Lipoprotein lipase deficiency due to a 3' splice site mutation in intron 6 of the lipoprotein lipase gene

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Abstract In a patient with primary hyperchylomicronemia as a result of lipoprotein lipase (LPL) deficiency, we sequenced all translated exons and intron-exon boundaries of the LPL gene. We found a C→A mutation in position −3 at the acceptor splice site of intron 6 which caused aberrant splicing. The major transcript showed a deletion of exons 6 through 9 and amounted to about 3% of the normal transcript of a healthy control individual. In addition to this major transcript, we found trace amounts of both a normally spliced LPL mRNA and a second aberrant transcript devoid of exon 7. On the same allele, we detected in the LPL gene of our patient four polymorphic variations, three of which have not as yet been described. A second patient from an unrelated family, but from the same geographic area, was also found to be homozygous for the same mutation. Of the relatives of the two probands studied, 11 were heterozygous and 5 were unaffected by the mutation. LPL activity in postheparin plasma was near zero in the probands and reduced in 4 of the 10 heterozygotes. A third hyperchylomicronemic patient from the same area was found to be a compound heterozygote who carried on one allele the 3' splice site mutation of intron 6 and on the other one an already described missense mutation resulting in Gly188→Glu substitution.—**Hölzl, B., R. Huber, B. Paulweber, J. R. Patsch, and F. Sandhofer.** Lipoprotein lipase deficiency due to a 3' splice site mutation in intron 6 of the lipoprotein lipase gene. *J. Lipid Res.* 1994. **35:** 2161–2169.

Supplementary key words hyperchylomicronemia • polymerase chain reaction • mismatch amplification mutation assay

Lipoprotein lipase (LPL, EC 3.1.1.34) hydrolyzes plasma triglycerides, transported in chylomicrons and very low density lipoproteins, thereby providing free fatty acids to serve numerous metabolic and structural tasks (1). The enzyme is expressed in various tissues, with adipose tissue, heart muscle, and lactating mammary gland showing the highest transcriptional activities (2). From the site of synthesis, the enzyme is translocated to its site of action on the luminal surface of the endothelium, from where it can be released by intravenous injection of heparin. The human LPL gene codes for a protein of 475 amino acids including a signal sequence of 27 amino acids. The gene is located on chromosome 8 (3). It spans more than 30 kb, and consists of 10 exons and 9 introns. Exons 1–9 are 105–276 bp long. The first base of exon 10, which consists of 1948 bp, is the last base of the stop codon, the rest being untranslated (4–6).

LPL deficiency is a rare autosomal recessive disorder, characterized by extremely high triglyceride levels in fasting serum due to accumulation of chylomicrons, often accompanied by eruptive xanthomas and recurrent episodes of pancreatitis. LPL activity in postheparin plasma is markedly decreased or absent. In the LPL gene, a large number of mutations responsible for LPL deficiency has been described (7), comprising various missense and nonsense mutations, one insertion, one duplication, and different splice site mutations. Three cases of single base substitution in exon–intron boundaries of the gene resulting in splicing defects have been reported so far: a G→C mutation at position −1 of intron 1 (8), a G→A mutation at the first nucleotide of intron 2 (9), and a G→A mutation at the last nucleotide of intron 2 (10). Thus, in all these cases the highly conserved two bases of donor or acceptor splice sites are affected.

A G→A mutation at codon 188 of exon 5 resulting in a Glu for Gly substitution has been observed in many families of various European and non-European ancestries (11, 12). We have also described three patients homozygous for this mutation in two families from a valley near the city of Salzburg (13). We now report on three patients presenting with primary hyperchylomicronemia due to LPL deficiency from three families from the same geographic area. Two of the probands are homozygous for an acceptor splice site mutation in intron 6 not described as yet, and one proband is a compound heterozygote carrying the newly described splicing mutation on one allele and the G→A mutation in codon 188 on the other.

Abbreviations: LPL, lipoprotein lipase; HDL, high density lipoprotein; PCR, polymerase chain reaction; MAMA, mismatch amplification mutation assay.

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SUBJECTS AND METHODS

Subjects

In this study, we investigated the molecular defect underlying type I hyperlipemia in three patients from a secluded valley near the city of Salzburg. All patients presented with markedly elevated plasma triglycerides and hepatosplenomegaly. One of them had a history of acute bouts of pancreatitis. None of the three patients had eruptive xanthomas.

Plasma lipids

Blood was drawn into EDTA after an overnight fast. Total serum cholesterol and triglycerides were determined enzymatically using the Monotest kit (Boehringer Mannheim, Germany). HDL cholesterol was determined after precipitation of apoB-containing lipoproteins.

Measurement of LPL activity

For determination of LPL activity, 60 IU of heparin per kg body weight was injected intravenously. Ten minutes after this injection blood was collected on ice. LPL activity was measured as described previously (13).

DNA preparation and sequencing

Total genomic DNA was prepared from blood leucocytes using the Triton X-100 method (14). Fragments containing the individual exons of the LPL gene including the exon–intron boundaries were amplified by polymerase chain reaction (PCR). The oligonucleotide primers used were the same as described by Gotoda et al. (15) and Paulweber et al. (13). PCR fragments containing exons 1, 2, 3, 4, 6, 7, 8 and a fraction of exon 10 were subcloned into plasmid pGEM-T (PROMEGA, Madison, WI) and sequenced by the dideoxy method with Sequenase (U.S. Biochemical Corporation, Cleveland, OH). Exon 9 was analyzed by direct sequencing of a double stranded PCR fragment with Taq polymerase (U. S. Biochemical Corp.). Exon 5 was analyzed by direct sequencing of a single-stranded template by the dideoxy method with Sequenase. Single-stranded template was produced by asymmetric PCR. cDNA fragments were sequenced after subcloning into plasmid pGEM-T (PROMEGA).

