Cloning of rat lysosomal acid lipase cDNA and identification of the mutation in the rat model of Wolman's disease

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Abstract Lysosomal acid lipase (LAL) is a hydrolase essential for the intracellular degradation of cholesteryl esters and triglycerides. We previously reported a rat model of Wolman's disease (Wolman rat) that is deficient for LAL activity. In this study, we cloned rat LAL (RLAL) cDNA and investigated abnormal LAL gene expression in the Wolman rat. We cloned the RLAL gene from a cDNA library made from normal rat liver mRNA using the human LAL cDNA as a probe, subcloned the RLAL cDNA into pBlueScript vector, and sequenced it. Next, we constructed a cDNA library from a Wolman rat liver, and used the RLAL cDNA as a probe to isolate the Wolman RLAL cDNA for comparison. The normal RLAL cDNA contains 3150 bp including an 1194 bp open reading frame and three poly A signals at the 3′ end. The deduced amino acid sequence contained 397 amino acids, showed 79.9% homology with human LAL, and had the same functional domains at the same sites as human LAL. Northern blot analysis revealed that the RLAL mRNA from normal rat was 3.2 kb in length, while the RLAL mRNA from Wolman rat was only 1.4 kb. Nucleotide sequence analysis showed that Wolman rat LAL cDNA had the same sequence as a RLAL cDNA from the 5′-untranslated region to nt 1101, followed by a 60 bp replacement from nt 1102 to nt 1161 with poly A signal and a 3′ 1.8 kb deletion. The deduced amino acid sequence demonstrated the substitution of ^{367}Ile to Asn, ^{368}Pro to stop codon, and deletion of the C-terminal 29 amino acids. Genomic Southern blot analysis disclosed a large deletion at the 3′ end of the gene. These results identify the molecular defect in the Wolman RLAL, and suggest that the C-terminus of RLAL is essential for the activity and/or stability of the enzyme.—**Nakagawa, H., S. Matsubara, M. Kuriyama, H. Yoshidome, J. Fujiyama, H. Yoshida, and M. Osame.** Cloning of rat lysosomal acid lipase cDNA and identification of the mutation in the rat model of Wolman's disease. *J. Lipid Res.* 1995. **36:** 2212–2218.

Supplementary key words cholesteryl ester storage disease • cholesteryl ester • triglyceride

Goldstein et al. (1) have established the role of lysosomal acid lipase (LAL) in the cellular degradation of low density lipoproteins (LDL). The LDL is taken up by peripheral cells through a specific receptor, the LDL receptor. Endocytic vesicles containing LDL then fuse with lysosomes, and the apolipoproteins, cholesteryl esters, and other lipid constituents undergo hydrolysis by lysosomal enzymes. The lysosomal degradation of cholesteryl esters is catalyzed by LAL. Liberated free cholesterol reduces cellular cholesterol synthesis and LDL receptor production, and stimulates cholesteryl ester formation. LAL also hydrolyzes triglycerides. In fact, LAL is one of the key enzymes in the regulation of triglycerides as well as being vital in lipoprotein and cholesterol metabolism (2, 3).

Wolman's disease (WD) and cholesteryl ester storage disease (CESD) are genetically distinct inborn errors of metabolism associated with a deficiency of LAL activity. Although both are characterized by massive accumulation of cholesteryl esters and triglycerides in most tissues of the body, the clinical phenotypes are quite different. The patients with WD manifest hepatomegaly, malabsorption, steatorrhea, abdominal distention, and adrenal calcification, and uniformly die in early infancy. Most patients with CESD have survived into adult life and many have been asymptomatic except for hepatomegaly with hyperbetalipoproteinemia. The molecular bases under-

Abbreviations: LAL, lysosomal acid lipase; Wolman rat, a model rat of Wolman's disease; LDL, low density lipoproteins; WD, Wolman's disease; CESD, cholesteryl ester storage disease; cDNA, complementary DNA; RLAL, rat lysosomal acid lipase; PCR, polymerase chain reaction; WRLAL, Wolman rat lysosomal acid lipase; HLAL, human lysosomal acid lipase.

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lying the distinctly different phenotypes in humans have not yet been clarified (3).

