACT **The genomic sequences encoding the human lysosomal acid lipase/cholesteryl esterase (sterol esterase; EC 3.1.1.13) have been isolated and sequenced, and the information has been used to identify mutations in both alleles of the gene from a patient with Wolman disease, an autosomal recessive lysosomal lipid storage disorder. The genomic locus consists of 10 exons spread over 36 kb. The 5′ flanking region is G+C-rich and has characteristics of a "housekeeping" gene promoter. One of the identified mutations involves the insertion of a T residue after position 634, resulting in the appearance of an in-frame translation stop signal 13 codons downstream. The second mutation is a T-to-C transition at nucleotide 638. This results in a leucine-to-proline substitution at amino acid 179 and is predicted to lead to the disruption of the α-helical structure in a highly conserved region of the protein. These mutations are each capable of completely disrupting the catalytic function of the lysosomal acid cholesteryl ester hydrolase; their presence can account for the extreme phenotype of the lysosomal lipid storage disorder manifested in members of this patient's family.**

Lysosomal acid lipase/cholesteryl ester hydrolase (sterol esterase; EC 3.1.1.13) is crucial for the intracellular hydrolysis of cholesteryl esters and triglycerides that have been internalized via receptor-mediated endocytosis of lipoprotein particles (1). This process is central to the supply of cholesterol to cells for growth and membrane function and in the regulation of processes that are mediated by cellular cholesterol flux. The importance of this enzyme in mediating the effect of low density lipoprotein (LDL) uptake on suppression of hydroxymethylglutaryl-CoA reductase and activation of endogenous cellular cholesteryl ester formation was established by Brown *et al.* (2) in the mid-1970s.

Consequences of a deficiency of this acid lipase activity are seen in two human genetic diseases, Wolman disease (WD) and cholesteryl ester storage disease (CESD). Both of these autosomal recessive conditions are characterized by severely depressed acid lipase/cholesteryl ester hydrolase activities, intralysosomal lipid accumulations, and derangements in the regulation of cholesterol production. Despite what are, in most instances, indistinguishably low levels of residual acid hydrolase activity, the clinical and biochemical phenotypes manifested by WD and CESD are dramatically dissimilar (1, 3). WD is lethal within the first year of life; infants die from complications of massive storage of lipids in most tissues before classic atherosclerotic pathology develops. Adrenal calcification is a hallmark of WD but is not common in CESD. Persons affected with the CESD variant show a broad spectrum of severity. Many present with only hepatic manifestations discovered adventitiously and the condition is compatible with survival into middle age and beyond (1, 4). Most of the CESD patients who have died before their 4th decade have shown advanced atherosclerotic vascular pathology at autopsy.

Tissue levels of both cholesteryl esters and triglycerides are markedly elevated in WD, while pathologic cholesteryl ester storage in CESD is accompanied by only a marginally abnormal intracellular accumulation of triglyceride. Normal plasma cholesterol and triglyceride levels can be found in infants with WD, but CESD patients characteristically are hypercholesterolemic with elevated LDL-cholesterol levels (1, 3).

Experimental observations consistent with the concept that WD and CESD arise from defects at the same genetic locus, presumably *LIPA*, the structural locus for the enzyme on chromosome 10q, include the findings that (*i*) extracts of cultured fibroblasts from patients with either WD or CESD exhibit similar very low to absent activities of the same isoform (lipase A) of acid lipase/cholesteryl ester hydrolase activity (5), (*ii*) the application of a secreted form of human lysosomal acid lipase (HLAL) with intact mannose-6-phosphate lysosomal targeting signals to cultured WD or CESD fibroblasts corrects lysosomal cholesteryl ester accumulation in the mutants (6), and (*iii*) as would be expected for allelic disorders, cocultivation of WD cells and CESD cells does not complement the deficient activities (2, 3). Until recently, however, there has been no direct proof that either condition arises from mutations in the structural gene.

To establish that defects at the structural gene locus are responsible for the acid hydrolase-deficient WD phenotype, we have isolated the HLAL gene from a human genomic library and used information from the cloned genomic sequences¶ to identify the causal mutations in the acid cholesteryl ester hydrolase alleles present in a patient with WD.

