A nonsense mutation in the 3-hydroxy-3-methylglutaryl-CoA lyase gene produces exon skipping in two patients of different origin with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency

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A novel nonsense mutation associated with the skipping of constitutive exon 2 of the 3-hydroxy-3-methylglutaryl-CoA lyase gene was found in two patients, from Portugal and Morocco, with 3-hydroxy-3-methylglutaric acidemia. By reverse transcriptase PCR and single-strand conformational polymorphism a G–T transversion was located, at nucleotide 109, of the 3-hydroxy-3-methylglutaryl-CoA lyase cDNA, within exon 2. Two mRNAs were produced as a result of this nonsense mutation: one of the expected size that contains the premature stop codon UAA, and the other with a deletion of 84 bp corresponding to the whole of exon 2. This deletion produced the loss of the last seven amino acids of the leader peptide and the first 21 amino acids of the mature protein. The nonsense mutation was found in a purine-rich GGAAG sequence, which is equal to, or similar to, others reported to be exonic splicing enhancers (ESE). We suggest that the nonsense mutation may affect a possible ESE on exon 2, which would hinder the splice site selection and facilitate an aberrant splice with the skipping of this exon. Determination by quantitative PCR shows that the ratio of mRNA with the nonsense mutation to the mRNA with the deletion is approx. 3:1.

INTRODUCTION

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase (HL; EC 4.1.3.4) is a mitochondrial matrix homodimer enzyme [1] that catalyses the cleavage of HMG-CoA to form acetoacetic acid and acetyl-CoA. This reaction is the common final step of leucine catabolism and the hepatic synthesis of ketone bodies that are used as alternative substrates to glucose during fasting [2].

HL deficiency (McKusick 24645) is a rare autosomal recessive metabolic disorder that usually appears either between 2 and 5 days or between 3 and 11 months of life. Clinical acute episodes include vomiting, hypotonia, lethargy and apnoea, that sometimes progress to coma [3,4]. Other symptoms are macrocephalia [5] or, less frequently, microcephalia, development delay and dilated cardiomyopathy with arrhythmia [6]. This disease is fatal in approx. 20 % of cases [7]. These symptoms are accompanied by metabolic acidosis with hypoketotic hypoglycaemia on fasting, variable hyperammonaemia and sometimes high plasma transaminase levels [8].

The characteristic excretory pattern of organic acids (3-hydroxyisovaleric, 3-methylglutaric, 3-methylglutaconic and 3-hydroxy-3-methylglutaric acids) of HL deficiency [3,9] (Figure 1) is insufficient for diagnosis, and also requires direct assay of the enzyme in leucocytes or fibroblasts [10].

The cDNA sequences that encode the HL for human, chicken, mouse and *Pseudomonas mevalonii* have been reported [11–13] and the HL gene has been assigned to the distal short arm of human chromosome 1 [12]. To date only three mutations of the HL gene have been reported: (i) frameshift mutation caused by deletion of two nucleotides [11]; (ii) frameshift mutation caused by the insertion of one nucleotide [14]; (iii) a donor splice site mutation in intron 8, which leads to two aberrantly spliced mRNAs. In one, several nucleotides of the downstream intron were added to exon 8, and in the other the upstream exon 8 was skipped entirely [15].

We now report a new mutation found in two homozygous patients of Portuguese and Moroccan origin with a confirmed biochemical diagnosis of HL deficiency. A nonsense mutation was found in a purine-rich sequence, in the middle of exon 2 of the HL gene: a G–T transversion that generated a stop codon UAA. The protein encoded by the mRNA with the nonsense mutation was heavily truncated and unable to catalyse the enzymic HL reaction. Besides, we also found a mRNA in which the whole of exon 2 had been deleted. We hypothesize that the nonsense mutation produces the skipping of exon 2. The occurrence of the mutation in the middle of the exon in a purine-rich sequence, far from the splice donor and acceptor sites, probably disrupts the effect of a positive exonic splicing enhancer found in this exon. The mRNA in which exon 2 had been skipped represents only 26–28 % of the two transcribed mRNAs. The catalytic efficiency of the encoded, partially deleted, protein is discussed.

MATERIALS AND METHODS

Case reports

One of the probands, P1, was a boy born of healthy non-consanguineous Portuguese parents after a normal pregnancy and delivery. Two previous children had died early in life. On the third day he was hospitalized with vomiting, hypotonia, hepatomegaly and metabolic acidosis without ketosis. No hypoglycaemia was noticed. He was fed with a leucine-deficient

Abbreviations used: HL, 3-hydroxy-3-methylglutaryl-CoA lyase; ESE, exonic splicing enhancers; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; RT-PCR, reverse transcriptase PCR; SSCP, single-strand conformational polymorphism; bGH, bovine growth hormone.