Patients with CESD show a predisposition to development of premature atherosclerotic disease, suggesting that LAL activity may be relevant in the pathogenesis of atherosclerosis. In freshly isolated mononuclear cells from a patient with atherosclerotic diseases (4, 5) and hypercholesterolemia (6), LAL activity was significantly reduced compared to cells from age-matched controls. Lipid accumulation in the arterial wall may possibly be the consequence of a relative deficiency of LAL.

We have previously reported a new animal model of LAL deficiency in a colony of Donryu rats (7). We described the disorder as an animal counterpart of WD (Wolman rat) on the basis of the severe manifestations, short lifespan, characteristic pathological findings, accumulation of cholesteryl esters and triglycerides, and the deficient activity of LAL (7, 8). In 1991, Anderson and Sando (9) reported cloning of 2.6-kilobase complementary DNA (cDNA) encoding human LAL (HLAL). Its amino acid sequence is 58% and 57% identical to those of human gastric and rat lingual lipase, respectively. Recently, Aslanidis et al. (10) reported the entire structure of the HLAL gene which consists of 10 exons, and also reported the DNA sequence of the putative promoter region. Moreover, Klima et al. (11) described a splice junction mutation producing a deletion of a 72-base exon from the mRNA for HLAL in a patient with CESD, and Anderson et al. (12) reported two different mutations at HLAL gene in patients with WD. These results indicated that CESD and WD can be caused by different defects in the same HLAL gene, but they have not yet explained the biochemical basis of different phenotypes in these two diseases. In this study, we cloned the normal rat LAL (RLAL) cDNA using a part of HLAL cDNA sequence as a probe, and then identified a mutation in the RLAL gene in the Wolman rat.

MATERIALS AND METHODS

Isolation of RNA and DNA

Total RNA and DNA were isolated from the livers of normal and Wolman rats including the heterozygous or homozygous rats. RNA was isolated by acidic phe-