MATERIALS AND METHODS

Library Screening. Standard molecular biology protocols (7) were used throughout unless specifically indicated. Six hundred thousand plaques from a commercially available genomic library constructed with human male placental DNA inserted into the λ FIX II vector (Stratagene) were screened twice with different ^{32}P-labeled HLAL cDNA fragments; 11 positive clones were obtained by screening with the 2.4-kb *Bam*HI fragment representing most of the acid lipase insert from pHLAL11 (8) and 4 more isolates were obtained by screening with the 145-bp *Bam*HI fragment encompassing the 5′ end of the coding sequence.

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Abbreviations: CESD, cholesteryl ester storage disease; HGL, human gastric lipase; HLAL, human lysosomal acid lipase/cholesteryl ester hydrolase; LDL, low density lipoprotein; WD, Wolman disease.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U04284–U04293).

λ DNA Isolation and Gene Structure Analysis. Small-scale isolation of λ clone DNAs from liquid cultures was performed as described by Grossberger (9). Multiple restriction endonuclease digests were analyzed on Southern blots probed sequentially with ^{32}P-labeled HLAL cDNA fragments encompassing (*i*) 145 bp from the 5′ end, (*ii*) 1.6 kb of the peptide coding region, and (*iii*) 0.9 kb of 3′ untranslated region. Only isolates that hybridized with the 3′ untranslated region probe or showed overlap in restriction site patterns with these clones were examined further; other clones were excluded so as to avoid confusion with sequences from the closely related human gastric lipase (HGL) gene. After this general selection and alignment, selected λ clones were digested with *Sac* I and *Bgl* II, and the resulting fragments were subcloned into pBluescript II SK(−) plasmids (Stratagene). Oligonucleotide primers previously used to sequence the HLAL cDNA (7) were used to definitively localize individual exons in the cloned inserts and characterize intron/exon splice junction sites by nucleotide sequence analysis. Plasmid clones were directly sequenced with the appropriate HLAL-specific oligonucleotides by using a double-stranded template and the Sequenase protocol (United States Biochemical).

Determination of Transcription Initiation Site. For primer extension, 30 μg of total RNA isolated from cultured normal human fetal foreskin fibroblasts was annealed with 20 pmol of 5′-end-labeled CMP2 oligonucleotide (5′-GTTTCAGGATCCACAGC-3′), which binds 82 nt downstream of the initial AUG in the published cDNA sequence (8). The reaction mixtures were incubated with Moloney murine leukemia virus reverse transcriptase (10). The S1 nuclease protection was performed with a 253-nt riboprobe synthesized on an *Xho* I–*Sac* I fragment subcloned into pBluescript from the 5′ end of λ-HLAL1311 (Fig. 1). The products of both reactions were electrophoresed on a 6% polyacrylamide denaturing gel beside a sequencing ladder originating with the CMP2 oligonucleotide priming the riboprobe vector insert.

PCR Sequence Analysis of Exon Fragments. Nucleotide sequences of the genomic regions flanking the intron/exon junction sites were used to design intron sequence-based oligonucleotide primers flanking each HLAL exon (see Fig. 2). Each "exon pair" of primers was used to initiate synthesis in a series of PCRs using human genomic DNA isolated from cultured fibroblasts as template. The fetal foreskin (normal control) and WD fibroblasts were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum, and DNA was isolated by using guanidine hydrochloride (11). The optimized concentration (1.0–2.5 mM) of $MgCl_2$ in a standard *Taq* DNA polymerase PCR buffer that led to the amplification of a single band was determined for each primer set. Reactions were programmed for 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C; they were run for 30 cycles and concluded with an extension step of 7 min at 72°C. PCR products were purified free of unincorporated primers by using Promega "Magic Prep" kits and sequenced directly by the CircumVent Thermal Cycle dideoxynucleotide sequencing protocol (New England Biolabs). Sequences were determined in both directions, with the same oligonucleotides used in the amplification step as sequencing primers. Sequence variations were confirmed by using at least three separate PCR amplification products and with the use of genomic DNA isolated on at least two separate occasions.