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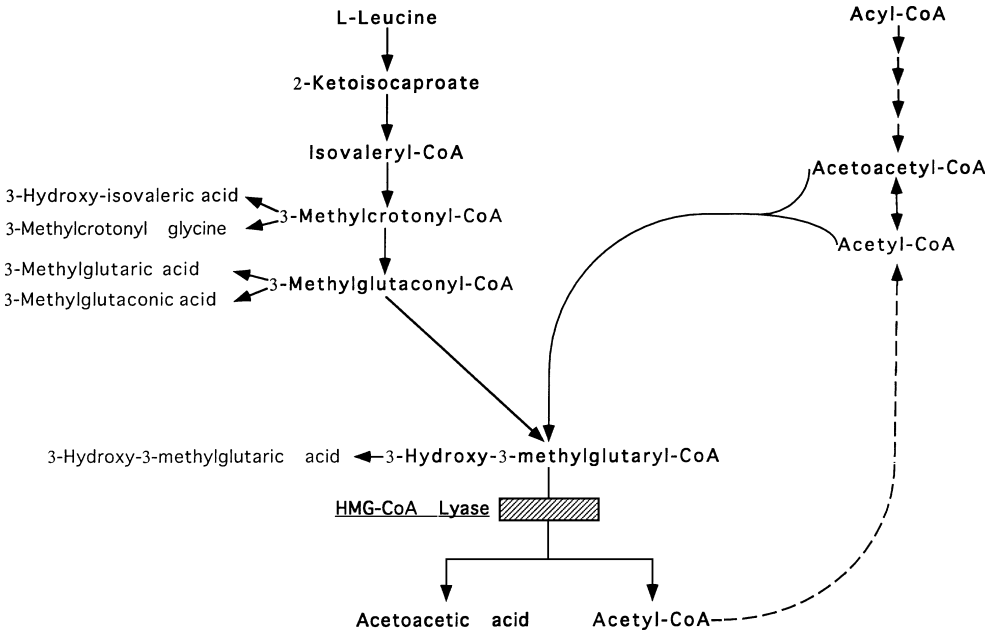

Figure 1 Metabolic interrelationships of HMG-CoA

The site of the defect in patients with HL deficiency is indicated by the cross-hatched box. In liver, HL, along with mitochondrial HMG-CoA synthase, plays a critical role in the production of ketone bodies. In other tissues lacking mitochondrial HMG-CoA synthase, HL is primarily involved in leucine degradation.

artificial milk, recovering shortly. At the age of 4 months he died, at home, of diarrhoea. The enzymic activity of HL was found to be 0 [16]. Gas-chromatographic analysis of volatile fatty acids revealed a peak in the position of 3-methylcrotonic acid. GC/MS analysis of urinary organic acids showed the occurrence of 3-hydroxyisovaleric acid, 3-glutaric acid, 3-methylglutaric acid, a huge peak of 3-hydroxy-3-methylglutaric acid and two unsaturated derivatives. Analysis of urinary amino acids revealed increased excretion of lysine metabolites (lysine itself, saccharopine and α-aminoadipic acid) and β-aminoisobutyric acid. The metabolic study was carried out at the Hospital Debrousse in Lyon (France).

The other proband, P2, was a girl born of healthy consanguineous Moroccan parents. She was breast fed. On the second day of life she presented vomiting and convulsions, hypotonia, slight hepatomegaly and hypoglycaemia (< 20 mg/dl), which was corrected by intravenous administration of glucose. The following morning she suckled well but started vomiting shortly afterwards. Blood ammonia was 375 μg/dl (normal 0–100). The organic acid pattern in urine was typical for HL deficiency [3,9], with lactic acidaemia 4.5 mM (normal up to 2.1), adipic acid (222 mmol/mol creatinine), suberic acid (27 mmol/mol creatinine), high levels of 3-methylglutaconic acid, 3-hydroxy-3-methylglutaric acid, 3-hydroxyisovaleric acid, 3-methylglutaric acid and 3-methylcrotonyl glycine. The enzyme activity in fibroblasts was very low (0.79 nmol/mg of protein per min), less than 3 % of the activity of healthy subjects. Two episodes of decompensation occurred, during a respiratory tract infection and gastroenteritis, which were treated by immediate administration of glucose intravenously at 9.5 mg of glucose/kg body weight per min to abolish the state of catabolism. During these episodes dicarboxylic acids reappeared in the organic acid chromatogram in urine. The baby, who is now 2 years old, is still alive. She was treated in the Provinciaal Centrum voor Opsporing van Metabole Aandoeningen in Antwerpen (Belgium).