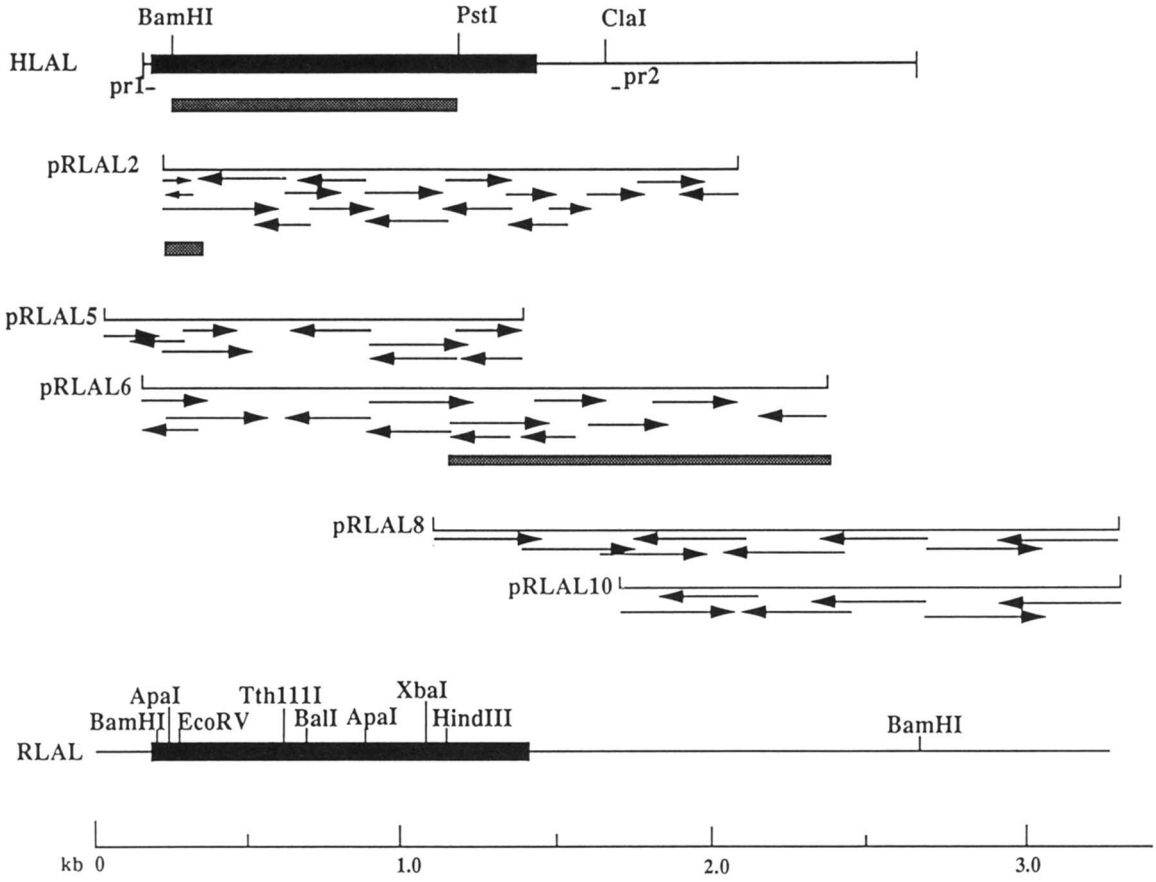

Fig. 1. Restriction map and sequencing strategy for rat lysosomal acid lipase (RLAL). The arrows indicate the direction and the extent of each sequence determination. In the cases in which arrows do not start at restriction sites, 18–20-mer oligonucleotide primers were constructed and used for the extensions. Solid bars represent the coding region. Stippled bars indicate probes used for screening.

