A two-base deletion in exon 6 of the 3-hydroxy-3-methylglutaryl coenzyme A lyase (HL) gene producing the skipping of exons 5 and 6 determines 3-hydroxy-3-methylglutaric aciduria

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A two-base deletion in exon 6 of the 3-hydroxy-3-methylglutaryl coenzyme A lyase (HL) gene producing the skipping of exons 5 and 6 determines 3-hydroxy-3-methylglutaric aciduria

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Abstract A novel two-base deletion in the 3-hydroxy-3-methylglutaryl coenzyme A lyase (HL) gene was found in a Spanish patient with homozygous 3-hydroxy-3-methylglutaric aciduria. Amplification by RT-PCR of the mRNAs showed that the gene was transcribed into three different mRNAs. One showed the complete deletion of exons 5 and 6 located between nucleotides 348 and 561 of the HL cDNA. The second transcript showed deletion of exon 6 only, and the third contained a two-base deletion CT in exon 6, corresponding to nucletotides 504 and 505 of the HL cDNA. These aberrant mRNAs are predicted to encode three abnormal HMG-CoA lyase proteins; the first (from skipped exons 5 and 6) lacks 71 amino acids, which represents 24% of the mature protein; the second, (from the skipping of exon 6, producing a frameshift) contains only 192 amino acids, the last 26 of which are missense amino acids preceding a stop codon; the third contains only 175 amino acids, the last 7 of which are missense. Northern blot analysis showed that the HL mRNA levels of the patient were 4% of the control. PCR quantitative analysis indicated that the mRNA lacking exons 5 and 6 was the most abundant, representing 88% of the total. The other two mRNAs represented 8% and 4%, respectively. In the genomic DNA only one CT deletion was found at positions +7 and +8 at beginning of exon 6. No mutations were observed in the splice donor, splice acceptor, or pyrimidine-rich sequences of the intronic regions flanking exons 5 and 6. All three aberrant mRNAs resulted only from the deletion of nucleotides CT. We suggest that this deletion may affect the interaction between the small nuclear ribonucleoproteins (snRNPs) and exon 6, and that, as a result, the abnormal splicing of the pre-mRNA produces two different aberrant transcripts.—**Casals, N., J. Pié, C. H. Casale, N. Zapater, A. Ribes, M. Castro-Gago, S. Rodriguez-Segade, R. J. A. Wanders, and F. G. Hegardt.** A two-base deletion in exon 6 of the 3-hydroxy-3-methylglutaryl coenzyme A lyase (HL) gene producing a skipping of exons 5 and 6 determines 3-hydroxy-3-methylglutaric aciduria. *J. Lipid Res.* 1997. **38:** 2303–2313.

Supplementary key words 3-hydroxy-3-methylglutaric aciduria • HMG-CoA lyase deficiency • ketone bodies • leucine metabolism • two base pair deletion • exon skipping

3-Hydroxy-3-methylglutaric aciduria is an autosomal recessive metabolic disorder that usually appears within the first year of life. The patients present acute clinical episodes with vomiting, lethargy, and hypotonia, which sometimes evolves to apnea and coma (1, 2). Many patients have metabolic acidosis with hypoketotic hypoglycemia on fasting, variable elevation of transaminases, and sometimes hyperammonemia (3). The disease is fatal in about 20% of cases (4).

The origin of the disease is a mutation in the gene encoding for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) lyase (HL). HL is a mitochondrial enzyme that catalyzes the last step of ketogenesis and the last reaction of leucine catabolism (5). Preliminary diagnosis is based on the analyses of the characteristic excretory pattern of organic acids in urine, which include 3-hydroxy-3-methylglutaric, 3-hydroxyisovaleric, 3-methylglutaconic, and others derived from the metabolism of leucine (1, 6). Some patients show dicarboxylic aci-

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HL, HMG-CoA lyase; TPE, 90 mM Tris-phosphate, 2 mM EDTA, pH 7.4; RT-PCR, reverse transcriptase-polymerase chain reaction.

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duria probably caused by the accumulation of free acids when their conversion through acetyl-CoA to ketone bodies is hindered (2). Confirmation of HL deficiency requires direct assay of the enzyme activity in leukocytes or fibroblasts (7).

The cDNA sequence coding for the human HMG-CoA lyase gene was reported in 1993, which led to the first genetic study of this disease in two Acadian French-Canadian siblings (8). To date, seven different mutations have been described, two of them by our group (8–13).

We report here a new mutation found in a homozygous Spanish girl in which the HMG-CoA lyase activity in fibroblasts was less than 4% of the control. A two-base deletion was found affecting nucleotides 7 and 8 of exon 6 of the HL gene. This mutation leads to three aberrantly spliced mRNAs, one in which only the CT is deleted, another in which exon 6 is skipped, and the third in which both exons 5 and 6 are skipped. Sequencing of genomic DNA of the patient's parents showed that both were carriers of the same CT deletion. The possible mechanism determining the generation of the skipped mRNAs and the catalytic efficiency of the different encoded proteins are discussed.

MATERIAL AND METHODS

Case report

The patient, a 17-year-old girl (C.G.L.), was born to healthy and non-consanguineous Spanish parents after a normal pregnancy and delivery. At 9 months of age she was admitted to a local hospital due to an acute episode diagnosed as a Reye-like syndrome. At the age of 5 years, she experienced a new episode triggered by vomiting and poor feeding followed by convulsions and coma. Carnitine treatment was started that was discontinued at the age of 9 years. Although the patient did not suffer other episodes at 15 years, she was admitted at the Galicia Xeral Hospital in Santiago de Compostela to study the origin of her previous Reye-like episodes. Physical examination was unremarkable. EEG and ECG were normal, but cerebral MRI revealed areas of hypersignals in T2 at the level of white cerebral matter (leukaryosis). The urinary organic acid profile showed increased excretion of 3-methylglutaconic, 3-hydroxy isovaleric, 3-hydroxy-3-methylglutaric, and 3-methylglutaric acids consistent with the diagnosis of HL deficiency. Ammonia, transaminases, lactate, and other usual blood analyses were normal. Total plasma carnitine was decreased (14.7 mmol/l; control range 31.1–74.4, n = 33) likewise plasma free carnitine was decreased (5.9 mmol/l; control range: 19.4–57.2, n = 33). Carnitine treatment (50 mg/kg/day) was again introduced, and the patient was advised to follow the usual dietetic rules for this disease. HMG-CoA lyase activity in fibroblasts measured using the method described in ref. 14 was very low, 0.8 nmol/min·mg protein, (normal range 12.0–27.0). The patient shows normal growth and development and no other episodes have been recorded.

Fibroblast culture

Primary fibroblast cell lines from the patient and controls were established from skin biopsies, using standard methods. Explants were cultured in 100-mm dishes in Dulbecco's Minimal Essential Medium (DMEM), 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 10% fetal calf serum, and 95% air–5% CO_2.