Fibroblast culture

A skin biopsy was taken from the probands and from a healthy control. Explants were cultured in 100-mm dishes in minimal essential medium, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 10 % (v/v) foetal-calf serum and 95 % air/5 % CO_2.

Reverse transcriptase (RT)-PCR conditions

Total RNA from cultured fibroblasts of each proband and control was purified with the Quick Prep total RNA extraction kit of Pharmacia Biotech, and a first-strand cDNA was synthesized at 37 °C for 1 h with the Ready to go T-primed First-Strand kit of Pharmacia Biotech. The oligonucleotides used in the RT-PCR experiments were synthesized by Genosys. Radioactive compounds were obtained from Amersham. The primers used in the amplifications were:

orf-f 5′GGCCAACATGGCAGCAATG3′ (bases -7 to $+12$ from human cDNA) [11]
orf-r 5′CCCTATTTCCACATCATCCC3′ (bases 1022 to 1002 from human cDNA)
utr-r 5′CGTAGCTCTCCACTTTCCAC3′ (bases 1455 to 1435 from human cDNA)
f1 5′ATAGACATGCTTTCTGAAGC3′ (bases 177 to 197 from cDNA)
f2 5′CTGCCTCAGAGCTCTTCACC3′ (bases 386 to 405 from cDNA)
f3 5′AGATCTCCCTGGGGGACACC3′ (bases 596 to 615 from cDNA)
f4 5′CTTGGAGGCTGTCCCTACGC3′ (bases 787 to 806 from cDNA)
r1 5′CTTAGGAGACACAAAGCTGG3′ (bases 240 to 221 from cDNA)

r2 5′CTGAAAACTCTCCTCTATGG3′ (bases 444 to 425 from cDNA)
r3 5′ATGTCTTTCATGATCCCTGG3′ (bases 650 to 631 from cDNA)
r4 5′TGGCCAAGTTTCCTGATGCC3′ (bases 832 to 813 from cDNA
F0 bis 5′TCGGTCTCCCTGGGAATTG3′ (3′ boundary of intron 1)
R0 bis 5′CACGTAATACTCAAAGCAGATG3′ (5′ boundary of intron 2)

The amplification conditions were as follows: 1 min at 94 °C, 30 s at 55 °C, 30 s at 72 °C, then 35 cycles and a final extension of 20 min at 72 °C. *Taq* DNA polymerase was purchased from Ecogen S.L. PCR products were separated from the unincorporated primers by electrophoresis on 2 % (w/v) agarose gel. After staining with ethidium bromide, the bands were excised and DNA was extracted from the gel using the Qiaquick Gel Extraction Kit of Qiagen.

PCR amplification of genomic DNA

Genomic DNA was extracted from cultured fibroblasts by a standard procedure [17]. To amplify the intron region of HL encompassing exon 2, 1 μg of genomic DNA was amplified in a 100 μl mixture containing 0.2 mM of each dNTP, 25 pmol of each primer, 2.5 units of *Taq* DNA polymerase in 1 × PCR buffer [20 mM Tris/HCl, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50 % (v/v) glycerol, 0.5 % (v/v) Tween-20, 0.5 % Nonidet P40, pH 8.0] and 2 mM $MgCl_2$. The upstream and downstream primers (F0 bis and R0 bis) complementary to flanking intronic sequences of exon 2 were taken from the literature [14]. They were slightly modified to remove restriction sites.

The conditions were: 98 °C for 10 min, and subsequently 94 °C for 1 min, 55 °C for 30 s, 72 °C for 30 s, then 35 cycles and a final extension of 20 min at 72 °C. PCR products were separated and purified (see above)

Single-strand conformation polymorphism (SSCP)

The SSCP method was performed essentially according to Orita et al. [18]. Overlapping fragments (A, B, C, D and E) from the HL cDNA for HL were amplified by PCR using the primers reported (see above). The amplified fragments were purified using the Quiaquick Gel Extraction Kit of Qiagen. Each purified overlapping fragment (350 ng) was mixed with 20 μl of 10 mM EDTA (pH 8.0)/0.05 % (v/v) xylene cyanol/0.05 % (v/v) Bromophenol Blue [in 98 % (v/v) formamide]. The reaction mixture was incubated at 80 °C for 10 min, placed immediately on ice, and then 10 μl was loaded on to the gel. The gels consisted of 10 % (w/v) acrylamide, 1 × TBE [0.18 M Tris/borate/0.8 mM EDTA (pH 8)], 0.08 % (w/v) ammonium persulphate and 6.6 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. The samples from control and the probands were electrophoresed at 250 V for 21 h at 4 °C. Gels were stained with $AgNO_3$ and then dried at 60 °C for 1–2 h.