WD Patient. The proband (FeD) from whom the fibroblasts used in this study were obtained was the fifth pregnancy in a family with two earlier siblings with WD. Those cases have been previously reported (12, 13). The clinical findings for the affected siblings included hepatospenomegaly, failure to thrive, and adrenal calcification, and the infants died at 6.5 and 3 months of age. Subsequently, two unaffected siblings were born; the second pregnancy was monitored by measuring acid lipase activity in cultured amniotic fluid cells. For the fifth pregnancy, analysis of chorionic villous samples showed less than 5% of control acid lipase activity as measured with [^{14}C]cholesteryl oleate as substrate, and no A isoenzyme of acid lipase was detectable after electrophoresis and staining with 4-methylumbelliferyl oleate. The pregnancy was terminated and the diagnosis was confirmed in cultured skin fibroblasts obtained from the abortus (P.M.C. and J. A. Cortner, unpublished observations). Of note is the observation that fibroblasts from the otherwise normal, presumably heterozygous, father of this child showed marked lipid accumulation; his measured acid lipase activity was less than 40% of controls (13). The parents were unrelated. Material from other members of the family is not available.

RESULTS

Structure of the HLAL Gene. A human genomic library in the λ FIX II vector was screened to isolate the HLAL gene after genomic Southern blot results (not shown) indicated HLAL sequences could be distinguished from homologous HGL sequences and suggested that the HLAL locus was of only moderate size. Fifteen clones, with inserts ranging in size from 12.5 to 18.8 kb, were successfully plaque purified to homogeneity. Restriction site maps of three of these clones, which cover the entire HLAL locus, are shown aligned in Fig. 1. As indicated, the gene spans ≈36 kb with 10 exons that account for all of the transcribed sequences. The restriction sites and exon/intron alignments shown here also account for all of the patterns that we have observed on Southern blots of human DNA probed with full-length HLAL cDNA.

The sequences of the exon/intron junctions (Fig. 2) for the 10 exons show the expected canonical splicing signals in all

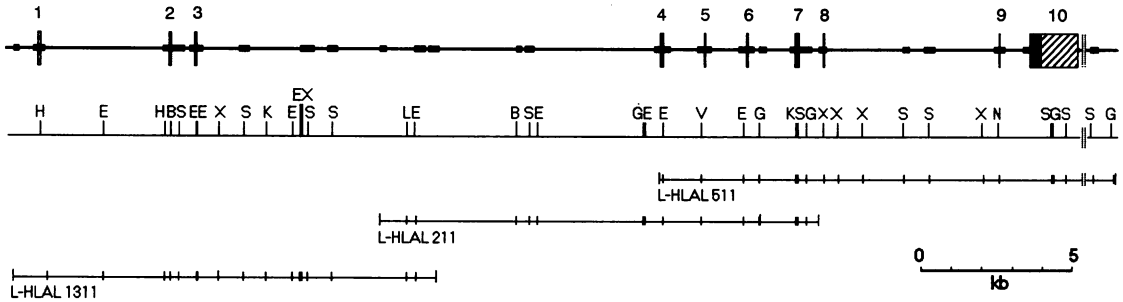

FIG. 1. Structure of the HLAL gene locus is shown on the first horizontal line; the exons are demarcated by vertical bars (solid for coding, hatched for 5′ and 3′ untranslated regions). Other areas that have been sequenced are indicated by thickened areas on the horizontal line. The combined restriction mapping data from the λ inserts are shown on the next line, and the alignment of overlapping λ clones covering the gene is shown in the lower area. Only the restriction sites used in mapping are shown: B, *Bam*HI; E, *Eco*RI; G, *Bgl* II; H, *Xho* I; K, *Kpn* I; L, *Bgl* I; N, *Nhe* I; S, *Sac* I; V, *Pvu* II; X, *Xba* I.