Sfc I fragment analyses

A fragment containing the 3' splice site of intron 6 of the LPL gene was PCR-amplified from genomic DNA using oligonucleotide primers A and C (**Table 1**). A single base mismatch of the 3' end of primer A generates an artificial Sfc I restriction site only when the wild type allele serves as a template. As complete digestion of the amplified fragment by Sfc I could not be obtained, this approach could be used only to distinguish between homozygous and heterozygous individuals. To distinguish between heterozygous and normal subjects a mismatch amplification mutation assay (MAMA) was used (see below).

TABLE 1. Oligonucleotide primers used in this study

A	5' CAA CAT GTT CGA ATT TCC TCC CCT A 3'
B	5' GTT CGA ATT TCC TCC CCA GA 3'
C	5' CAG GAG AGG GAC TGG TGC CAT GAT G 3'
D	5' CCT AAC TTT GAG TAT GCA GAA GCC 3'
E	5' CTT CCT CCA CTT CAT TCT TCA CAG 3'
F	5' GAG TCG TCT TTC TCC TGA TGA TGC 3'
G	5' CAC ATG CCG TTC TTT GTT CTG TAG 3'
H	5' GAG CTT CAA CAT GAG TAG TTC TCC 3'
I	5' GGC AGA CCG AGA TGA ATC CTC A 3'
J	5' CAG GTC CAG GGG TCT TGG TCC 3'
K	5' CAC TAG AGA ATA TTT TCT CTC TCT TAC C 3'
L	5' GAT GGA CAT TGT CCA GAG GGT AGT TGA A 3'
M	5' AAG TTT CCA CAA ATA AGA AA 3'
N	5' GAG CTT CAA CAT GAG TAG TTC TCC 3'

Use of primers A–N for a particular application is referred to in the body of the text.

Ava II fragment analyses

The previously described G→A missense mutation in exon 5 (cDNA position 818) abolishes an AvaII restriction site (13). To detect this mutation, digestion of PCR fragments with AvaII was performed.

EcoR I fragment analyses

For analysis of the polymorphic site at cDNA position 609 of the LPL gene, a 190 bp fragment containing this site was amplified using PCR primers K and L (Table 1). A single mismatch at the 3' end of primer G results in an artificial EcoR I restriction site with the mutant allele.

Mismatch amplification mutation assay (MAMA) (16)

To detect the splice site mutation in intron 6 and the silent mutation in exon 8, a mismatch amplification mutation assay was performed. Using primers B and C (Table 1) a 190 bp fragment containing the acceptor splice site of intron 6 was amplified. The mutated allele could be amplified selectively, whereas no amplification of the wild type allele was obtained (15 cycles with denaturing at 96°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min, followed by 15 cycles with annealing at 58°C for 1 min, denaturing and extension as in the first 15 cycles). This assay allowed us to distinguish normal subjects from heterozygous ones on one hand and from homozygous individuals on the other. To detect the silent mutation in exon 8 an analogous approach was taken by using primers M and N (Table 1) under the following PCR conditions: 30 cycles with denaturation at 96°C for 1 min, annealing and extension in one step at 50°C for 1 min.

RNA isolation

Total cellular RNA was isolated from about 200 mg of adipose tissue using the acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (17). Adipose tissue from one patient was obtained by biopsy of subcutaneous fat. Adipose tissue obtained during abdominal surgery was used to isolate RNA from a control individual.

cDNA synthesis and RT-PCR analyses (18)

cDNA synthesis was performed from 1.0 μg of total RNA using the Superscript Preamplification System (Gibco BRL, Gaithersburg, MD). Oligo(dT) was used for priming. First strand cDNA was extracted with Tris-EDTA saturated phenol-chloroform, precipitated with ethanol and diluted in 50 μl water. Five μl cDNA was amplified in a first PCR using primers D and E (Table 1) at a final concentration of 250 nM, thus creating a cDNA fragment extending from exon 5 to exon 10 of the LPL gene. PCR amplification was performed under the following conditions: 1 min denaturation at 96°C, 1 min primer annealing at 55°C, 1 min extension at 72°C, 15 cycles. To amplify target regions, 3/100 of the first PCR product was subjected to a second PCR with a pair of internal primers (F, G or F, H in Table 1). Two ng of P^{32}-labeled primer F was added to each reaction. PCR was performed for 20 or 25 cycles under the same conditions as described above. To ensure that the PCR was done in a quantitative fashion, normal cDNA template was used in a series of dilutions. Amplification of a 143 bp S14 ribosomal protein cDNA fragment served as an internal control (primers I and J in Table 1). Reaction products were analyzed on a denaturing 8 M urea-5% polyacrylamide gel.

RESULTS

Plasma lipids and LPL activity

Lipid values and LPL activities are summarized in **Table 2.** The three hyperchylomicronemic patients showed markedly elevated plasma triglycerides and markedly decreased HDL cholesterol. LPL activity in postheparin plasma of the three patients was absent or severely reduced. The LPL activities of four heterozygous family members were below the range of the control group; six heterozygotes were within the normal range giving rise to a considerable overlap between heterozygous and normal individuals. The pedigrees of the three families are shown in **Fig. 1.**

TABLE 2. Characteristics of members studied from three families with LPL deficiency