DNA sequencing

Purified PCR amplification products were sequenced either by the dideoxynucleotide chain-termination method [19] with modified T7 DNA polymerase (Sequenase, United States Biochemical), or automatically with an Applied Biosystems 373 DNA sequencer ABI Prism using the DNA sequencing kit of Perkin–Elmer.

Quantification of the two HL mRNA levels by PCR kinetic analysis

The relative initial amount (N_0) of HL mRNA of the two species found was quantified by PCR kinetic analysis. To this end, values of the ^{32}P in (c.p.m.) associated with the PCR-amplified cDNAs were determined for a number of consecutive amplification cycles (n) for the two mRNAs in the same sample. To determine the relative difference in N_0 between the two mRNAs, a value of n is chosen at a point where the two curves are parallel (suggesting equal values for the efficiency in amplification), and the log c.p.m. is extrapolated to the intercept for each line ($n = 0$). We performed this according to the following protocol.

(a) Total RNAs from the probands were converted into cDNAs by using the reverse transcriptase; (b) we amplified the HL cDNAs using orf-f and utr-r as primers; (c) using PCR again we re-amplified fragment A using 2.5 μCi (0.25 μl) of [α-^{32}P]dCTP (3000 Ci/mmol) and the mixture of reagents used in the PCR reaction. The amplification was carried out in different tubes with a variable number of cycles (from 18 to 30). Aliquots of 15 μl were analysed on a 3 % agarose metaphor gel. For the quantification of relative c.p.m., the two amplified gel bands of each lane were cut out and their radioactivity was analysed in a liquid-scintillation counter. For the kinetic analysis, values of log c.p.m. were plotted against the number of cycles, in both mRNA species. The lines obtained are consistent with the equation:

$$\log[^{32}\text{P radioactivity (c.p.m.)}] = [\log(1+E)]\cdot n + \log N_0$$

where E is the efficiency of the amplification, n is the number of cycles and N_0 is the initial amount of the mRNAs. This method allowed us to determine the difference in the initial number of target molecules, i.e. the HL mRNA with the stop codon and the HL mRNA lacking exon 2, but not the absolute number of the starting target molecules [20,21].

RESULTS

RT-PCR

Fibroblast cultures obtained from skin biopsies on the two probands were used for the extraction of RNA, which was then used as a substrate for the first strand cDNA synthesis. We amplified the orf-f/utr-r (ORF-0, 1455 bp) fragment and then we re-amplified the sample using orf-f and orf-r as primers (fragment ORF, 1024 bp) (Figure 2ii). The orf-f/orf-r cDNA was used as a template for the PCR amplification of different overlapping fragments called A, B, C, D and E, as summarized in Figure 2(ii). While in the control the amplified fragment orf-f/orf-r generated a single fragment of 1024 bp, an additional fragment of approx. 940 bp appeared in the probands (Figure 2iii). The amplified fragments A–E obtained from the control and the proband fibroblast mRNAs are shown in Figure 2(iiii). The amplification of fragment A (orf-f/r1) showed two bands, one of approx. 247 bp and the other of approx. 163 bp in each proband (Pb1 and Pb2). The other amplified fragments, B–E, were of the expected size. Thus these findings indicated that two HL mRNA species were present in the fibroblasts of both probands.

SSCP analysis

SSCP analysis of the different purified fragments A–E was carried out in the control and in the proband fibroblasts. Fragment A (247 bp) of the probands had a different band