Southern blot analysis

Genomic DNA was extracted from cultured fibroblasts by a standard procedure (15). Fifteen μg DNA was digested using 5–10 U/μg of different restriction enzymes, and electrophoresis was performed in a 0.8% agarose gel in 1 × TPE buffer (90 mM Tris-phosphate, 2 mM EDTA, pH 7.4). The samples were transferred to nylon membranes, and hybridized to human HL cDNA probes.

cDNA synthesis and PCR amplifications

Total cellular RNA, obtained from cultured fibroblasts of the patient and control with the Quick Prep total RNA extraction kit from Pharmacia Biotech, were used for the first strand cDNA synthesis using the *ready to go* T-primed First-Strand kit from Pharmacia Biotech (Uppsala, Sweden). The primers used in the amplifications are represented in **Table 1** (12, 13) and were supplied by Genosys Technologies (Cambridge, U.K.). Radioactive compounds were obtained from Amersham (Little Chalfon, Buckinghamshire, U.K.). Amplification by the polymerase chain reaction (PCR) was performed under the following conditions: 1 min at 95°C, 30 sec at 55°C, 30 sec at 72°C, then 35 cycles, and a final extension of 7 min at 72°C. PCR products were separated from each other and from the unincorporated primers by electrophoresis on a 2% agarose gel, and then purified using Qiaex from Qiagen (Hilden, Germany). Taq DNA polymerase was supplied by Ecogen S.L. (Barcelona, Spain).

Genomic PCR

To amplify the HL intron regions flanking exons 4, 5, 6 and 7, genomic DNA was obtained from cultured fibroblasts from patient and control by a standard procedure (15), and 250 ng of DNA was amplified in a 100-μl mixture containing 0.2 mM of each dNTP, 25 pmol

TABLE 1. Primers of PCR amplification of cDNA

Name	Position[a]	Sequence
orf-f	Sense: −7–12	5′ GGCCAACATGGCAGCAATG 3′
f1	Sense: 177–197	5′ ATAGACATGCTTTCTGAAGC 3′
f2	Sense: 386–405	5′ CTGCCTCAGAGCTCTTCACC 3′
r1	Antisense: 650–631	5′ ATGTCTTTCATGATCCCTGG 3′
r2	Antisense: 832–813	5′ TGGCCAAGTTTCCTGATGCC 3′
orf-r	Antisense: 1022–1002	5′ CCCTATTTCCACATCATCCC 3′
utr-r	Antisense: 1436–1455	5′ CGTAGCTCTCCACTTTCCAC 3′

[a]Positions refer to the numbering of the HL cDNA sequence in Mitchell et al. (8).

of each primer, 2.5 units of Taq DNA polymerase in 1 × PCR buffer, and 2 mM $MgCl_2$; these reagents were supplied by Ecogen S.L. (Barcelona, Spain). PCR was performed as described above. PCR products were separated and purified (see above). The primers used in the amplifications were taken from the literature (10) and were supplied by Genosys Tech. (Little Chalfont, Buckinghamshire, U.K.) (**Table 2**).

DNA sequencing

Purified PCR amplification products were sequenced automatically with an Applied Biosystems 373 DNA sequencer ABI Prism (Warrington, U.K.), using the DNA sequencing kit from Perkin Elmer, (Nieukerke, Holland). All sequences reported were obtained at least twice, once in each direction.

Analysis of the HL mRNA levels

Northern blot analysis. Total RNA from cultured fibroblast was isolated as described (15). RNA samples were fractionated in 1% agarose/formaldehyde gels, transferred to Nytran-N membranes (Schleicher & Schuell, Dassel, Germany) and cross-linked at 80°C for 2 h. Hybridizations were carried out as described (16) using the full-length HL cDNA as a probe, and washes were performed at 68°C in 0.2× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), and 0.1% sodium dodecyl sulfate. mRNA levels were measured by densitometry of the autoradiograms with a Vilber Dourmat (Marne La Vallée, France) computing densitometer. Densitometry values were corrected using human β-actin as a constitutive probe. Filters were dehybridized either in water at 100°C for 10 min or in 50% formamide/6× SSPE at 70°C for 2 h (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 5 mM Na_2 EDTA).

Quantification of the HL mRNA levels. The initial amount (No) of the three species of HL mRNA found, was quantified by PCR kinetic analysis. We performed this according to the following protocol: *a*) by using the reverse transcriptase, total RNAs from the patient were converted into cDNAs; *b*) the HL cDNAs were amplified with the oligonucleotides orf-f and orf-r; *c*) again by PCR, the fragment f1-r2 was amplified using 2.5 μCi (0.25 μl) of [α-^{32}P]dCTP (3000 Ci/mmol) and a mixture of the PCR reagents. The amplification was carried out in different tubes with a variable number of cycles (from 13 to 25). The reaction products (15 μl) of the cDNAs were size-selected on a 2% agarose metaphor gel. The three amplified gel bands of each line were cut out and their radioactivity was measured. For kinetic analysis, values of log cpm were plotted against the number of cycles, in the three mRNA species. The lines obtained are consistent with the equation:

$$\text{Log c.p.m.}(^{32}\text{P}) = [\text{Log } (1 + \text{E})] \cdot \text{n} + \text{Log No}$$

where E is the efficiency, n the number of PCR cycles, and No the initial amount of the mRNAs. This method did not allow us to determine the absolute number of the starting target molecules, but we were able to calculate the percentages of the initial target molecules of the three mRNAs detected (17, 18).

TABLE 2. Amplification primers for human HL exons[a]

Name	Position	Sequence
fE4	5′ exon 4	5′ TCTCTGCTCTTGGTGATGACT 3′
fE5	5′ exon 5	5′ TCGCAAGACTCCATCTCAAACA 3′
fE6	5′ exon 6	5′ TCGCCCTGCCTCAGTTCT 3′
fE7	5′ exon 7	5′ TCATTCTGTATCCTCCCAAGTGCC 3′
rE4	3′ exon 4	5′ CAAGACAAGGCAGGGAC 3′
rE5	3′ exon 5	5′ GAACGGTACAGAGGAAAGGA 3′
rE6	3′ exon 6	5′ ACCCTCACCAAACCCC 3′
rE7	3′ exon 7	5′ CGTGACCTTTGGGAGAAT 3′

[a]Taken from Wang et al. (10).

RESULTS

Southern blot analysis

Genomic DNA from the patient and control was digested with *Bam* H1, *Sac* I, and *Bgl* II to exclude the presence of major structural defects in the HL gene. After hybridization with the HL cDNA as a probe, no major HL gene rearrangements were detected (data not shown).