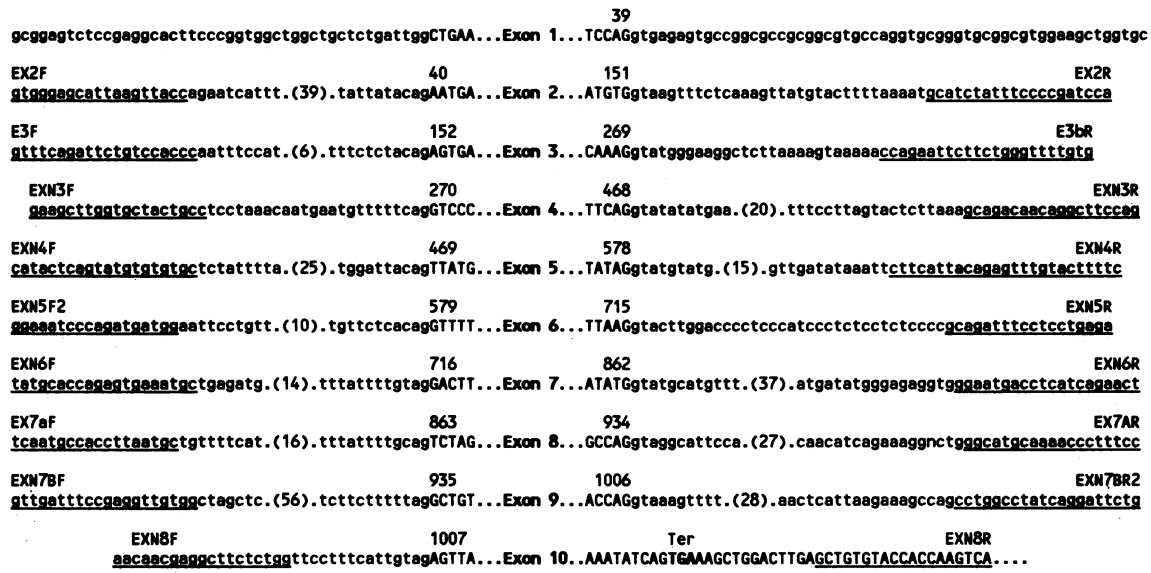

FIG. 2. Exon/intron junctions. Partial intron sequences appear in lowercase letters surrounding the exons in uppercase letters (only the junction nucleotides are shown). Nucleotide positions of the junctions as numbered in the cDNA (8) appear on the upper lines. Numbers in parentheses represent nucleotides deleted so that the sequences and positions of the primers (underlined) used in PCR amplifications can be shown. The translation termination site is labeled (Ter).

cases. The exon sizes and structures are remarkable only for the length of exon 10 (1487 nt), which contains extensive protein coding sequences as well as all of the sequence for the long 3′ untranslated region. Exons 8 and 9 are both 72 bp long and end with intact codons; the near identity of the distal 12 amino acids in each suggests that they resulted from a duplication event. The translation start site lies at the beginning of exon 2 and the conserved esterase pentapeptide motif which appears to be an active site candidate in many lipases (14) is in exon 5.

Primer extension of normal fibroblast RNA with the HLAL-specific CMP2 primer and nuclease protection of a 113-nt fragment beginning at the *Xho* I site by an antisense riboprobe indicate a major transcription initiation site consistent with a 140-nt 5′ untranslated segment for the HLAL mRNA (Fig. 3). There is also a rarely used initiation site at position −20. The 5′ flanking sequences, as well as the exon 1 and early first intron sequences shown in Fig. 3 have a 66% G+C content. The only consensus binding sites are for general transcription factors such as Sp1 (15).

Identification of WD Mutations. There was no evidence of deletions or rearrangements in the HLAL alleles of any of the WD and CESD DNAs examined by Southern blotting when compared with normal fibroblast DNA or the cloned sequences (data not shown). When the size and levels of HLAL mRNA were examined on Northern blots of total cellular RNA from 12 acid lipase-deficient fibroblast lines, almost all showed relatively low, but detectable, levels of approximately full-length HLAL message. A representative result is shown for the WD fibroblast line, FeD, in Fig. 4.