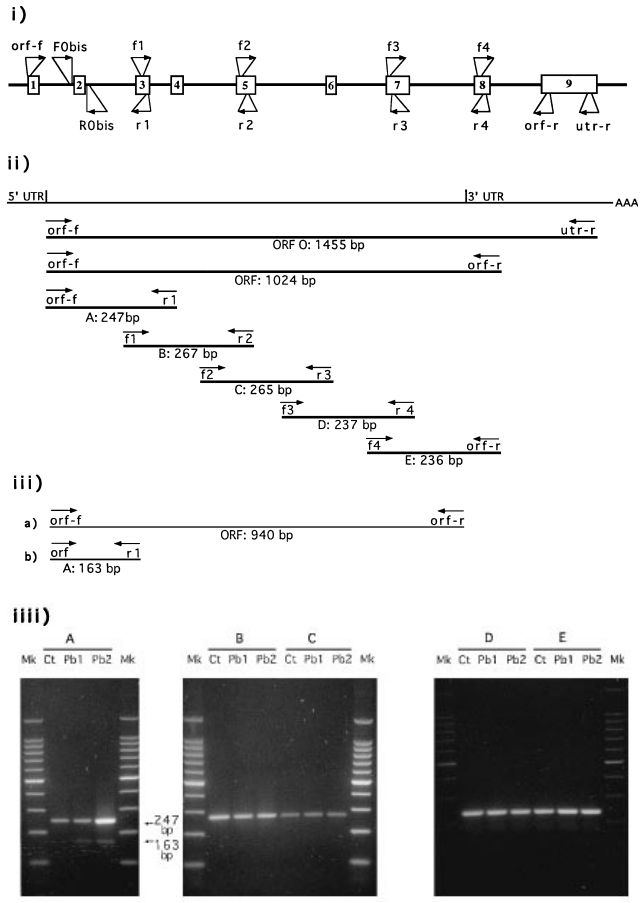

Figure 2 Schematic structure of the human HL gene

(i) The exons (shown as boxes) and introns (shown as lines) and primers used are indicated. Redrawn from [38]. (ii) Diagram of the ORF-0, ORF and different overlapping fragments A, B, C, D and E amplified by RT-PCR using specific sets of oligonucleotides described in the Materials and methods section. (iii) The abnormal RT-PCR products obtained in the probands are indicated with (a) an ORF cDNA fragment with 940 bp instead of 1024 bp of the control and (b) fragment A with 163 bp instead of 247 bp of the control. (iiii) The different amplified fragments A, B, C, D and E were separated by electrophoresis in 2% agarose gels. RT-PCR products from control (Ct) and probands (Pb1 and Pb2) are shown. Lanes Pb1 and Pb2 of fragment A show two bands, one normal, of 247 bp, and the other of 163 bp. Mk, molecular-mass markers; UTR, untranslated region; ORF, open reading frame.

pattern from that of the control (Figure 3). Moreover, SSCP analysis of fragment D in the probands and in the control revealed two different band patterns. The other fragments, B, C and E, did not show any difference between control and probands (results not shown).

Sequence of the RT-PCR fragments

PCR fragments A from the probands and control were purified and sequenced. The smaller fragment, A, of the probands revealed an 84-bp deletion from nucleotides 61 to 144 of the HL cDNA. The size and location of this deletion coincided with exon 2 [14]. The sequence of the normal-sized fragment A of the probands showed a point mutation: a G–T transversion at nucleotide 109 of the HL cDNA, which changed from triplet 37 (GAA) to UAA, i.e. a stop codon (Figure 4).

The sequence of all fragments D from control and probands showed the presence of an A–G transition at nucleotide 727 of HL cDNA, in relation to the reported human cDNA [11].

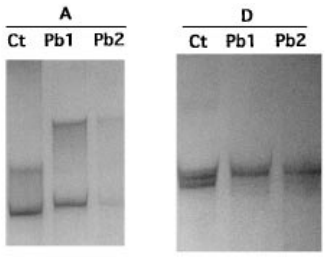

Figure 3 SSCP analysis of the amplified HL fragments A and D, in probands and controls

Ct, control; Pb1, proband 1; Pb2, proband 2.

Moreover, the sequence of fragment D from the control also revealed another A–G transition at nucleotide 654 of the HL cDNA, which did not, however, affect the enzymic activity of the encoded protein.

PCR amplification and sequence of the deleted exon and the flanking intron boundaries

To explore the origin of the deletion seen in one of the A fragments, we amplified the exon 2 and the intron region that encompassed it. Using the modified primers fo bis/ro bis, a fragment of approx. 200 nucleotides was amplified by PCR using genomic DNA as a template. The fo bis/ro bis amplification always yielded a fragment of the same size whether control or proband genomic DNA was used. After purification, this fragment was sequenced. The probands were homozygous for the mutation 109 G–T, which produced a stop codon. No point mutations were found in the 3′ acceptor or 5′ donor splice sites or in the pyrimidine-rich domain. These results suggest that the nonsense mutation could be the cause of partial alternative abnormal splicing in which exon 2 is skipped.

Predicted translation products of HL mRNAs

The nonsense mutation G–T at nucleotide 109 of the HL cDNA causes the change of the codon GAA-37 (Glu) to UAA, i.e. a stop codon. The translation of the mRNA generates a truncated mature protein formed by the leader peptide to target the protein into mitochondria and the first nine amino acids TLPKRVKIV (Figure 5) of the mature HL protein. The resulting small peptide does not contain the important Cys-266 of the catalytic site [22] nor Cys-323, which is responsible for the formation of the homodimer protein found in eukaryotes [23], and thus it cannot catalyse the HL enzymic reaction.