Genotype of 3'ss Mutation in Intron 6	Subject Number[a]	Age	Sex	Triglyceride	Cholesterol	HDL-Chol	LPL-activity	G→A Exon 4 Pos. 609	C→A Exon 8 Pos. 1338
		yr			*mg/dl*		*mmol FFA min^{-1} ml^{-1}*		
Homozygous	11	27	M	2180	277	18	5	+/+	+
	18	7	M	6560	427	15	17	+/+	+
Compound heterozygous	20	23	F	3170	225	13	0	+/−	+
Heterozygous	2	66	F	128	250	77	264	+/−	+
	3	59	M	228	294	44	320	+/−	+
	4	61	M	162	255	42	257	+/−	+
	5	63	F	160	267	48	250	+/−	+
	7	34	M	177	246	38	134	+/−	+
	8	32	F	142	208	39	280	+/−	+
	10	33	M	130	228	43	191	+/−	+
	12	24	M	147	206	41	171	+/−	+
	13	33	M	219	188	37	n.d.	+/−	+
	16	27	F	72	157	62	163	+/+	+
	17	29	M	117	198	48	261	+/−	+
Unaffected by mutation	1	63	F	106	289	63	228	−/−	−
	6	35	M	61	233	59	305	−/−	−
	9	26	F	96	236	82	257	−/−	−
	14	3	F	62	190	63	n.d.[b]	−/−	−
	15	3	F	86	194	64	n.d.	−/−	−
	19	27	F	41	162	72	n.d.	−/−	−
Controls (n = 50)							287.3 ± 71.0[c]		

[a]The numbers of the subjects correspond to the numbers in Fig. 1.
[b]n.d., not determined.
[c]Mean ± SD.

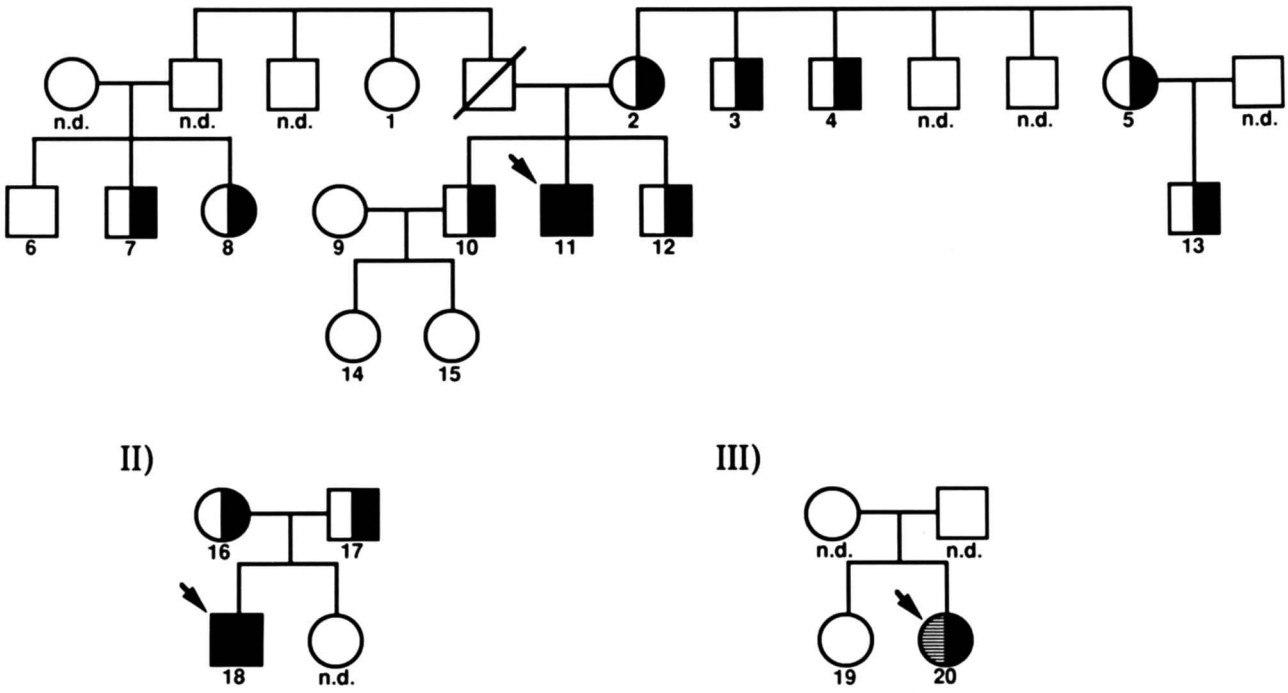

Fig. 1. Pedigrees of the three families. Black symbols identify the splice site mutation in intron 6 and the shaded symbol represents the Gly→Glu 188 missense mutation in exon 5. The numbers below the symbols correspond to the numbers of the probands listed in Table 2. The arrows indicate the three patients with hyperchylomicronemia. Patients 11 and 18 are homozygous for the splice site mutation; patient 20 is a compound heterozygote carrying the splice site mutation on one allele and the Gly→Glu 188 missense mutation on the other; n.d., not determined.

Genetic analyses

The entire coding region of the LPL gene including the exon–intron boundaries of patient number 11 was sequenced. The following point mutations were found: A C→A mutation of intron 6 (position −3) affecting the acceptor splice site (**Fig. 2**), an A→C mutation in intron 7 not altering splice site (position −23), a silent G→A mutation in exon 4 (cDNA position 609), and a silent C→A mutation in exon 8 (cDNA position 1338) (**Fig. 3**). In the untranslated region of exon 10 (cDNA position 1661) an A was found instead of a G, which has been described as a common polymorphism (5).

To investigate the effect of the mutation at the acceptor splice site of intron 6, RNA was isolated from adipose tissue of patient number 11 and a healthy control subject. In the patient and the control, cDNA was synthesized from equal amounts of total RNA as assessed by amplification of an internal control (S14 ribosomal protein). When cDNA was amplified with primers located in exons 5 and 10 (D and E for 15 cycles followed by 20 cycles with nested primers F and G), in the control the expected fragment of 880 bp was obtained as a strong band (**Fig. 4**). In the patient, however, only a smaller fragment of 229 bp was detected (Fig. 4). This band was much weaker than the normal band. When cDNA from the control was diluted 32-fold, the bands of the normal and abnormal transcript showed similar intensity (Fig. 4). From these results we concluded that the amount of the abnormal transcript was about 3% of that of the normal transcript from a control subject.