A detailed analysis for mutations in the genomes of cultured HLAL-deficient fibroblasts was performed by directly sequencing exons, using genomic DNA as template. The oligonucleotide primers shown in Fig. 2 were first used to PCR amplify specific genomic fragments encompassing each exon, and then the same primers were used to determine the nucleotide sequences of the amplified fragments. The priming sites were chosen to show splice junctions as well as the peptide encoding regions. To obtain representative sequence data with minimal interference due to the infidelity of *Taq* polymerase, the entire uncloned population of linear molecules obtained in each exon PCR was subjected to direct sequencing.

Sequence variations were detected in both HLAL alleles of the FeD line with this technique. The two mutations in the FeD line that significantly alter HLAL peptide structure are shown in the sequencing gel autoradiographs in Fig. 5. The changes are close to each other and, by the nature of the insertion mutation, produce an initially difficult to dissect pattern in the sequencing gel of the uncloned mix of PCR products from the two different alleles (Fig. 5 *Upper Left*). The sequencing analyses of subclones of isolated PCR fragments encompassing FeD exon 6 that are shown in Fig. 5 *Lower* clarify the patterns and demonstrate that the sequence

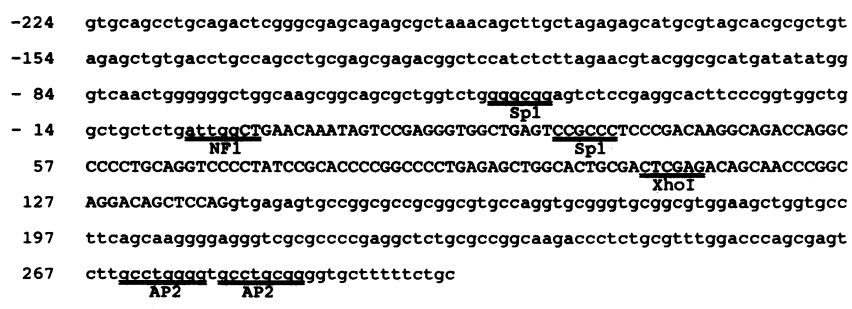

FIG. 3. The 5′ region of the HLAL gene. Transcribed sequences to the end of exon 1 appear in uppercase letters and 5′ flanking and intron sequences are in lowercase letters. Consensus binding sequences for transcription factors Sp1, NF1, and AP2 and the *Xho* I restriction site in exon 1 are underlined.

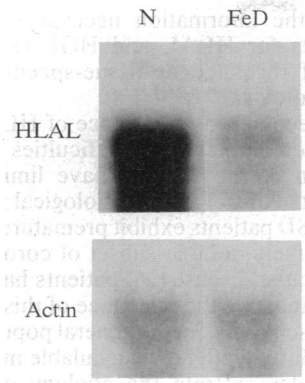

FIG. 4. Autoradiograph of Northern blot loaded with 20 μg of total cell RNA from normal (N) foreskin and the FeD WD fibroblast line probed consecutively with a full-length HLAL insert followed by an α-actin sequence. The control sample was left with film as long as was required to get the FeD signal, resulting in its marked overexposure.

variations are on different alleles. Sequencing of the opposite strands confirmed the results shown here (data not shown).

The first mutation is a nucleotide insertion in exon 6; a seventh T residue is inserted after residue 634 at the end of a run of 6 Ts. This leads to the creation of an in-frame termination codon 12 amino acids downstream (Fig. 6*A*). This same change is present in the proband and a parent in 1 of the 11 unrelated acid cholesteryl ester hydrolase-deficient families examined.

The second mutation is a T-to-C transition at nucleotide residue 639 producing a nonconservative leucine-to-proline coding change (Fig. 6*B*). This nucleotide substitution also creates a new *Sma* I restriction site which we have demonstrated in the PCR product and on Southern blots of genomic DNA (data not shown). The restriction site polymorphism has permitted the rapid screening of DNA samples from 13 normal subjects, none of whom showed the restriction pattern seen in FeD DNA. This mutation has not been seen in any of the other cholesteryl ester hydrolase-deficient fibroblast lines screened.

The only other sequence variation seen in a peptide coding region is a common C-to-A polymorphism in one allele at position 86. This results in the presence of a threonine instead of a proline as the 16th amino acid residue from the amino terminus of the signal peptide.