The mRNA with the 84 bp in-frame deletion leads to the loss of 28 amino acids (Val-21 to Lys-48) in the mature protein (Figure 5). This deleted region includes the last seven amino acids, VSTSSMG, of the leader peptide of the precursor HL protein and 21 amino acids of the N-terminus of the mature protein.

The A–G transition observed in the amplified fragment D at nucleotide 654 of the HL cDNA produces no change in the amino acid encoded, leucine (a silent mutation). The A–G transition at nucleotide 727 was observed in both mRNAs of probands and controls. This transition causes a change from the codon ACC-243 (Thr) to GCC (Ala), which does not modify the catalytic efficiency of the enzyme.

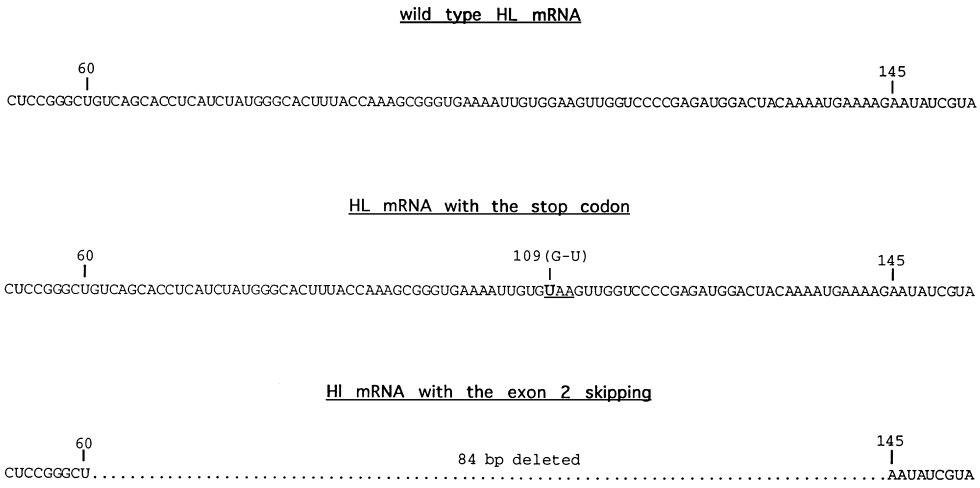

Figure 4 mRNA sequences of HL wild-type, HL with the stop codon and HL with the exon 2 missing are shown

mRNA with the stop codon has a G–U transversion at nucleotide 109 of the HL cDNA, which produces the change of the triplet 37 GAA (Glu) to UAA (stop codon). The mRNA with exon 2 missing presented a 84 bp deletion from nucleotides 61 to 144 of the HL cDNA that coincided with the location and size of exon 2.

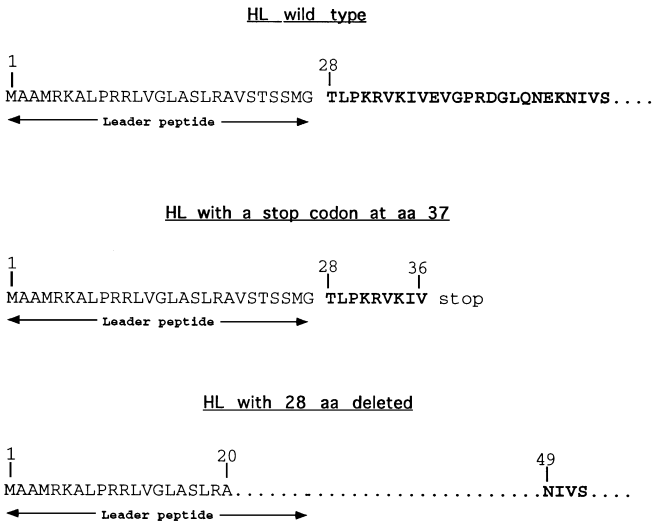

Figure 5 Peptide sequences of HL wild-type, HL with the stop codon in amino acid 37 and HL with the deletion of 28 amino acids

The HL with the stop codon at amino acid (aa) 37 produces a truncated protein. The HL with the deletion of 28 amino acids has lost the last seven aa of the leader peptide to import the protein into mitochondria, and the 21 aa of the HL N-terminus. The predicted site of cleavage of the mitochondrial leader peptide is also lost.