To suppress amplification of the 229 bp fragment, in the second PCR primer E (Table 1), which is located in exon 10, was substituted by a primer located in exon 8

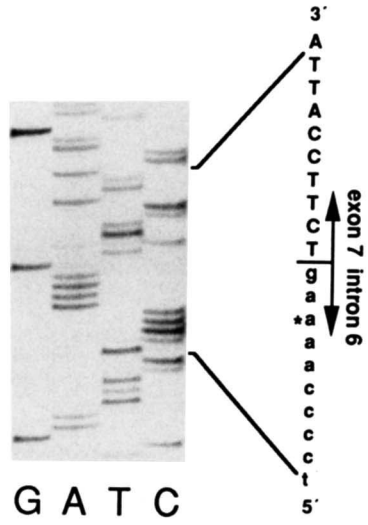

Fig. 2. Genomic sequences showing the C→A transition in the 3' splice site of intron 6. The position of the mutation is marked by an asterisk.

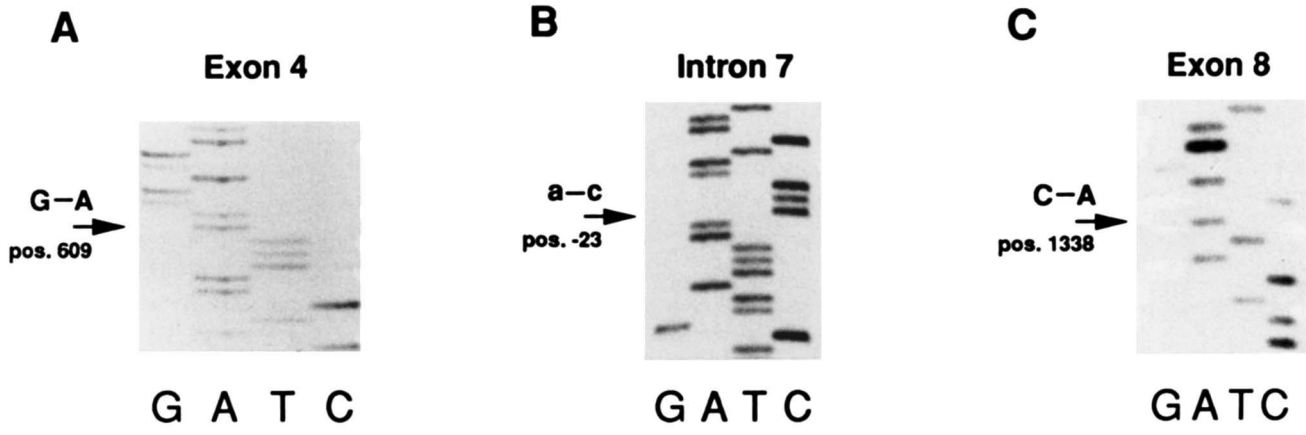

Fig. 3. Sequences demonstrating the silent mutation in exon 4 (A), the mutation in intron 7 (B), and the silent mutation in exon 8 (C). All these mutations turned out to be polymorphic variations.

(primer G), and amplification was performed for 5 more cycles. In a normal control the expected fragment of 579 bp was obtained (**Fig. 5**). In the patient, amplification resulted in either a fragment of normal length or a shorter fragment of 456 bp, both at very low amounts (Fig. 5). The second aberrant transcript and the normal transcript could be detected only by using the strategy described, i.e., suppressing the major transcript and adding 5 more cycles of amplification. From these results we conclude that both the transcript devoid of exon 7 and the normal transcript are expressed at an even lower level than the transcript lacking exons 6 through 9.

The two aberrant fragments were subcloned and sequenced. Sequence analysis of the 229 bp fragment indicated deletion of exons 6 through 9 (**Fig. 6**), thereby abolishing the normal stop codon and producing instead 12 codons downstream a new TGA stop codon in exon 10. Analysis of the 456 bp fragment indicated deletion of exon 7 (Fig. 6) causing a shift in the reading frame. The deletion creates 12 codons downstream a TAA stop codon in exon 8.

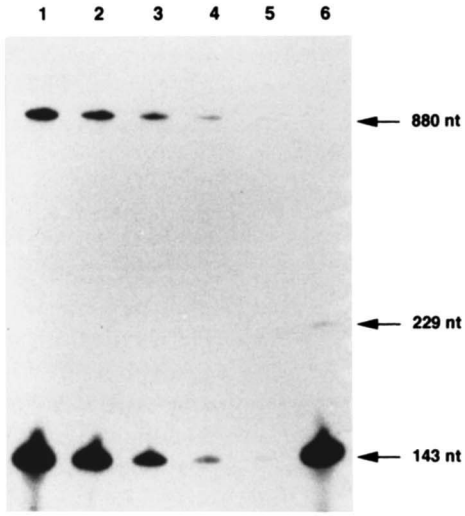

Fig. 4. Quantitative RT-PCR analyses with primers in exons 5 and 10. First strand cDNA was synthesized from 1.0 μg of total RNA using the Superscript Preamplification System (Gibco BRL, Gaithersburg, MD). This first strand was amplified by PCR using primers D and E (Table 1) at a final concentration of 250 nM; 3/100 of this first PCR product was subjected to a second PCR using 250 nM of nested primers F and G (Table 1). Two ng of ^{32}P-labeled primer F was added to each reaction. PCR products were analyzed on a denaturing 8 M urea 5% polyacrylamide gel and visualized by autoradiography. In a control subject, an 880 nt fragment was obtained as a strong band (lane 1), whereas in patient No. 11 (Table 2) only a smaller fragment of 229 nt was detected as a faint band (lane 6). To ensure that the PCR was performed in a quantitative range, normal cDNA was used in 4-fold (lane 2), 16-fold (lane 3), 32-fold (lane 4), and 64-fold (lane 5) dilutions, respectively. When the cDNA template of the control was diluted 32-fold, bands of the normal and abnormal transcript showed similar intensity. A 143 nt fragment of S14 ribosomal protein cDNA was used as an internal standard. This fragment was coamplified in each reaction using primers I and J (Table 1). Primer I was labeled with ^{32}P. As can be appreciated from this figure, the 143 nt bands in lane 1 (undiluted control cDNA) and in lane 6 (cDNA from patient No. 11) appear to be very similar. The size of the fragments was assessed by comparison with an ethidium-bromide-stained molecular weight standard run on the same gel.