DISCUSSION

The results presented here demonstrate that the WD phenotype arises from HLAL structural gene mutations. Prior to this, biochemical, coculture, and genetic complementation experiments were not able to exclude the possibility that a defect in a nondiffusible factor encoded at another locus but necessary for HLAL maturation or transport was the cause of acid lipase deficiency phenotypes.

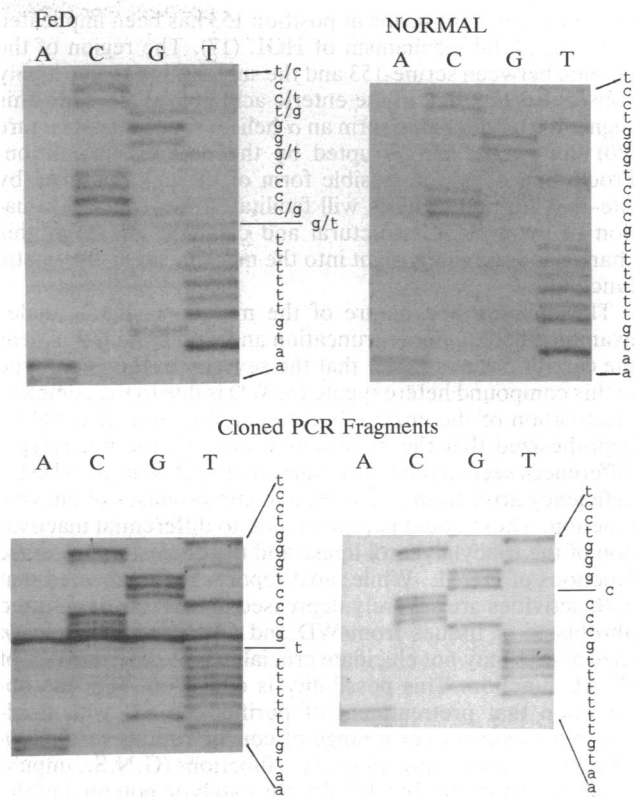

FIG. 5. Nucleotide sequences for FeD, exon 6. Upper gels are from direct cycle sequencing of PCR fragments. The result for the WD line is at the left and the normal at the right. Lower gels show sequences from PCR fragments cloned in pBluescript, with examples of each of the two alleles found.

It is likely that we have identified both of the mutations that produced the disease in this patient (Fig. 6). The insertion frameshift mutation in one HLAL allele results in the loss of half of the protein and will almost certainly lead to an enzymatically inactive unstable product. This type of frameshift mutation with premature termination of translation would also be expected to lead to destabilization of the mRNA (16) and explain the lowered HLAL message level observed in this WD patient's fibroblasts.

The nonconservative leucine-to-proline substitution predicted from the sequence of the second allele would also be expected to compromise enzymatic function. It occurs 26 amino acids away from what is presumably the active-site serine of HLAL, on the basis of the residue's position in a conserved esterase pentapeptide motif and the observation

A INSERTION OF "T" RESIDUE : truncation of the protein

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                    634
FeD    : ...ATGTTTTTTTGCCCTGGGTCCTGTGGCTTCCGTCGCCTTCTGTACTAGCCCT...
         ... M  F  F  C  P  G  S  C  G  F  R  R  L  L  Y  TER

Normal: ...ATGTTTTTTGCCCTGGGTCCTGTGGCTTCCGTCGCCTTCTGTACTAGCCCT...
         ... M  F  F  A  L  G  P  V  A  S  V  A  F  C  T  S  P ...
```

B MUTATION OF "T" -> "C" : proline for leucine substitution

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                    153
HLAL: ... Y  Y  V  G  H  S  Q  G  T  T  I  G  F  I  A  F  S  Q
HGL : ... H  Y  V  G  H  S  Q  G  T  T  I  G  F  I  A  F  S  T

                                                179
                                                 P
         I  P  E  L  A  K  R  I  K  M  F  F  A  L  G  P  V  A  S ...
         N  P  S  L  A  K  R  I  K  T  F  Y  A  L  A  P  V  A  T ...
```