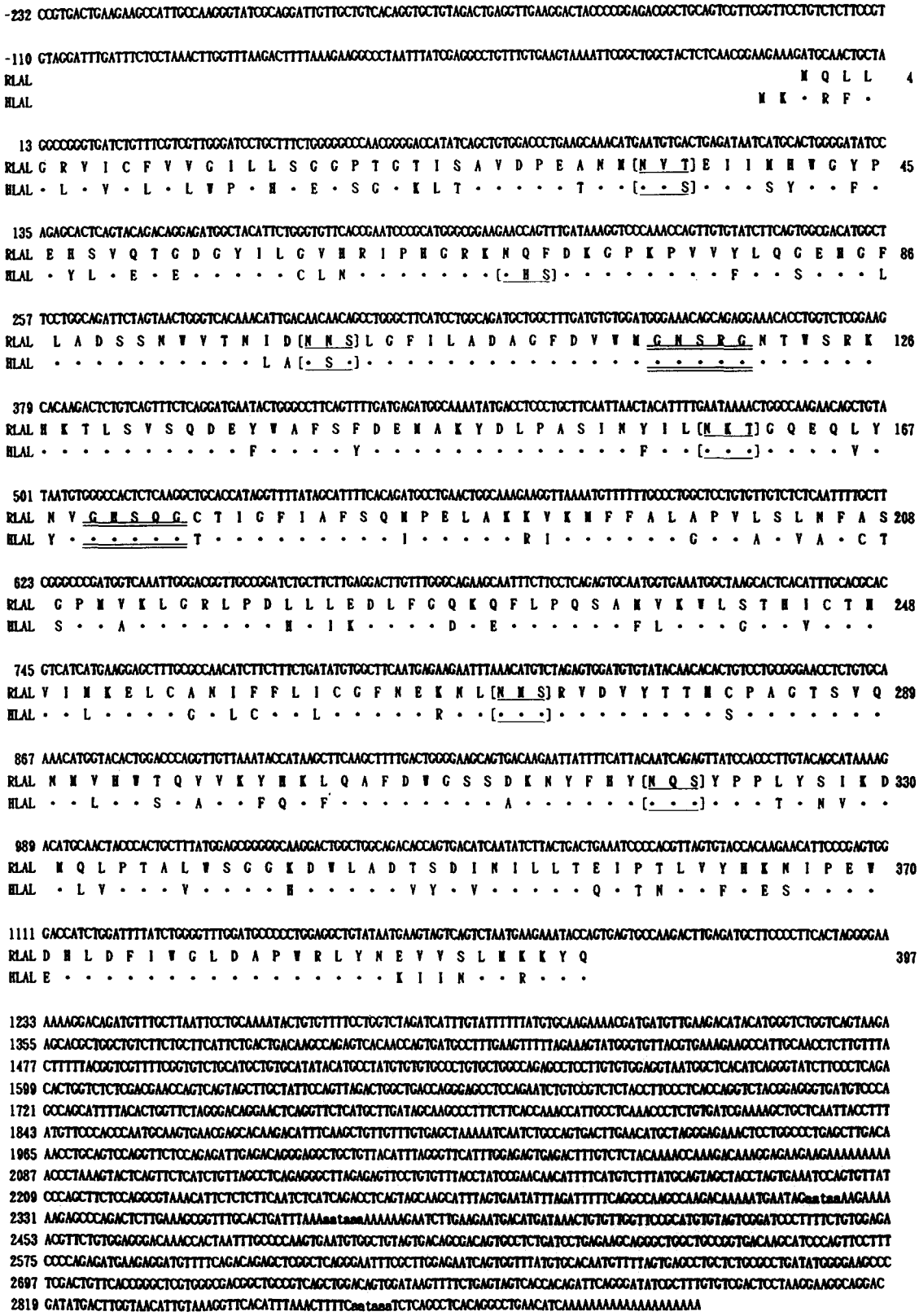

Fig. 2. Nucleotide and deduced amino acid sequence of cDNA encoding rat lysosomal acid lipase (RLAL). The top line represents the nucleotide sequence of the insert in the RLAL cDNA clones; the deduced amino acid sequence (single-letter code) is shown immediately below. On the next line is shown the reported amino acid sequence of related human lysosomal acid lipase (HLAL) proteins; dots (.) denote that amino acid residues identical to those in RLAL are present. Nucleotides and amino acids are numbered starting with the first methionine in the open reading frame; negative numbers represent 5′-untranslated sequence. Nucleotides are numbered on the left, and amino acid residues are numbered on the left. Underlined tripeptides in brackets, [_], represent potential N-glycosylation sites. Doubly underlined pentapeptides represent the potential function motifs discussed in the text. Lower case nucleotides indicate the polyadenylation signals.

nol/chloroform extraction in guanidinium isothiocyanate (13). Poly(A)$^+$ RNA was separated by passing through an oligo(dT) cellulose column (Stratagene Inc., La Jolla, CA) as described (14). Isolation of genomic DNA was carried out as described (15).

Cloning of rat LAL (RLAL) cDNA

Isolated Poly(A)$^+$ RNA (5 μg) from the livers of normal or Wolman rat was primed with oligo(dT) and/or pd(N)$_6$ (Pharmacia LKB Biotechnology Inc., Sweden) and the double-stranded cDNA was synthesized by the method of Gubler and Hoffman (16). cDNA libraries of normal and Wolman rat livers were contracted using λ ZAP and Uni-ZAP (Stratagene Inc.), respectively, according to the suppliers' instructions. Avian myeloblastosis virus reverse transcriptase (Gibco BRL Inc.) with a pd(N)$_6$ was used to synthesize the first-strand cDNA from 5 μg of purified poly(A)$^+$ RNA that had been isolated from HepG2 cells. Oligonucleotide primers for the polymerase chain reaction (PCR) were synthesized using Gene Assembler Plus (Pharmacia LKB Biotechnology Inc.). The 5′-oligonucleotide primer (pr1 in Fig. 1) used was a 19-mer oligonucleotide that corresponded to nucleotide 1-19 of HLAL cDNA. The 3′-primer (pr2 in Fig. 1) used was a 22-mer oligonucleotide specific to nucleotide 1502–1533 of the cDNA (9). PCR was carried out in a 50 μl reaction volume containing 50 ng of the first-strand cDNA as a template, 50 pmol of each primer, and 5 nmol of 4 dNTPs, using 1.5 units of thermostable Taq polymerase (Boehringer Mannheim GmbH, Germany). After a denaturing step, 30 thermal cycles were programmed for 1 min at 94°C, 2 min at 58°C, and 3 min at 72°C. The reactions were terminated and electrophoresed into a 1.0% low melting temperature agarose gel. An approximately 1.5-kb base band was detected by staining with ethidium bromide and excised. After gel purification, the fragment was digested with BamHI and PstI, mixed with vector pBlueScript (Stratagene Inc.) digested with the same restriction enzymes, and then ligated with T4 DNA ligase. Transformed colonies that represented recombinants were screened with nucleotide sequencing using [α-^{35}S]dATP in a double-stranded template version of the Sequenase dideoxy sequencing protocol (United States Biochemicals, Cleveland, OH). cDNA clones of RLAL were isolated from 10^6 recombinant phages by the method of Benton and Davis (17), under low stringency conditions using a BamHI/PstI restriction fragment containing 923 bp of coding sequence of HLAL cDNA as a hybridization probe (Fig. 1). Plaque hybridization was performed with nick-translated cDNA (radiospecific activity, 8.0×10^7 cpm/μg) as the probe. The reaction was carried out at 65°C in 20 mM Tris buffer (pH 7.5) containing 1 M NaCl, 10 mM EDTA, 0.1% sodium N-laurylsarcosinate, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% bovine serum albumin, and 100 μg/ml salmon sperm DNA. The filters were washed twice at 50°C for 30 min in 0.5 × NaCl/Cit (NaCl/sodium citrate; 0.15 M/0.015 M) containing 0.1% sodium N-laurylsarcosinate. Positive clones were purified by four rounds of plaque purification and DNA inserts were subcloned into pBlueScript vector at the EcoRI site. For cloning of Wolman rat LAL (WRLAL) cDNA, clones (5×10^5) were plated without amplification and screened with a EcoRI/HindIII restriction fragment of the RLAL cDNA as a probe. After plaque purification, three clones were isolated and subcloned into the EcoRI/XhoI site of pBluescript SK(-).