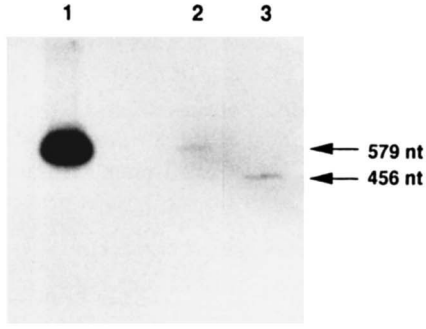

Fig. 5. RT-PCR analyses with primers in exons 5 and 8. cDNA synthesis, PCR, and gel electrophoresis were performed as described in Fig. 4 with the following modifications. Primer H (Table 1) was used instead of primer G, and 5 more cycles were performed for the second PCR. The transcript of the normal subject (579 nt) is shown in lane 1. A fragment of the same size (lane 2) and a smaller fragment of 456 nt (lane 3) could be obtained in the patient. These two bands from the patient were extremely weak and could be detected only after repeated PCR experiments, indicating extremely low abundance of these transcripts. Both fragments were never obtained in the same PCR experiment.

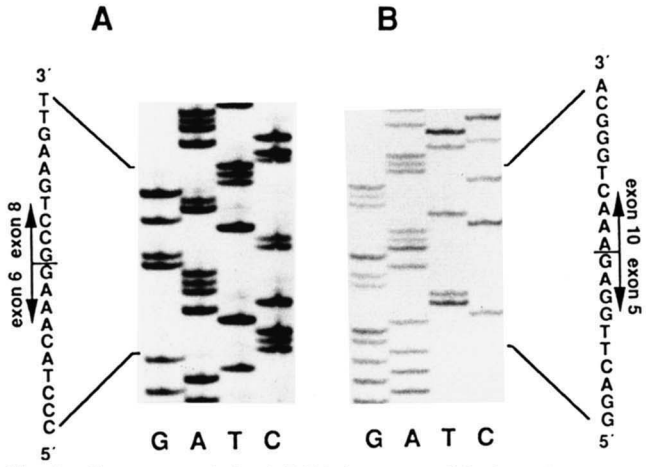

Fig. 6. Sequence analysis of cDNA fragments of the hyperlipemic patient (No. 11 in Table 2). After PCR amplification the fragments were subcloned in plasmid pGEM-T and sequenced. In the fragment of 456 bp, exon 7 was found to be skipped (A), in the 229 bp fragment exons 6 through 9 were found to be deleted (B).

To examine the other two patients and the relatives of all three patients for the 3' splice site mutation in intron 6, Sfc I restriction fragment analysis and MAMA were used. Combining the two methods allowed us to distinguish between homozygous, heterozygous, and unaffected individuals. Homozygous carriers of the splice site mutation have no Sfc I restriction site, but show a positive result with MAMA. Heterozygotes are characterized by an Sfc I restriction site and amplification of a specific fragment with MAMA. Homozygous carriers of the wild type allele have an Sfc I restriction site and a negative result with MAMA. All three hyperlipidemic patients had the C→A splice site mutation in intron 6. Hyperlipidemic probands numbers 11 and 18 were homozygous while hyperlipidemic proband number 20 was heterozygous for the mutation (Fig. 1). Of the 17 relatives available for our study, 11 were heterozygous and 6 were unaffected (Fig. 1). In 50 unrelated control individuals the mutation could not be detected.

To evaluate the other mutations found in subject 11, unrelated normolipidemic individuals were studied. The silent A for G substitution at residue 609 in exon 4 was found in 3 of 36 controls and the C→A mutation in exon 8 (cDNA position 1338) in 9 of 36 control subjects. For the A→C mutation in intron 7 (position −23) neither a suitable enzyme for restriction fragment analysis was available, nor a mismatch amplification mutation assay could be developed. Therefore, PCR fragments containing this residue amplified from genomic DNA of two unrelated healthy individuals were sequenced. The A→C mutation could be found in one. These findings indicate that the mutations in exon 4, exon 8, and intron 7 represent DNA polymorphisms, which have not been described as yet.

If one assumes that the splice site mutation observed in subjects from the restricted geographic area originated only once on a given allele, the homozygous carriers of the splice site mutation should also be homozygous for the four polymorphic variations found in these subjects. The other family members were tested only for the polymorphisms in exon 4 and 8. These polymorphic variations could indeed be detected in all patients and in all relatives heterozygous for the splice site mutation. None of these variations could be observed in any of the family members not carrying the splice site mutation. The distribution of these two polymorphisms in the heterozygotes and normals is consistent with the assumption that the splice site mutation and the polymorphisms reside on the same allele.

The hyperlipemic proband number 20 was heterozygous for the splice site mutation in intron 6 and for the Gly→Glu 188 missense mutation in exon 5. As this proband had no LPL activity in the postheparin plasma, it can be postulated that she has no normal LPL allele and therefore is a compound heterozygote. Her sister (No. 19) was unaffected by either of the mutations.