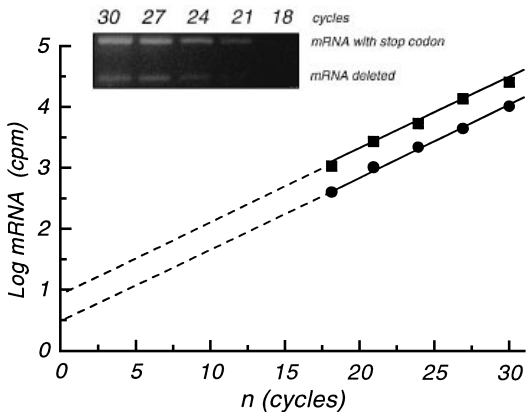

Figure 6 Quantitative determination of the two HL mRNA species by PCR kinetic analysis

The initial amounts of the two HL mRNAs species observed in the probands were determined by kinetic analysis as described in the Materials and methods section. The electrophoretic pattern of the two amplified mRNA species in relation to the number of cycles is shown in the inset. The log c.p.m. of ^{32}P from [α-^{32}P]CTP incorporated into each of the two amplified species was plotted against the number of cycles of amplification. Two straight lines were obtained with the same slope, indicating that the efficiency of amplification was the same. The antilog of the respective intercepts shows the ratio of the two mRNAs [the nonsense mutated mRNA (●) and the mRNA with the exon missing (■)], which equals 1/2.73. The data correspond to the patient P1. The ratio corresponding to the patient P2 (not shown) is 1/2.57.

Quantification of the two HL mRNAs observed in the probands

To determine the relative amount of each species of HL mRNA in the probands, kinetic analysis by PCR was performed on fragments A, which are different in size in the two species analysed. As shown in Figure 6, which represents data for the proband P1, the slopes of the two straight lines are identical, showing that the efficiency of the amplification in the two fragments is the same. The determination of the intercept for both lines indicates that the ratio of mRNA with the exon missing to mRNA with the nonsense mutation is 1:2.73, which means that the deleted mRNA is 26.8% of the total HL mRNA species. Identical determination carried out on the patient H.C. gave a ratio of 1:2.57, i.e. the deleted mRNA is 28.0% of the total mRNA.

DISCUSSION

The HL deficiency is a rare autosomal recessive inborn error. We report a novel point mutation of the HL gene that is identical in two patients, of Portuguese and Moroccan origin, with HL deficiency. This is the first HL mutation described

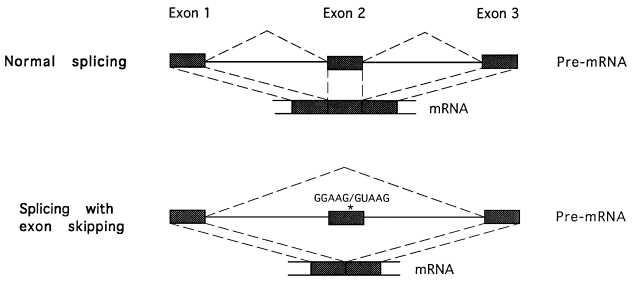

Figure 7 Schematic representation of normal splicing and aberrant splicing with exon skipping of the HL pre-mRNA in the probands

The G–U transversion, located in the middle of the exon (marked with an asterisk), affects a probable ESE (GGAAG) in exon 2 and allows the formation of another mRNA species, present in lower amounts, as a result of the skipping of the whole of exon 2.

in two patients of different geographical origin. Only a nonsense mutation in exon 2 was found in the HL cDNA, and two mRNA species were derived as a consequence of this unique mutation, one with the stop codon, identical in size with control, and the other lacking exon 2. Other mutations within exon 2 were not observed either in the donor or in the acceptor splicing sites, or in the pyrimidine-rich domain. As described in other cases [24–26], the nonsense mutation can produce an aberrant splicing with the skipping of the constitutive exon in which this mutation is found.

The mRNA with exon 2 missing has a deletion of 84 nucleotides. The translation product of this mRNA causes the loss of 28 amino acids in the N-terminal domain of the HL enzyme. There is no kinetic evidence that this deletion can produce a protein with low but measurable enzymic activity; however, certain findings reinforce this interpretation. First, seven amino acids of the leader peptide are lost. This may hinder the passage of the enzyme into the mitochondria. Second, even if the protein enters, it may not be fully processed, because the lack of seven amino acids in the C-terminus of the leader peptide could produce a decrease in the signal for intra-mitochondrial proteolysis in the truncated leader peptide. Third, the expression of the mRNA with exon 2 missing is low (26–28 % of both HL mRNAs).

The mRNA with a premature stop codon at triplet 37 generates a truncated protein that has only the leader peptide and the nine amino acids of the N-terminus of the mature protein. A high percentage of the amino acid sequence is lost ($> 97\,\%$), including the domain that contains the active catalytic centre of the enzyme [22] and Cys-323, which is responsible for the formation of the homodimer protein found in eukaryotes [23]. In this case the truncated mRNA could not codify for a functional enzyme.