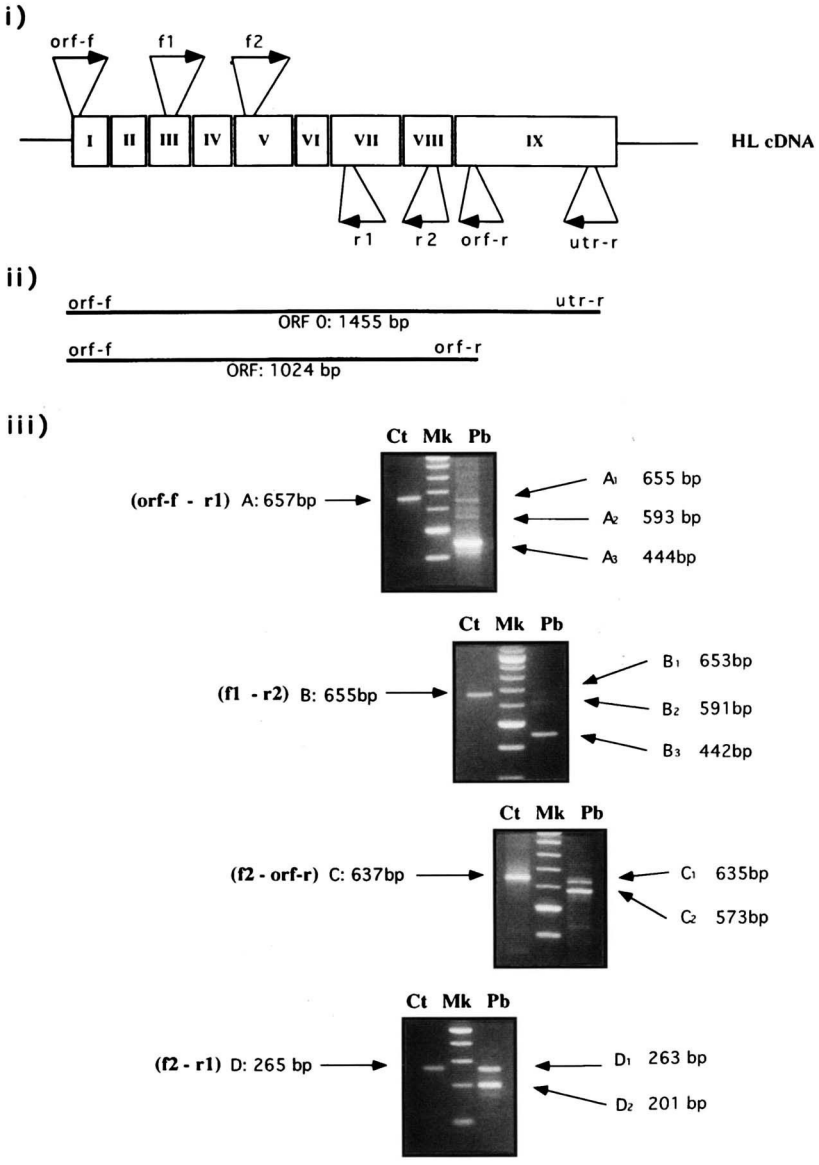

Fig. 1. i) Schematic representation of the HL cDNA showing the exons (I to IX) to scale. Primers are shown by arrows and are described in Table 1. ii) Diagram of the amplified ORF-0 (1455 bp) and the ORF (1024 bp) fragments. iii) The A, B, C, and D overlapping fragments of the control (Ct) are represented by bold lines. In the patient (Pb), the different abnormal bands for each amplified fragment are indicated with capital letters and a subindex number. The amplified fragments of the control and patient were separated by electrophoresis in 2% agarose gels. MK, molecular weight markers.

Reverse transcription and PCR amplification

The schematic representation of the HL cDNA showing the exons at scale and also the primers used is seen in **Fig. 1, i.** First-strand cDNA of HL from the patient and control was used to amplify the orf-f/utr-r (ORF-0, 1455 bp) fragment. It was then reamplified using orf-f and orf-r (ORF fragment, 1024 bp) primers (Fig. 1, ii). The amplified ORF cDNA fragment was used as a template for the PCR amplification of different overlapping fragments called A, B, C, and D (Fig. 1, iii). The results show marked differences between patient and control in the number and size of the bands. While the amplified ORF fragment generated a single band of 1024 bp in the control, two additional bands of about 960 and 811 bp appeared in the patient (data not shown). The amplification of the control fragment A (orf-f/r1) gave a band of about 657 bp, but in the patient we obtained three bands of 655, 593 and 444 bp (Fig. 1, iii). The size of fragment B (f1-r2) was similar in the control and in the patient (655 bp and 653 bp); in addition, two more bands of 591 and 442 bp appeared in the patient. The amplifications of fragments C (f2/orf-r) and D (f2-r1) showed bands of 637 and 265 bp respectively in the control. In the patient we found two bands in fragment C of 635 and 573 bp and also two bands in fragment D of 263 and 201 bp (Fig. 1, iii). These findings suggested the presence of three HL mRNA species in the fibroblasts of the patient. Two of the mRNAs showing deletions of 64 and 213 bp were located in the cDNA