DISCUSSION

Three subjects presenting with the clinical phenotype of primary hyperchylomicronemia were studied. They belong to three families living in the same geographic area, a secluded valley in Austria. No relation between these families is known. LPL activity in their postheparin plasma was near zero, explaining the extremely high triglyceride concentration in their fasting serum. To study the molecular basis of LPL deficiency in these patients, all exons of the LPL gene, except the noncoding part of exon 10, and all the donor and acceptor splice sites were sequenced in one of the hyperlipemic probands (No. 11). A C→A mutation was found in position −3 in the acceptor splice site of intron 6 in both alleles of the LPL gene. The proband was also homozygous for a silent G→A mutation in exon 4 (position 609), a silent C→A mutation in exon 8 (position 1338), and an A→C mutation in intron 7 (position −23) which turned out to be polymorphic variations as they were also observed in unrelated control individuals. In position 1611 of the untranslated region of exon 10, the proband had a G instead of an A nucleotide known to be a polymorphic variation (5). The hyperlipidemic proband number 20 was also found to be a homozygous carrier of the same mutant allele. In the families of these two probands, 11 members were heterozygous for this mutant allele and 5 members were homozygous for the wild type of the LPL gene. The third hyperlipemic proband (No. 20) turned out to be a compound heterozygote carrying on one allele the above described mutation and on the other allele a previously described G→A mutation at position

818 in exon 5 (11, 12) causing a Glu for Gly substitution at residue 188. Finding a compound heterozygote of this type was not entirely unexpected, as the missense mutation Gly→Glu 188 had been found previously in three other patients with type I hyperlipidemia from the same area (13).

The 3' splice site mutation in intron 6 resulted in the loss of either exon 7 alone or of exons 6, 7, 8, and 9 from the patient's mRNA. A transcript of normal size could be detected only in trace amounts. Skipping of exon 7 alone caused a frameshift and the creation of a stop codon 12 codons downstream of the 5' end of exon 8. Skipping of exons 6 through 9 abolished the normal stop codon and created a new stop codon 12 codons downstream within the normally untranslated part of exon 10. The levels of all these transcripts were extremely low. Postheparin LPL activity, confirmed by repeated assays, was zero in the compound heterozygote but not completely absent in the two patients homozygous for the splice site mutation. This finding is explained by the extremely low amounts of the normal transcript. In the compound heterozygote the completely undetectable LPL activity could be explained by an even lower abundance of normal transcript generated by one splice site mutant allele. The Glu188 for Gly mutation from the other allele is known to completely abolish the activity of the enzyme (13). Among the heterozygotes, four subjects had reduced LPL activities in the postheparin plasma, while six had LPL activities in the normal range. This finding contrasts with our observation with heterozygotes carrying the 188 mutation where postheparin LPL activities averaged only 40% of those of the unaffected family members (13, 19). These differences regarding the heterozygotes are no doubt due to the entirely different nature of the two mutations.

The mechanisms of normal and aberrant splicing due to splice site mutations are not yet fully understood. In the nuclear genes of perhaps all eukaryotes, the nucleotides at position +1 and +2 at the 5' splice sites and −2 and −1 of the 3' splice sites are invariant. With regard to these invariant nucleotides, three splice site mutations have been described for the LPL gene so far, one involving the first nucleotide of intron 1 (8), one the first nucleotide of intron 2 (9), and one involving the last nucleotide of intron 2 (10). When compared to the nucleotides at positions 1 and 2, those at position −3 are less well conserved with C being present in 74% and A in 3% (20). In our patients, the C→A mutation in position −3 of the 3' splice site in intron 6 reduced the degree of homology with 3' splice sites from other genes from 89.2% to 77.8% (consensus values were scored by the method of Shapiro and Senapathy (20)).

Mutations in 3' splice sites most frequently result in utilization of the acceptor splice site in the next intron downstream with loss of the respective exon (21–24) or in utilization of a cryptic splice site in the next exon downstream from the mutation with loss of parts of the exon (25). Steingrimsdottir et al. (26) studied the consequences of various 3' splice site mutations in the introns of the hprt gene in transfected fibroblasts. Most of these mutations caused skipping of the next exon downstream or utilization of cryptic splice sites within the next exon downstream, except for one mutation causing loss of the following two exons downstream. These results, however, should be extrapolated to the in vivo situation with some caution because gene expression in transfected cells may differ from that in cells in vivo (27).

According to a recently published review (28), 87% of the 3' splice site mutations affect the invariant AG dinucleotide. To our knowledge, only two examples of mutations in position −3 of the 3' splice site causing several splicing errors have been described to date, one for the β-globin gene (29) and one for the thyroglobulin gene (30). In the β-globin gene, mutations in the acceptor splice site have been observed in intron 2 in an Iranian and Egyptian family, and in intron 1 in a Saudi Arabian family (29). In the case of the intron 2 mutation, both normal splicing and utilization of a cryptic splice site in intron 2 were observed. The total level of β-globin RNA was not markedly different from that of normal β-globin gene. The consequences of the other mutation at position −3 of the acceptor splice site in intron 1 have not been characterized in detail (29). In the thyroglobulin gene, the major transcript lacked only the one exon downstream the mutated 3' splice site, but a minor transcript lacked one exon upstream plus two exons downstream from the mutation (30). This type of aberrant splicing is similar to that described in our study.

The mechanisms leading to the selection of particular splice sites are not completely understood. In the LPL gene, the consensus values for 3' splice sites in introns 5 (94.3%), 7 (88.3%), 8 (96.9%), and 9 (91.3%) are higher than that of the mutated splice site in intron 6 (77.8%) of our proband. The distance from the authentic splice site is another determinant in splice site selection (9). Different scanning mechanisms for splicing appear to exist. Recently, an exon recognition sequence (ERS) has been identified, which together with the 3' splice site (including branch-point, polypyrimidine tract, and the invariant AG motive at the splice site) and the downstream 5' splice site seems to influence splice site selection (31). This downstream stimulation was suggested to play an important role in pre-mRNA with suboptimal consensus sequences at the 3' splice site (31).

In our proband, the abundance of both aberrant transcripts, the one devoid of exon 7 and the one devoid of exons 6–9, was extremely low when compared to normal transcripts. As described above, skipping of exon 7 produced a stop codon in exon 8. Nonsense mutations usually result in decreased levels of mRNA (32–35). The amount of mRNA transcribed from genes with nonsense

mutations, however, can vary considerably (33) and appears not to be related to the position of the mutation (36). On the other hand, nonsense mutations with normal amounts of the specific mRNA have been described for the insulin receptor gene (37) and the LDL receptor gene (38). The mechanisms underlying defective metabolism of nonsense mutated mRNA are not understood (32, 36).