Northern analysis

Purified poly(A)$^+$ mRNA (5 μg) was denatured, and electrophoresed on 0.8% agarose containing 2.2 M formaldehyde, and then transferred onto nylon mem-

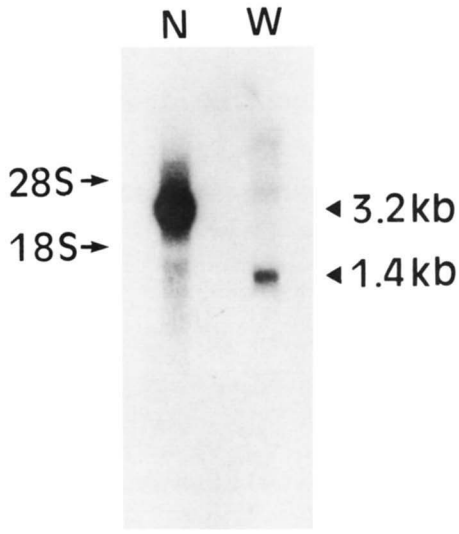

Fig. 3. Northern blot analysis of liver poly(A)$^+$ RNA. Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography and separated by electrophoresis as described under Materials and Methods. Quantities of poly(A)$^+$ RNA used were 5 μg. Filter was hybridized with the ^{32}P-labeled cDNA of rat lysosomal acid lipase (RLAL) as probe. The autoradiographic signal resulted from a 36-h exposure. The positions of rRNA size markers are shown on the right; N, normal rat liver; W, Wolman rat liver.

branes. The filters were hybridized with a ^{32}P-labeled 108 bp BamHI/RsaI restriction fragment with the 5′-coding region of RLAL cDNA (radiospecific activity, 1.2 × 10^8 cpm/μg) as described (18), then were washed twice at 55°C for 30 min in 0.1 × NaCl/Cit solution containing 0.2% sodium N-laurylsarcosinate.

Southern blot analysis of chromosomal DNA

Purified genomic DNAs (5 μg) were digested with several restriction enzymes, and electrophoresed in a 1.0% agarose gel and blotted to nylon membrane in 0.4 M NaOH. The blots were prehybridized, hybridized with ^{32}P-labeled probes, and then washed as described in Northern blot analysis.