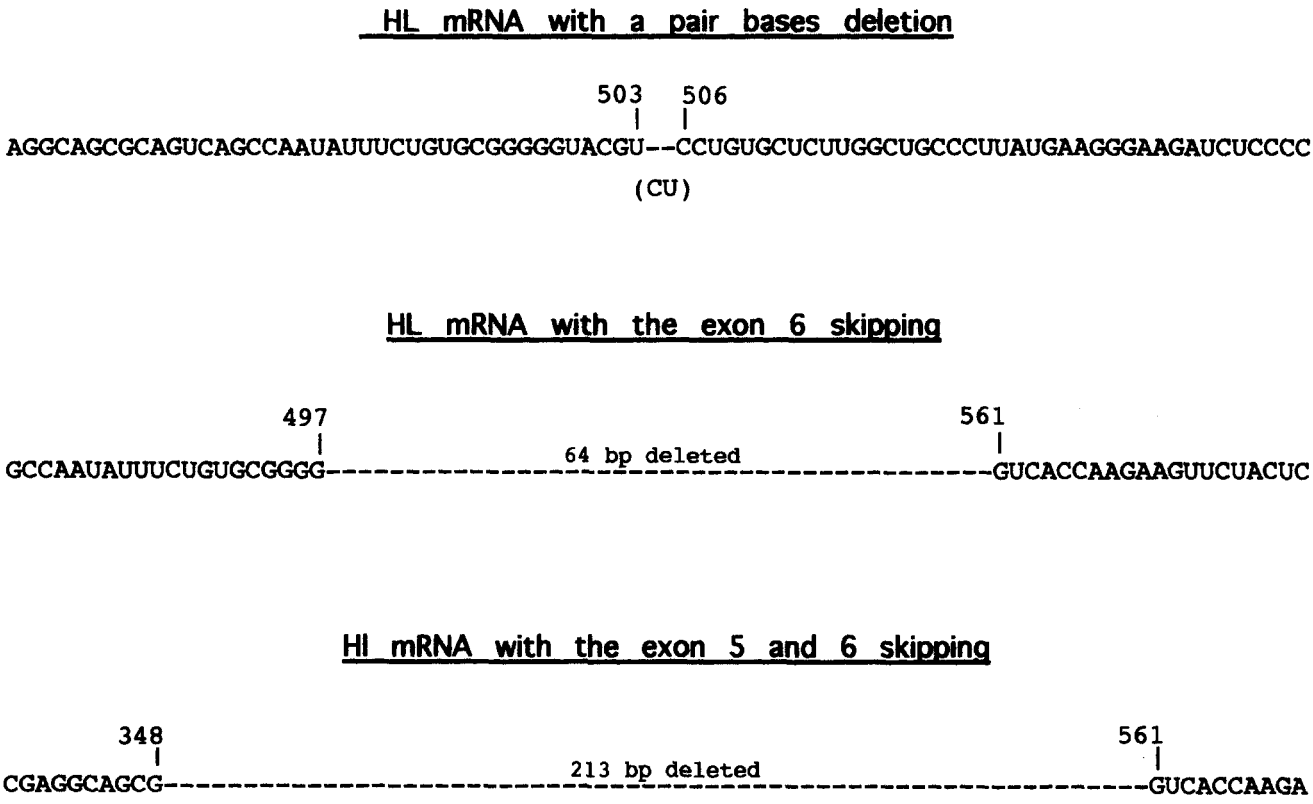

Fig. 2. The mononucleotide sequences of the mRNAs species at the boundaries of the deleted regions are represented. Top: HL mRNA with a 2bp deletion (CU) causes a frameshift. Middle: HL mRNA with a 64 bp deletion corresponding to exon 6 also causes a frameshift. Bottom: HL mRNA with a 213 bp deletion from nucleotide 348 to 561 of the HL cDNA is an in-frame deletion that coincided with the location and size of exons 5 and 6.

sequence between the primers f1 and r1. The third mRNA was similar in size to the control.

Sequence of the RT-PCR fragments

The differents bands of the A, B, C, and D PCR fragments from the patient and control were purified and sequenced. The sequence of the medium-sized band of A and B and the smaller band of C and D fragments revealed a 64 bp deletion from nucleotide 497 to 561 of the HL cDNA. The size and location of this deletion coincided with exon 6 (10). In addition, the sequence of the smaller bands of the A and B fragments showed a 213 bp deletion from nucleotides 348 to 561 of the HL cDNA. The size and location of this deletion coincided with exons 5 and 6 of the HL cDNA (10). Moreover, when the apparently normal fragments A, B, C, and D were sequenced, a two-base CT deletion corresponding to nucleotides 504 and 505 of the HL cDNA appeared. The results of the nucleotide sequences of the different RT-PCR fragments are shown in **Fig. 2.**

Genomic PCR amplification and sequence of the DNA comprising exons 5 and 6 and their flanking intron boundaries

The Southern blot analysis (see above) did not indicate that the skipping of exons 5 and 6 corresponded to a large deletion. Accordingly, we attempted to identify the origin of the deletion of exon 6 and exons 5 and 6. To this end, we amplified the DNA genomic region comprising exons 5 and 6 and the intronic regions that encompassed them. Using the modified primers shown in Table 2 and a genomic DNA as a template, fragments fE5-rE5 (including exon 5) and fE6-rE6 (including exon 6) were amplified. The amplified genomic fragment that included exon 5 always yielded the same size (270 bp), whether control or patient genomic DNA was used. However, the amplified fragment that contained exon 6 in the patient showed a two-base deletion CT (**Fig. 3**) corresponding to nucleotides 504, 505 of the HL cDNA (Fig. 2). This deletion affected nucleotides 7 and 8 of the 5′ region of exon 6. No mutations were observed in the splice donor, splice acceptor, or pyrimi-

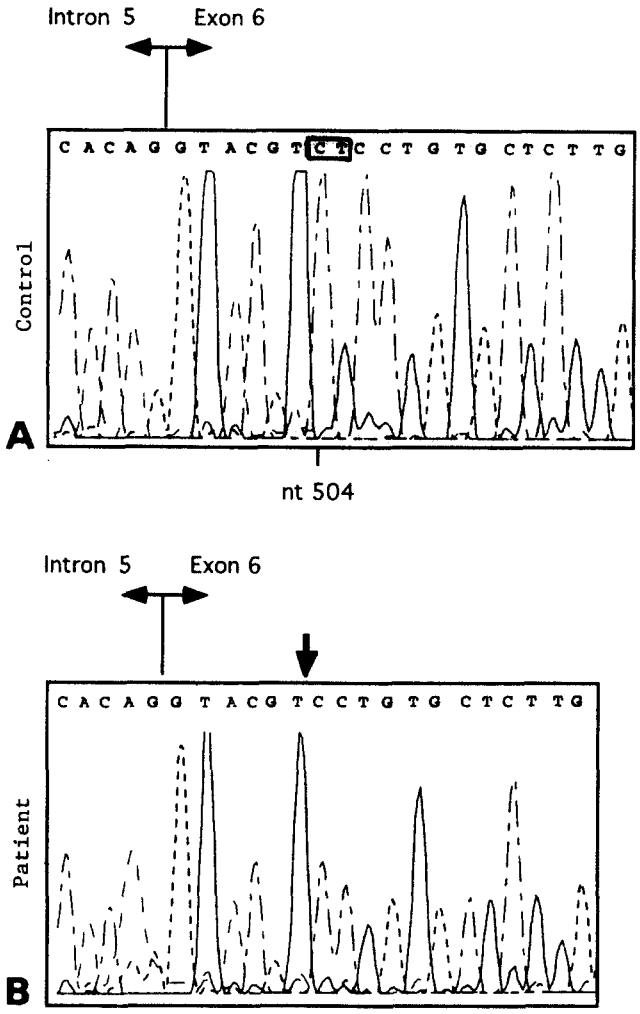

Fig. 3. The genomic sequence from the last five nucleotides of intron 5 to position 517 of exon 6 in the control (A) and in the patient (B) is shown. The 2bp deletion CT is boxed. The arrow denotes the change in the frameshift produced by the deletion.

dine-rich sequences of the intronic regions flanking exons 5 and 6. To rule out the presence of mutations in other exon sequences or in their flanking intron boundaries, exons 4 and 7 were sequenced and no mutation was detected. The localization of the amplification primers for human HL exons is indicated in **Fig. 4.**

Predicted translation products of HL mRNAs

The CT deletion affecting nucleotides 504 and 505 of the HL cDNA is a frameshift mutation that generates a truncated version of the mature protein. The deletion produces missense codons starting at Val 168, which leads to a stop codon 8 triplets downstream (**Fig. 5**). The mature protein has 175 amino acid residues, the last 7 of which are missense amino acids lacking the last 158 sense amino acid residues. The mRNA lacking exon 6 produces a deletion of 64 nucleotides at 497 to 561, which also causes a change in the open reading frame. The translation of this mRNA generates a 192 amino acids truncated mature protein. This deletion produces the absence of 21 sense amino acids and a change in the open reading frame. The mature protein has 26 missense amino acids that follow Gly-166 and lacks the last 160 sense amino acid residues. The mRNA in which exons 5 and 6 are skipped has a 213 bp in-frame deletion located between nucleotides 348 and 561. This deletion leads to the loss of 71 amino acids (from Ala-116 to Val-187) in the mature protein.