SSCP analyses of fragment D of the HL cDNA reveal a different band pattern in the control and in the probands. The sequencing of fragment D of the control shows the A–G transition at nucleotide 654. This mutation leads to a change of codon 218 from CTA (CUA) to CTG (CUG), both encoding the same amino acid, leucine. It is a case of a polymorphic mutation that produces a silent mutation, without functional reflection in the protein. Moreover, in all cases including controls, the sequence of fragment D of the HL cDNA reveals an A–G transition at the nucleotide 727. This mutation changes the codon 243 from ACC (Thr) to GCC (Ala). The change does not seem to affect the activity of the enzyme. In fact, both the chicken and *P. mevalonii* HL have alanine at this position, and both are catalytically active. Our finding suggests either the occurrence of a polymorphism or an error in the sequence of the HL cDNA reported previously [11].

The occurrence of a nonsense mutation in a gene is frequently associated with (i) a decrease in the steady-state level for the point-mutated mRNA and (ii) the skipping of the constitutive exon that contains the mutation [24–26]. The decrease in mRNA levels in other genes could be related to the premature ending of translation due to the presence of a stop codon [27]. However, this is not the case in the mutation studied. The point-mutated mRNA species is more abundant (72–74 %) than the mRNA species with the exon missing (26–28 %).

Partial skipping of exon 2, which contains the nonsense mutation, takes place in both probands. This aberrant splicing involves a lack of recognition of the correct splice sites [28] (Figure 7). It has been reported that skipping in a constitutive exon could depend on the change that a nonsense mutation produces in the structure of the exon [27].

The classical splice-site consensus sequences are less well conserved in the genes of the higher eukaryotes than in the lower eukaryotes, such as yeast [29]. On the other hand, the alternative splicing occurs in at least one out of every 20 genes [30]. This mechanism allows the production of more than one protein for every gene, owing to the recognition of different splice sites in the same pre-mRNA [28,30]. These circumstances suggest that the splice donor, the splice acceptor, the branch point and the pyrimidine-rich sequences are not the only factors that regulate the selection of the splice sites in the higher eukaryotes [31]. It has been demonstrated that purine-rich sequences included in the exons, called exonic splicing enhancers (ESE) [32,33], may affect the selection of the splice sites [33–35]. It is admitted that these exonic enhancers may act as *cis* elements regulating either the inclusion of the exon in the splicing or the intron retention [31]. A number of examples have been published in which the ESE sequences modulate the inclusion of an exon in the mature mRNA species. This has been observed in HIV type I, which has an ESE and an exonic splicing silencer able to *cis*-acting modulate the 3′ *tat rev* splice site [36,37].

The consensus sequences of eukaryotic ESE are not yet well known. It has been shown that different purine sequences present in exon 5 of the bovine growth hormone (bGH) of the pre-mRNA may modulate the splicing machinery. The sequences GGAA and GGAAG are of this kind, although the highest efficiency occurs in a sequence of 13 mononucleotides [30]. Another typical example is the cardiac troponin T pre-mRNA, which contains an ESE that is required for the inclusion of the alternative exon 5 [31,34]. The sequence of this ESE is the 9-nucleotide motif GAGGAAGAA. Besides, a series of point mutations in this ESE have been described *in vivo* that increase or decrease the exon 5 inclusion [31].

The nonsense mutation found in the HL exon 2 is a G–T transversion that affects a purine-rich sequence that changes from GGAAG to GTAAG. The non-mutated sequence is equal to that mentioned above in the pre-mRNA bGH, functioning as an ESE [30]. Furthermore, this sequence is also analogous to the ESE of cardiac troponin T, and identical with the central five purine bases present in the sequence GA**GGAAG**AA shown above [31]. This last sequence is one of the few to have been well defined as an ESE to date. We suggest therefore that the nonsense mutation could affect a possible positive ESE sequence seen in the middle of exon 2, thus hindering the selection of its splice sites and facilitating a partial aberrant splicing that would lead to the skipping of this exon.

The skipping of exon 2, subsequent to suppression of an ESE sequence after the G–T transversion, may represent an advantage for these patients. It is clear that if the skipping was not

produced, the enzyme could not be active in these probands, since the premature truncation of the protein, which resulted in only nine amino acids, eliminated all catalytic activity. Through the mechanism described, the nonsense mutation is not present in the exon 2-deleted mature protein, which conserves 277 amino acids, with 26–28 % of occurrence. Although small, the enzymic activity due exclusively to this protein showed a residual value estimated as less than 3 % in the Moroccan patient, which was probably enough to facilitate her survival.

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