RESULTS

Isolation and characterization of RLAL cDNA clones

Figure 1 shows the restriction map of RLAL cDNA which was constructed by analysis of overlapping cDNA clones. Regions of clones that were sequenced on both or either strand are shown by arrows. A search in the GenBank and EMBL databases indicated that the RLAL amino acid sequence has similarities to those published for human LAL, human gastric lipase, and rat lingual lipase. **Figure 2** shows the nucleotide sequence and the amino acid sequence deduced from the longest open reading frame of the cDNAs. RLAL cDNA consisted of 3150 bp nucleotides including 1194 bp open reading frame and three poly A signals at the 3′ end. The RLAL message had a nucleotide sequence that was 75.5% identical to HLAL. The deduced amino acid sequence of 397 amino acids showed 79.9% identity with the deduced HLAL peptide sequence and had two similar functional domains at the same sites as the HLAL.

Northern blot analysis

The radiolabeled partial RLAL cDNA hybridized to a mRNA with a length of approximately 3.2 kb in normal rats. On the other hand, the same probe hybridized to a mRNA with a length of approximately 1.4 kb in Wolman rats. The hybridization to Wolman rat mRNA was weaker than that to normal rat mRNA (**Fig. 3**).

Isolation and characterization of Wolman rat LAL (WRLAL) cDNA clones

Three different clones were obtained and analyzed with several restriction enzymes. The restriction maps of these three cDNAs showed the same cleavage sites in their