Quantification of the mRNA levels observed in the patient

In Northern blot experiments different expression was observed in patient and control when their respective mRNAs were hybridized to the cDNA probe. Only the patient's transcript corresponding to the mRNA lacking exons 5 and 6 was observed. The other transcripts were not detected. Quantitative determination by densitometry of the bands, after normalization with β-actin showed that the mRNA levels of the patient were 4% of the control (**Fig. 6A**).

As Northern blot analysis did not allow quantification of the relative proportion of the different mRNA species of the patient, we determined this by PCR kinetic analysis. To this end, we used the fragment f1-r2, whose predicted amplified bands are different in size at the three species analyzed. The slope of the three straight lines was identical, indicating a similar efficiency in the three mRNAs amplified (Fig. 6B). The percentage of the three mRNAs species, calculated by the intercept of the each line, was 4/8/88. The results showed that mRNA lacking exons 5 plus 6 was the most abundant, representing 88% of the total. The mRNA lacking exon 6 was 8% and the mRNA with the CT deletion represented only 4% of the total.

PCR amplifications of the relatives of the patient

We also performed PCR amplifications of exon 6, using the genomic DNA as a template and using as primers fE6 and rE6, i.e., primers located in introns 5 and 6 respectively (Table 2), in the father, the mother, and one brother of the patient. With the same primers as those used in the patient, an identical 187-bp fragment was amplified. The sequence of the fragment revealed that both the mother and father had the same 2-bp deletion in one of the two alleles, as starting at position 504 there was a mixture of the wild type HL sequence and the 2-bp deleted sequence. This result indicated that both parents were carriers of the same deletion. The HL sequence of the patient's brother was identical

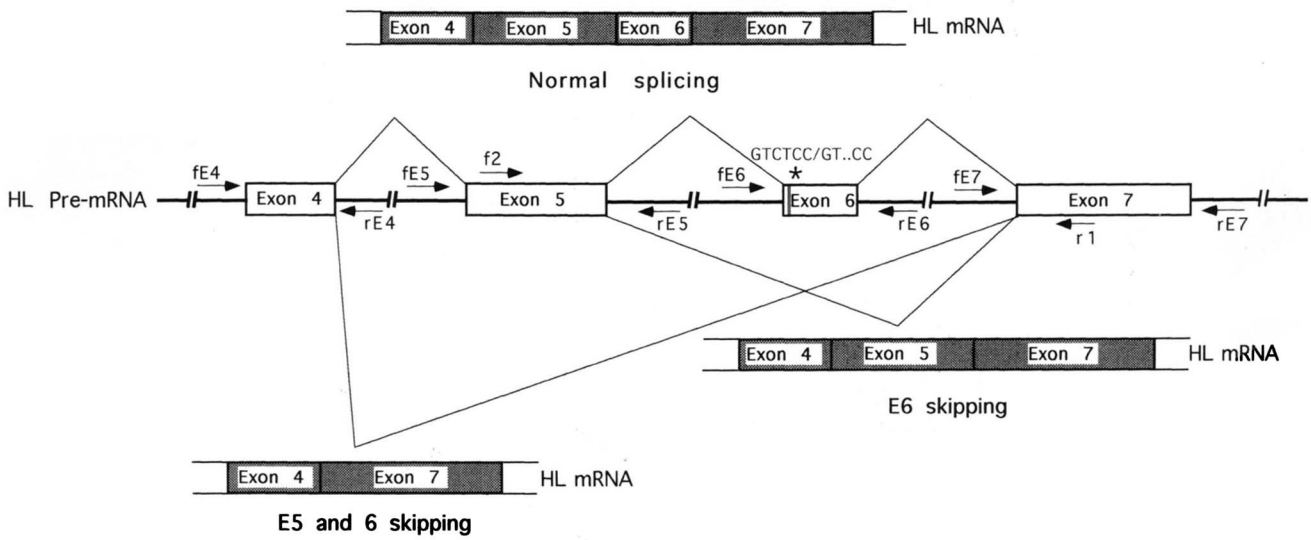

Fig. 4. Schematic representation of the normal splicing and two aberrant splicings in the HL gene of the patient, one that produces the skipping of exon 6 and another the skipping of exon 5 plus 6. The primers are represented by arrows. The asterisk mark indicates the localization of the 2-bp deletion at the positions 7 and 8 of exon 6.

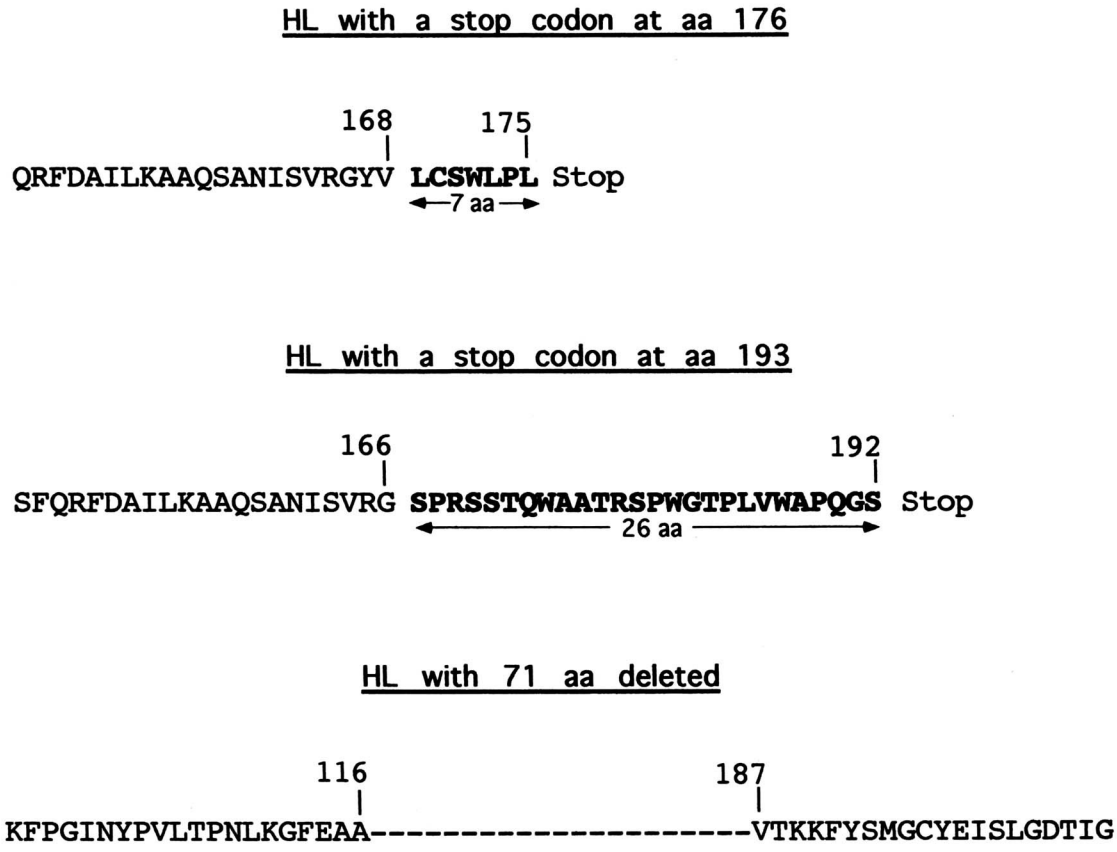

Fig. 5. The predicted peptide sequences of the three mature proteins are shown. Top: The CT deletion is expected to produce 7 missense codons starting at Val-168 that end in a stop codon 8 triplets downstream. Middle: The skipping of exon 6 would cause 26 missense codons starting at Gly-166, which lead to a stop codon 27 triplets downstream. Bottom: The skipping of exon 5 plus 6 would produce a 71 amino acid deletion in the middle of the protein.