In our patient, therefore, the low level of the aberrantly spliced mRNA lacking exon 7 could be explained by the creation of the nonsense codon in exon 8. Skipping of exons 6–9 abolished the normal stop codon and produced a new stop codon in the normally untranslated exon 10. The low level of this aberrantly spliced mRNA, however, cannot be explained by the new stop codon. We hypothesize that the loss of four exons causes mRNA instability most likely because of profound alteration in the secondary structure of the molecule.

Clearly, mutations of splice sites of introns can have various consequences, which cannot be predicted by the primary sequence alone. Other less well-defined factors such as secondary pre-mRNA structure or mRNA protein interactions may play a role. It is well established that the splicing reaction does not proceed sequentially along the precursor RNA, but seems to follow a preferred pathway. Apparently, mutations in splice sites can disturb this order. The analysis of naturally occurring mRNA splicing errors such as the one described herein may help us to better understand the factors that influence the pathways used by splicing reactions in vivo.

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REFERENCES

1. Olivecrona, T., and G. Bengtsson-Olivecrona. 1987. Lipoprotein lipase from milk: the model enzyme in lipoprotein lipase research. *In* Lipoprotein Lipase. J. Borensztajn, editor. Evener Publishers, Inc., Chicago. 15–77.
2. Semenkovich, C. F., S. H. Chen, M. Wims, C. C. Luo, W. H. Li, and L. Chan. 1989. Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation, and evolution. *J. Lipid Res.* **30:** 423–431.
3. Sparkes, R. S., S. Zollman, I. Klisak, T. G. Kirchgessner, M. C. Komaromy, T. Mohandas, M. C. Schotz, and A. J. Lusis. 1987. Human genes involved in lipolysis of plasma lipoproteins: mapping of loci for lipoprotein lipase to 8p22 and hepatic lipase to 15q21. *Genomics.* **1:** 138–144.
4. Kirchgessner, T. G., K. L. Svenson, A. J. Lusis, and M. C. Schotz. 1987. The sequence of cDNA encoding lipoprotein lipase. A member of a lipase gene family. *J. Biol. Chem.* **262:** 8463–8466.
5. Wion, K. L., T. G. Kirchgessner, A. J. Lusis, M. C. Schotz, and R. M. Lawn. 1987. Human lipoprotein lipase complementary DNA sequence. *Science.* **235:** 1638–1641.
6. Deeb, S. S., and R. L. Peng. 1989. Structure of the human lipoprotein lipase gene [published erratum appears in *Biochemistry* 1989 Aug 8; 28(16):6786]. *Biochemistry.* **28:** 4131–4135.
7. Hayden, M. R., and Y. Ma. 1992. Molecular genetics of human lipoprotein lipase deficiency. *Mol. Cell Biochem.* **113:** 171–176.
8. Chimienti, G., A. Capurso, F. Resta, and G. Pepe. 1992. A G---C change at the donor splice site of intron 1 causes lipoprotein lipase deficiency in a southern-Italian family. *Biochem. Biophys. Res. Commun.* **187:** 620–627.
9. Gotoda, T., N. Yamada, T. Murase, T. Inaba, S. Ishibashi, H. Shimano, S. Koga, Y. Yazaki, Y. Furuichi, and F. Takaku. 1991. Occurrence of multiple aberrantly spliced mRNAs upon a donor splice site mutation that causes familial lipoprotein lipase deficiency. *J. Biol. Chem.* **266:** 24757–24762.
10. Hata, A., M. Emi, G. Luc, A. Basdevant, P. Gambert, P. H. Iverius, and J. M. Lalouel. 1990. Compound heterozygote for lipoprotein lipase deficiency:Ser---Thr244 and transition in 3' splice site of intron 2 (AG---AA) in the lipoprotein lipase gene. *Am. J. Hum. Genet.* **47:** 721–726.
11. Emi, M., D. E. Wilson, P. H. Iverius, L. Wu, A. Hata, R. Hegele, R. R. Williams, and J. M. Lalouel. 1990. Missense mutation (Gly---Glu188) of human lipoprotein lipase imparting functional deficiency. *J. Biol. Chem.* **265:** 5910–5916.
12. Monsalve, M. V., H. Henderson, G. Roederer, P. Julien, S. Deeb, J. J. Kastelein, L. Peritz, R. Devlin, T. Bruin, M. R. Murthy, C. Cagne, J. Davignon, P. J. Lupien, J. D. Brunzell, and M. R. Hayden. 1990. A missense mutation at codon 188 of the human lipoprotein lipase gene is a frequent cause of lipoprotein lipase deficiency in persons of different ancestries. *J. Clin. Invest.* **86:** 728–734.
13. Paulweber, B., H. Wiebusch, G. Miesenboeck, H. Funke, G. Assmann, B. Hoelzl, M. J. Sippl, W. Friedl, J. R. Patsch, and F. Sandhofer. 1991. Molecular basis of lipoprotein lipase deficiency in two Austrian families with type I hyperlipoproteinemia. *Atherosclerosis.* **86:** 239–250.
14. Kunkel, L. M., K. D. Smith, S. H. Boyer, S. D. Borgoankar, S. S. Wachtel, O. J. Miller, W. R. Breg, H. W. Jones, and J. M. Rary. 1977. Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. *Proc. Natl. Acad. Sci. USA.* **74:** 1245–1249.
15. Gotoda, T., N. Yamada, M. Kawamura, K. Kozaki, N. Mori, S. Ishibashi, H. Shimano, F. Takaku, Y. Yazaki, Y. Furuichi, and T. Murase. 1991. Heterogeneous mutations in the human lipoprotein lipase gene in patients with familial lipoprotein lipase deficiency. *J. Clin. Invest.* **88:** 1856–1864.
16. Cha, R. S., H. Zarbl, P. Keohavong, and W. G. Thilly. 1992. Mismatch amplification mutation assay (MAMA): application to the c-H-ras gene. *PCR Methods Applications.* **1:** 14–20.
17. Chomczynski, P., and N. Sacchi. 1986. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
18. Foley, K. P., M. W. Leonard, and J. D. Engel. 1993. Quantitation of RNA using the polymerase chain reaction. *Trends Genet.* **9:** 380–385.
19. Miesenbock, G., B. Hölzl, B. Foger, E. Brandstatter, B.