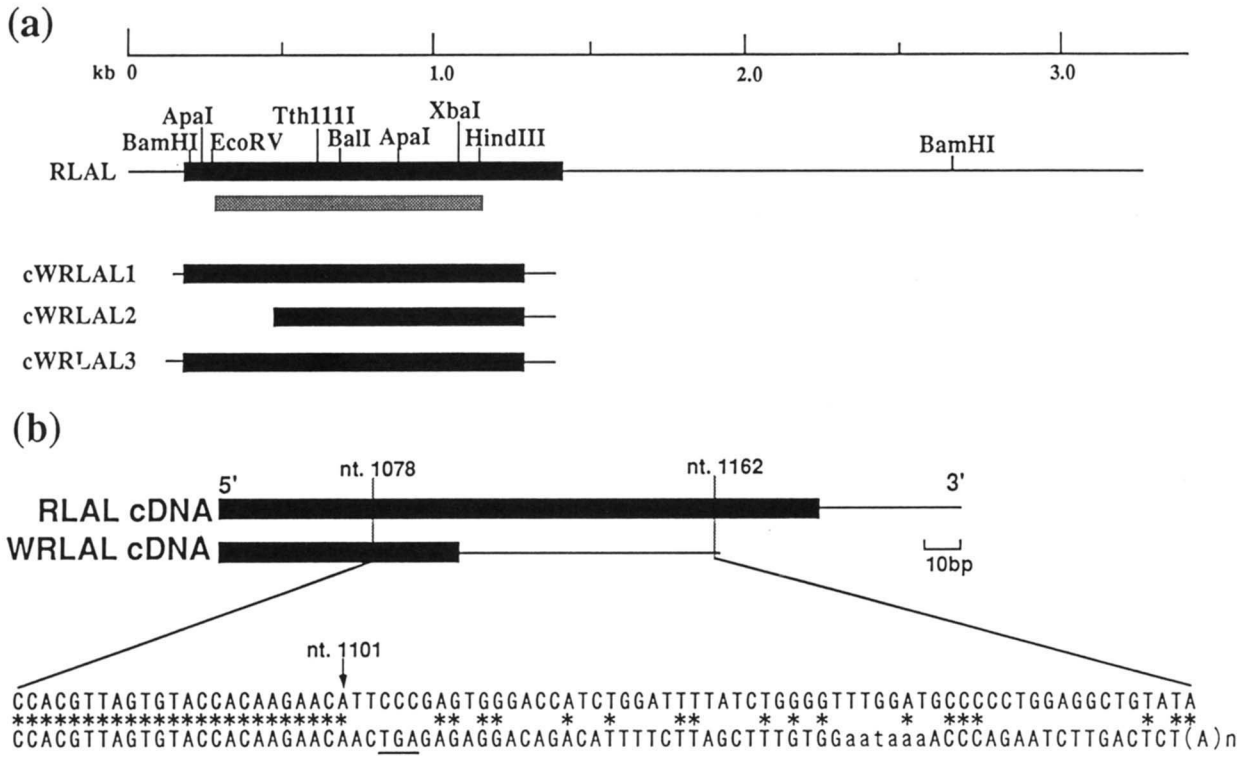

Fig. 4. Structures of RLAL cDNA (normal rat) and WRLAL cDNA (Wolman rat). A thick portion in each bar shows coding region of each cDNA. (a) A hatched bar indicates the probe for screening of cDNA from Wolman rat liver. This 1.0 kb probe is the EcoRV-HindIII fragment of normal rat LAL cDNA. Three positive cDNA clones from the Wolman rat liver cDNA library are shown as cWRLAL1 to 3. (b) A 3′-part of the open reading frame of RLAL cDNA and WRLAL cDNA is shown. Nucleotide sequence from nt. 1078 to nt. 1162 of the normal RLAL cDNA (upper line) and that of WRLAL cDNA (lower line) are aligned. Asterisks indicate the same nucleotides between each sequence.

overlapping regions. Two clones were sequenced in their entirety. The relationship between RLAL and WRLAL cDNAs is shown in **Fig. 4.** Nucleotide sequence analysis showed that the WRLAL cDNA had the same sequence as the RLAL cDNA from 5′-untranslated region to nt 1101. The analysis also revealed 60 bp replacement from nt 1102 to nt 1161 with poly A signal and 1.8 kb deletion. The sequence of the replaced 60 bp was not found in any other reported nucleotide sequences in GenBank and EMBL. Deduced amino acid sequence demonstrated the substitution of ^{367}Ile to Asn, ^{368}Pro to stop codon, and a deletion of 29 amino acids (Fig. 4b).

Southern blot analysis of chromosomal DNA

Two probes (probes 1 and 2) were used for the analysis of genomic southern blot. Probe 1 hybridized to both RLAL and WRLAL DNAs while probe 2 hybridized to only RLAL DNA. As shown in **Figure 5,** probe 1 hybridized with EcoRI or PstI digested DNA from normal, heterozygous and homozygous Wolman rats. Probe 2 could not detect any band in both EcoRI and PstI digested DNA from a Wolman rat. While an approximately 10-kb band was seen in EcoRI-digested normal rat DNA that hybridized with both probes 1 and 2, a 5.5-kb band was observed in EcoRI-digested Wolman rat DNA that hybridized only with probe 1. These observations indicated that around 4.5-kb DNA was deleted in RLAL gene. To confirm these results, we also utilized several other probes and restriction enzymes which gave similar results (data not shown).

DISCUSSION

The goal of this study was to characterize the molecular genetic basis for the rat lipid storage disorder. As genomic organization and sequence of the RLAL had not yet been elucidated, we cloned and sequenced the normal RLAL cDNA. To do this, we screened the rat liver cDNA library using the PCR product from a part of the HLAL cDNA sequence (9) as a probe. Evidence that the cDNA clone isolated (pRLAL6) represented the full coding sequence for RLAL included: *1)* the length of insert corresponded to that of the unique hybridizing band on a Northern blot of poly(A)$^+$ RNA from a cell expressing the gene; *2)* the open reading frame was the size expected to encode a protein the size of RLAL, and it showed homology along the entire coding sequence for HLAL; *3)* the deduced amino acid sequence showed 79.9% homology with HLAL and had the same two functional domains at the same sites as HLAL; and *4)* expression of the recombinant plasmid in COS cells produced enzymes with acid hydrolase activity toward triolein (data not shown).

Next, we analyzed a cDNA library from a Wolman rat liver using a part of RLAL cDNA as a probe. Our analysis revealed that RLAL mRNA in Wolman rat had a 1.8-kb deletion and a 60-bp replacement from nt 1102 to nt 1161 with poly A additional signal. This 60 bp replacement, which was not seen in any other sequence reported in data bank, made an unexpected stop codon at codon 368 in normal RLAL, which resulted in a truncated enzyme protein missing the C-terminal 29 amino acids. Further-