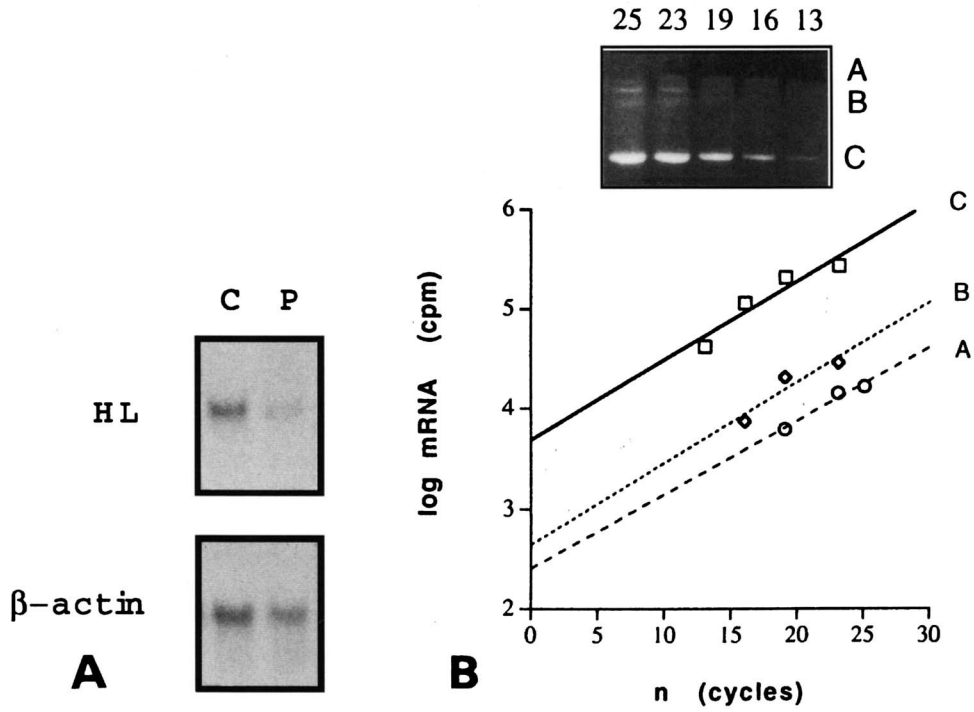

Fig. 6. Northern Blot analysis and quantification of the three HL mRNA species by competitive PCR kinetics. A: Northern blot analysis of the HMG-CoA lyase mRNAs of a control and the patient. The two lanes were hybridized with the full-length cDNA of the human HMG-CoA lyase as a probe. The size of the mRNA was deduced by comparison with markers (not shown). The amounts of the RNA samples applied to the gel were compared by determining the mRNA levels of β-actin. B: The relative amount of the three mRNA species observed in the patient was determined by PCR kinetic analysis as described in Material and Methods. The electrophoretic pattern of the three mRNA species in relation to the number of cycles is shown in the inset. A, mRNA with the CT deletion; B, mRNA lacking exon 6; C, mRNA lacking exons 5 and 6. The log cpm of ^{32}P from [α-^{32}P]dCTP incorporated to each of the three mRNAs was plotted against the number of cycles of amplification. Three straight lines with the same slope were obtained, indicating that the efficiency of amplification was the same. The anti-log of the respective intercepts shows the proportion of the three mRNA species.

to the control; therefore we deduced that the boy had not inherited either mutated allele.

DISCUSSION

To date, few articles have been published on the mutations of the HL gene that could explain hereditary methylglutaric aciduria. In the current study, a novel mutation in the HMG-CoA lyase gene from a homozygous Spanish female patient has been identified. We have found at the beginning of exon 6 of HMG-CoA lyase a CT deletion at positions 504–505, which determines the occurrence of three mature transcripts: *i*) the mRNA corresponding to the CT deletion itself; *ii*) an mRNA transcript containing an in-frame deletion of exon 5 plus 6; *iii*) an mRNA transcript containing a frameshift deletion of exon 6 (64 bp). Other mutations within exon sequences or in donor or acceptor splice sites or in pyrimidine-rich sequences of the introns 4, 5, and 6 were not observed. Moreover, the Southern blot analysis did not detect rearrangements of the HL gene. It can be assumed that the CT deletion, by itself, can produce aberrant splicings that lead to the three transcripts mentioned.

The mRNA species having the 2-bp CT deletion and also that with the skipping of exon 6 caused frameshifts that produced premature stop codons at amino acids 176 and 193, respectively. In both cases, these mRNAs generated two truncated proteins with a substantial loss of the amino acid sequence (>53%). The missing sequences include the domains that contain the catalytically important histidine 233 (11), the catalytic site of the cysteine 266 (19) and of the cysteine 323, which is responsible for the formation of the homodimeric protein found in eukaryotes (20). The overall consequences are the absence of enzyme properties in the proteins encoded by these mutated mRNAs.

The mRNA in which exons 5 and 6 are skipped, which is the most abundant mRNA, has an in-frame deletion of 213 nucleotides, and its translation product

causes the loss of 71 amino acids out of a total of 298 in the mature protein. This loss represents approximately 24% of the protein and it is located in the middle of the structure of the HL enzyme. It seems probable that the deletion of 71 amino acids results in a non-catalytically active protein, unable to sustain the synthesis of mitochondrial HMG-CoA lyase.

The conservation of the 5′ and 3′ splice site nucleotide sequences, the branch point and the pyrimidine-rich sequences in the intron sequences flanking the exons, is critical for accurate splicing (21, 22). It has been shown that mutations at donor and acceptor splice sites at intron regions produce skipping of the flanked exons (23). However, mutations in exon sequences leading to exon skipping are unusual. The CT deletion in exon 6 reported here could be one of this class. It seems that the lack of recognition of the correct splice sites (24), produced by the incorrect recognition of this exon domain, determines the aberrant splicings observed in this patient.