FIG. 6. Structural implications of the mutations found in the FeD HLAL alleles. (*A*) Nucleotide and predicted amino acid sequences near the site of insertion of an extra T residue after nt 634 in the HLAL cDNA sequence (8). The insertion creates an in-frame termination codon downstream. Normal sequences are shown for comparison. (*B*) Amino acid sequences, showing the implication of the C-to-T transition in the codon for amino acid 179. A proline for leucine substitution (double underlines) occurs 26 amino acids away from the candidate active site serine (position 153) in the conserved pentapeptide esterase motif (single underline). The homologous sequence of HGL is shown on the lower line.

that the equivalent serine at position 153 has been implicated in the catalytic mechanism of HGL (17). The region of the peptide between serine-153 and the substitution is also highly conserved (Fig. 6*B*) in the enteric acid lipases (18, 19). This segment is predicted to form an α-helical secondary structure (20) that would be disrupted by the proline substitution. Production of an expressible form of the mutated gene by site-specific mutagenesis will facilitate biochemical evaluation of the specific structural and catalytic effects of this change and provide insight into the mechanism of enzymatic function.

The location and nature of the mutations in the alleles examined here, a major truncation and a point mutation near the catalytic site, suggest that the severity of the phenotype in this compound heterozygote for WD is due to the complete inactivation of the enzyme's catalytic function. It could be hypothesized that the mutations producing the phenotypic differences seen in the CESD variant of acid cholesteryl ester deficiency arise from less profound compromises of enzyme function. These could perhaps relate to differential inactivation of the triacylglycerol lipase and the cholesteryl esterase functions of HLAL. While most reports have indicated that both activities are severely depressed in extracts of cultured fibroblasts or tissues from WD and CESD patients, the *in vitro* assays may not elucidate crucial *in vivo* determinants of HLAL function. This possibility is consistent with the observation that pretreatment of purified HLAL with thiol-reactive reagents over a range of concentrations resulted in differential inactivation of the two functions (G.N.S., unpublished observation). In addition, the catalytic potential of the enzyme has been reported to be differentially altered in a tissue-specific manner (3) in some cases of HLAL deficiency. It has not been excluded that some WD and CESD allele mutations could affect lysosomal targeting (21) or RNA splicing in distinct ways in different tissues.

While preliminary work in this laboratory has suggested that structural gene defects are also present in CESD cells, experimental results have not ruled out the possibility that some variants of WD or CESD could be partially caused or exacerbated by a defect in another gene product necessary for the structural maturation or subcellular transport of the lipase as opposed to a mutation in the primary structure of the peptide. This could be akin, for example, to the "protective protein" that interacts with lysosomal β-galactosidase and neuraminidase in the endoplasmic reticulum (22).

There are precedents among lysosomal storage disorders for the occurrence of markedly variant phenotypes for allelic disorders, even to the appearance of phenotypic normality in persons with little to no measurable *in vitro* activity of a particular lysosomal enzyme. Many of these effects probably arise from the kinetic interactions in multienzyme pathways (23). In the case of HLAL, however, the reaction involves only a few recognizable substrates, and differences in activity levels should correlate with the alterations in the gene and protein structures provided by these "experiments of nature."

The 5′ flanking sequence of the gene provides no obvious indications of transcriptional regulatory effectors, and the G+C-rich character and the prominence of the Sp1 consensus sequences suggest a "housekeeping" promoter with constitutive expression (24). Restriction site mapping suggests that some of the initially positive clones that did not hybridize with the 3′ untranslated region probe are from the HGL locus, and eventual characterization of these clones could provide the information necessary to contrast the control elements for HLAL and HGL to understand the determinants of regulated or tissue-specific expression of these similar proteins.

Despite the recognized importance of HLAL in the regulation of cholesterol metabolism, difficulties with experimental manipulation of the enzyme have limited progress in understanding its control and physiological role. The observations that CESD patients exhibit premature atherosclerosis and that, conversely, a large subset of coronary artery (25) and cerebrovascular disease (26) patients have low levels of HLAL activity indicate the relevance of this enzyme's function to cardiovascular risk in the general population. This risk can now be explored with newly available molecular genetic tools that can demonstrate the etiology of the enzyme's dysfunction.

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