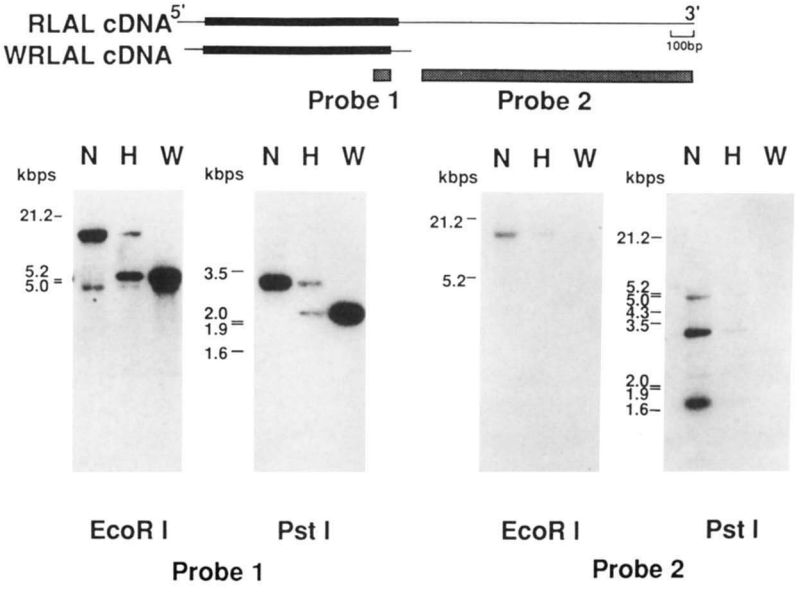

Fig. 5. Southern blot analysis of genomic DNAs from rat livers. Digestion of genomic DNAs (5 μg) and Southern blot hybridization analysis were performed as described under Materials and Methods. RLAL and WRLAL cDNA structures are shown in the top of this figure. Probe 1 and probe 2, both hatched, are designed to distinguish RLAL from WRLAL genomic sequences; N, normal rat DNA; H, heterozygous Wolman rat DNA; W, homozygous Wolman rat DNA.

more, genomic Southern blot analysis showed a corresponding deletion in genomic DNA. Transfection study in COS-cells confirmed that no detectable enzyme activity was expressed from pWRLAL1. Usually, an abnormal mRNA results from aberrant splicing or deletion of genomic DNA. Abnormally short mRNA usually use poly A signals that exist in the original mRNA. The exact mechanism of acquisition of the additional poly A signal in this genomic deletion remained unclear. The deletion did not include the region encoding the putative functional site of the esterase activity, the Gly-Xaa-Ser-Xaa-Gly sequence. Also, the mutation did not affect potential sites for N-glycosylation, and no cysteine residue was included within the 29 amino acid deletion. Nevertheless, there appeared to be a significant reduction of the activity of the truncated protein. This reduced activity might be related to conformational changes in the protein structure. Alternatively, the defective protein might be degraded more rapidly or not be transported properly to the lysosome.

Consequences of a deficiency of LAL are seen in two human genetic diseases, WD and CESD. Both are characterized by severely reduced LAL and intralysosomal lipid accumulations. However, the clinical manifestations of the two disorders are dramatically different. Recently, Klima et al. (11) reported that a combination of one allele which is abnormally spliced due to a point mutation, leading to a 72 bp deletion from the mature mRNA and resulting in deletion of the codons for amino acids 254-277, with a null allele caused the clinical expression of CESD in one patient. Subsequently, Anderson et al. (12) found two different mutations in the LAL gene in patients with WD; one mutation involved the insertion of a T residue after position 634, resulting in the appearance of an in-frame translation stop signal 13 codons downstream. Another mutation, a T-to-C transition at nucleotide 638, results in a leucine-to-proline substitution at amino acid 179. These results indicated that CESD and WD can be caused by different mutations in the HLAL gene, but have not yet accounted for the different phenotypes in these two disorders.

The 10-kb genomic deletion in the RLAL gene of the Wolman rat model induced an abnormal mRNA possessing an additional poly A signal. The unexpected emergence of the additional poly A signal allows the mRNA to be processed and translated. Deletion of 29 amino acids from the carboxy terminal of the product of this mRNA apparently causes the reduction in RLAL enzyme activity, resulting in the rat animal model of Wolman's disease.

Manuscript received 8 June 1995 and in revised form 7 July 1995.

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