Recently, in the higher eukaryotes it has been demonstrated that purine-rich sequences included in exons, called either exon recognition sequences (ERS) (25) or exon splicing enhancers (ESE) (26, 27), may also affect the selection of splice sites. In a previous paper we reported that a nonsense mutation in exon 2 of the HMG-CoA lyase gene could affect a possible positive ESE sequence, thus facilitating aberrant splicing which could lead to skipping of this exon (13). In the case reported here, the patient has a 2-bp deletion in the HMG-CoA lyase gene in a sequence poor in purines and not coincident with the ESE sequences, suggesting that the mutation described does not affect an ESE, and accordingly, the abnormal splicings observed could not be explained by the mutation of an ESE sequence.

Recent studies have also shown that the secondary structure of the mRNA and the exon sequences at the 5′ end of the exon could play a role in the accurate splice site selection (28). Wakamatsu et al. (29) showed that a single base C/T substitution at position 8 of exon 11 of the human β-hexosaminidase β subunit produced exon skipping and the activation of a cryptic splice site in the same exon, determining the generation of three different mature mRNAs. Similarly, a single A/G nucleotide polymorphism in exon 2 of the episialin gene, 8 nucleotides downstream of the splice acceptor site, was shown to affect 3′ splice site selection (30). They proposed the interesting hypothesis that the A-to-G substitution results in the formation of an altered secondary structure of the pre-mRNA, which makes the splice acceptor site inaccessible to the splicing machinery. Previous studies have also suggested that the structure of stable hairpins of pre-mRNA can influence splice site selection (31–37). It is surprising that the mutation that determines the hereditary diseases in the two cases mentioned has been produced in nucleotide 8 of an exon. In the present case, in which a deletion of nucleotides 7 and 8 of the exon is observed, there is a possibility that the mutated exon 6 affects the secondary structure of HMG-CoA lyase pre-mRNA and thus impairs the correct splicing.

In vitro studies of pre-mRNA splicing in higher eukaryotes have demonstrated that splicing of individual introns takes place in two steps after assembly of the pre-mRNA into the large multicomponent complex, the spliceosome, which is composed of multiple small ribonucleoprotein particles (snRNPs) (reviewed in ref. 38). Decisive interactions for the accurate splice-site selection and formation of the active spliceosome are produced by the contacts of a conserved loop in U5 small nuclear RNA (snRNA) with the sequences of the previous exon near the 5′ donor splice site and with the first nucleotides of the exon considered.

In the case described here, it is possible that the CT deletion located at the nucleotides 7 and 8 in exon 6 may affect the interaction between the U5 snRNP and the exon itself. This, on the one hand, would hinder the tethering of the free exon 5 to the complex intron–3′exon lariat, which would lead to its loss. On the other hand, the lack of recognition of the 3′ splice site would produce the loss of exon 6 as it would be considered as part of an intron.

The three mature HL transcripts determined in the patient by RT-PCR are in unequal proportions: 4:8:88%, respectively, for the CT-deleted, exon-6-skipped, and exon-5-and-6-skipped mRNAs. While the third mature transcript maintains the open reading frame (the deletion is of 243 bp), in the first two a frameshift has been produced (the CT deletion and the skipping of 64 bp). The low level of the frameshifted mRNAs could be attributed to the loss of stability in the transcribed mRNA produced by the premature stop codon. It is well known that mutations that cause premature stop codons may produce loss of stability in the mature mRNAs (39–41).

The development of disease in the patient studied here can now be understood from the data presented at the molecular level. On the one hand, the low levels of the HL mRNAs found in the patient (4%) cannot sustain the synthesis of normal levels of HL protein. On the other hand, the mature mRNAs produced after the abnormal splicings described so far after the translational process will not be able to act as efficient catalytically active proteins.