Paulweber, F. Sandhofer, and J. R. Patsch. 1993. Heterozygous lipoprotein lipase deficiency due to a missense mutation as the cause of impaired triglyceride tolerance with multiple lipoprotein abnormalities. *J. Clin. Invest.* **91:** 448–455.
20. Shapiro, M. B., and P. Senapathy. 1987. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res.* **15:** 7155–7174.
21. Tromp, G., and D. J. Prockop. 1988. Single base mutation in the pro alpha 2(I) collagen gene that causes efficient splicing of RNA from exon 27 to exon 29 and synthesis of a shortened but in-frame pro alpha 2(I) chain. *Proc. Natl. Acad. Sci. USA.* **85:** 5254–5258.
22. Dunn, J. M., R. A. Phillips, X. Zhu, A. Becker, and B. L. Gallie. 1989. Mutations in the RB1 gene and their effects on transcription. *Mol. Cell Biol.* **9:** 4596–4604.
23. Horowitz, J. M., D. W. Yandell, S. H. Park, S. Canning, P. Whyte, K. Buchkovich, E. Harlow, R. A. Weinberg, and T. P. Dryja. 1989. Point mutational inactivation of the retinoblastoma antioncogene. *Science.* **243:** 937–940.
24. Naylor, J. A., P. M. Green, A. J. Montandon, C. R. Rizza, and F. Giannelli. 1991. Detection of three novel mutations in two haemophilia A patients by rapid screening of whole essential region of factor VIII gene. *Lancet.* **337:** 635–639.
25. Su, T. S., and L. H. Lin. 1990. Analysis of a splice acceptor site mutation which produces multiple splicing abnormalities in the human argininosuccinate synthetase locus. *J. Biol. Chem.* **265:** 19716–19720.
26. Steingrimsdottir, H., G. Rowley, G. Dorado, J. Cole, and A. R. Lehmann. 1992. Mutations which alter splicing in the human hypoxanthine-guanine phosphoribosyltransferase gene. *Nucleic Acids Res.* **20:** 1201–1208.
27. Treisman, R., S. H. Orkin, and T. Maniatis. 1983. Specific transcription and RNA splicing defects in five cloned beta-thalassemia genes. *Nature.* **302:** 591–596.
28. Krawczak, M., J. Reiss, and D. N. Cooper. 1992. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum. Genet.* **90:** 41–54.
29. Wong, C., S. E. Antonarakis, S. C. Goff, S. H. Orkin, B. G. Forget, D. G. Nathan, P. J. Giardina, and H. H. Kazazian, Jr. 1989. Beta-thalassemia due to two novel nucleotide substitutions in consensus acceptor splice sequences of the beta-globin gene. *Blood.* **73:** 914–918.
30. Ieiri, T., P. Cochaux, H. M. Targovnik, M. Suzuki, S. Shimoda, J. Perret, and G. Vassart. 1991. A 3' splice site mutation in the thyroglobulin gene responsible for congenital goiter with hypothyroidism. *J. Clin. Invest.* **88:** 1901–1905.
31. Watakabe, A., K. Tanaka, and Y. Shimura. 1993. The role of exon sequences in splice site selection. *Genes & Dev.* **7:** 407–418.
32. Urlaub, G., P. J. Mitchell, C. J. Ciudad, and L. A. Chasin. 1989. Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. *Mol. Cell Biol.* **9:** 2868–2880.
33. Atweh, G. F., H. E. Brickner, X-X. Zhu, H. H. Kazazian, Jr., and B. G. Forget. 1988. New amber mutation in a beta Thalassemic gene with nonmeasurable levels of mutant messenger RNA in vivo. *J. Clin. Invest.* **82:** 557–561.
34. Fojo, S. S., A. F. Stalenhoef, K. Marr, R. E. Gregg, R. S. Ross, and H. B. Brewer, Jr. 1988. A deletion mutation in the ApoC-II gene (apoC-II Nijmegen) of a patient with a deficiency of apolipoprotein C-II. *J. Biol. Chem.* **263:** 17913–17916.
35. Kadowaki, T., H. Kadowaki, and S. I. Taylor. 1990. A nonsense mutation causing decreased levels of insulin receptor mRNA: detection by a simplified technique for direct sequencing of genomic DNA amplified by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA.* **87:** 658–662.
36. Baserga, S. J., and E. J. Benz, Jr. 1988. Nonsense mutations in the human β-globin gene affect mRNA metabolism. *Proc. Natl. Acad. Sci. USA.* **85:** 2056–2060.
37. Kadowaki, T., C. L. Bevins, A. Cama, K. Ojamaa, B. Marcus-Samuels, H. Kadowaki, L. Beitz, C. McKeon, and S. I. Taylor. 1988. Two mutant alleles of the insulin receptor gene in a patient with extreme insulin resistance. *Science.* **240:** 787–790.
38. Lehrman, M. A., W. J. Schneider, M. S. Brown, C. G. Davis, A. Elhammer, D. W. Russell, and J. L. Goldstein. 1987. The Lebanese allele at the low density lipoprotein receptor locus. Nonsense mutation produces truncated receptor that is retained in endoplasmic reticulum. *J. Biol. Chem.* **262:** 401–410.