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REFERENCES

1. Wysocki, S. J., and R. Hahnel. 1986. 3-Hydroxy-3-methylglutaryl coenzyme A lyase deficiency: a review. *J. Inherit. Metab. Dis.* **9:** 225–233.
2. Gibson, K. M., J. Breuer, K. Kaiser, W. L. Nyhan, E. E. McCoy, P. Ferreira, C. L. Greene, M. G. Blitzer, E. Shapira, F. Reverte, C. Conde, P. Bagnell, and D. E. C. Cole. 1988. 3-Hydroxy-3-methylglutaryl coenzyme A lyase deficiency: report of five new patients. *J. Inherit. Metab. Dis.* **11:** 76–87.
3. Sweetman, L., and J. C. William. 1995. 3-Hydroxy-3-methyl glutaric aciduria: 3-hydroxy-3-methylglutaryl CoA lyase deficiency. *In* The Metabolic and Molecular Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. Vol I. 7th Ed. McGraw-Hill, New York, NY. 1400–1402.
4. Ozand, P. T., A. Al Aqeel, G. Gascón, J. Brismar, E. Thomas, and H. Gleispach. 1991. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) lyase deficiency in Saudi Arabia. *J. Inherit. Metab. Dis.* **14:** 174–188.
5. Robinson, A. L., and D. H. Williamson. 1980. Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiol. Rev.* **60:** 143–187.
6. Faull, K. F., P. D. Bolton, B. Halpern, J. Hammond, and D. M. Danks. 1976. The urinary organic acid profile associated with 3-hydroxy-3-methylglutaric aciduria. *Clin. Chim. Acta.* **73:** 553–559.
7. Sovik, O., L. Sweetman, K. M. Gibson, and W. L. Nyhan. 1984. Genetic complementation analysis of 3-hydroxy-3-methylglutaryl coenzyme A lyase deficiency in cultured fibroblasts. *Am. J. Hum. Gen.* **36:** 791–801.
8. Mitchell, G. A., M. F. Robert, P. W. Hruz, S. Wang, G. Fontaine, C. E. Behnke, L. M. Mende-Mueller, K. Schappert, C. Lee, K. M. Gibson, and H. M. Miziorko. 1993. 3-Hydroxy-3-methyl glutaryl coenzyme A lyase (HL). Cloning of human and chicken liver HL cDNAs and characterization of a mutation causing human HL deficiency. *J. Biol. Chem.* **268:** 4376–4381.
9. Mitchell, G. A., C. Jakobs, K. M. Gibson, M. F. Robert, A. Burlina, C. Dionisis-Vici, and L. Dallaire. 1995. Molecular prenatal diagnosis of 3-hydroxy-3-methylglutaryl CoA lyase deficiency. *Prenatal Diagn.* **15:** 725–729.
10. Wang, S. P., M. F. Robert, K. M. Gibson, R. J. A. Wanders, and G. A. Mitchell. 1996. 3-Hydroxy-3-methylglutaryl CoA lyase (HL): mouse and human HL gene (HMGCL) cloning and detection of large gene deletions in two unrelated HL-deficient patients. *Genomics.* **33:** 99–104.
11. Roberts, J. R., G. A. Mitchell, and H. Miziorko. 1996. Modeling of a mutation responsible for human 3-hydroxy-3-methylglutaryl CoA lyase deficiency implicates histidine 233 as an active site residue. *J. Biol. Chem.* **271:** 24604–24609.
12. Buesa, C., J. Pié, A. Barceló, N. Casals, C. Mascaró, C. H. Casale, D. Haro, M. Duran, J. A. M. Smeitink, and F. G. Hegardt. 1996. Aberrantly spliced mRNAs of the 3-hydroxy-3-methylglutaryl coenzyme A lyase (HL) gene with a donor splice-site point mutation produce hereditary HL deficiency. *J. Lipid Res.* **37:** 2420–2432.
13. Pié, J., N. Casals, C. H. Casale, C. Buesa, C. Mascaró, A. Barceló, M. O. Rolland, T. Zabot, D. Haro, F. Eyskens, P. Divry, and F. G. Hegardt. 1997. A nonsense mutation in the 3-hydroxy-3-methylglutaryl coenzyme A lyase (HL) gene produces exon skipping in two patients of different origin with HL deficiency. *Biochem. J.* **323:** 329–335.
14. Wanders, R. J. A., R. B. H. Schutgens, and P. H. M. Zoeters. 1988. 3-Hydroxy-3-methylglutaryl-CoA lyase in human skin fibroblasts: study of its properties and deficient activity in 3-hydroxy-3-methylglutaric aciduria patients using a simple spectrophotometric method. *Clin. Chim. Acta.* **171:** 95–102.
15. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Extraction, purification, and analysis of messenger RNA from eukaryotic cells. *In* Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 7.3–7.84.
16. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* **18:** 5294–5299.
17. Salomon, R. N., R. Underwood, M. V. Doyle, A. Wang, and P. Libby. 1992. Increased apolipoprotein E and c-fms gene expression without elevated interleukin 1 or 6 mRNA levels indicates selective activation of macrophage functions in advanced human atheroma. *Proc. Natl. Acad. Sci. USA.* **89:** 2814–2818.
18. Dallman, M. J., R. A. Montgomery, C. P. Larsen, A. Wanders, and A. F. Wells. 1991. Cytokine gene expression: analysis using Northern blotting, polymerase chain reaction and in situ hybridization. *Immunol. Rev.* **119:** 163–179.
19. Roberts, J. R., C. Narasimhan, and H. M. Miziorko. 1995. Evaluation of cysteine 266 of human 3-hydroxy-3-methylglutaryl CoA lyase as a catalytic residue. *J. Biol. Chem.* **270:** 17311–17316.
20. Roberts, J. R., C. Narasimhan, P. W. Hruz, G. A. Mitchell, and H. M. Miziorko. 1994. 3-Hydroxy-3-methylglutaryl CoA lyase: expression and isolation of the recombinant human enzyme and investigation of a mechanism for regulation of enzyme activity. *J. Biol. Chem.* **269:** 17841–17846.
21. Mount, S. M. 1982. A catalogue of splice junction sequences. *Nucleic Acids Res.* **10:** 459–472.
22. Smith, C. W., E. B. Porro, J. G. Patton, and B. Nadal-Ginard. 1989. Scanning from an independently specified branch point defines the 3′ splice site of mammalian introns. *Nature.* **342:** 243–247.
23. Nakai, K., and H. Sakamoto. 1994. Construction of a novel database containing aberrant splicing mutations of mammalian genes. *Gene.* **141:** 171–177.
24. Dibb, N. J. 1993. Why do genes have introns? *FEBS Lett.* **325:** 135–139.
25. Watakabe, A., K. Tanaka, and Y. Shimura. 1993. The role of exon sequences in splice site selection. *Genes Dev.* **7:** 407–418.
26. Sun, Q., A. Mayeda, R. K. Hampson, A. R. Krainer, and F. M. Rottman. 1993. General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer. *Genes Dev.* **7:** 2598–2608.
27. Humphrey, M. B., J. Bryan, T. A. Cooper, and S. M. Ber-

get. 1995. A 32-nucleotide exon-splicing enhancer regulates usage of competing 5′ splice sites in a differential internal exon. *Mol. Cell. Biol.* **15:** 3979–3988.
28. Reed, R., and T. Maniatis. 1986. A role for exon sequences and splice-site proximity in splice-site selection. *Cell.* **46:** 681–690.
29. Wakamatsu, N., H. Kobayashi, T. Miyatake, and S. Tsuji. 1992. A novel exon mutation in the human beta-hexosaminidase beta subunit gene affects 3′ splice site selection. *J. Biol. Chem.* **267:** 2406–2413.
30. Fu, X-D., R. A. Katz, A. M. Skalka, and T. Maniatis. 1991. The role of branchpoint and 3′-exon sequences in the control of balanced splicing of avian retrovirus RNA. *Genes Dev.* **5:** 211–220.
31. Solnick, D. 1985. Alternative splicing caused by RNA secondary structure. *Cell.* **43:** 667–676.
32. Eperon, L. P., J. P. Estibeiro, and I. C. Eperon. 1986. The role of nucleotide sequences in splice site selection in eukaryotic pre-messenger RNA. *Nature.* **324:** 280–282.
33. Eperon, L. P., I. R. Graham, A. D. Griffiths, and I. C. Eperon. 1988. Effects of RNA secondary structure on alternative splicing of pre-mRNA: is folding limited to a region behind the transcribing RNA polymerase? *Cell.* **54:** 393–401.
34. Solnick, D., and S. I. Lee. 1987. Amount of RNA secondary structure required to induce an alternative splice. *Mol. Cell Biol.* **7:** 3194–3198.
35. Halfter, H., and D. Gallwitz. 1988. Impairment of yeast pre-mRNA splicing by potential secondary structure-forming sequences near the conserved branchpoint sequence. *Nucleic Acids Res.* **16:** 10413–10423.
36. Chebli, K., R. Gattoni, P. Schmitt, G. Hildwein, and J. Stevenin. 1989. The 216-nucleotide intron of the E1A pre-mRNA contains a hairpin structure that permits utilization of unusually distant branch acceptors. *Mol. Cell. Biol.* **9:** 4852–4861.
37. Watakabe, A., K. Inoue, H. Sakamoto, and Y. Shimura. 1989. A secondary structure at the 3′ splice site affects the in vitro splicing reaction of mouse immunoglobulin mu chain pre-mRNAs. *Nucleic Acids Res.* **17:** 8159–8169.
38. Krämer, A. 1996. The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annu. Rev. Biochem.* **65:** 367–409.
39. Baserga, S. J., and E. J. J. Benz. 1988. Nonsense mutations in the human beta-globin gene affect mRNA metabolism. *Proc. Natl. Acad. Sci. USA.* **85:** 2056–2060.
40. Humphries, R. K., T. J. Ley, N. P. Anagnou, A. W. Baur, and A. W. Nienhuis. 1984. Beta O-39 thalassemia gene: a premature termination codon causes beta-mRNA deficiency without affecting cytoplasmic beta-mRNA stability. *Blood.* **64:** 23–32.
41. Belgrader, P., and L. E. Maquat. 1994. Nonsense but not missense mutations can decrease the abundance of nuclear mRNA for the mouse major urinary protein, while both types of mutations can facilitate exon skipping. *Mol. Cell. Biol.* **14:** 6326–6336.