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

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Abstract A novel point mutation in the 3-hydroxy-3-methylglutaryl coenzyme A lyase gene was found in a Turkish patient with homozygous 3-hydroxy-3-methylglutaric acidemia. Amplification by RT-PCR of the mRNA using six different pairs of oligonucleotides produced no differences in four of the fragments amplified with respect to the control, but generated two fragments of different size. One was representative of a deletion of 126 bp and the other of an insertion of 78 bp. These abnormal mRNAs resulted from a G → C transversion at the nucleotide +1 of an intron, which changed the invariant GT dinucleotide of the 5' donor splice site. This was associated with the occurrence of an alternative splicing, which led to the skipping of the whole exon of 126 bp, and also with the activation of one cryptic donor splice site in the same intron. These aberrant spliced mRNAs are predicted to encode two abnormal HMG-CoA lyase proteins: the first results in a protein with an internal deletion of 42 amino acids, whose enzyme activity is largely abolished, as the catalytic site was completely removed; the second contains 17 missense amino acids that precede a stop codon. Northern blot analysis showed that the overall content of these aberrantly spliced mRNAs in proband fibroblasts was the same as that found in control fibroblasts. However, hardly any transcript was observed corresponding to the inserted mutated mRNA when it was examined by a specific probe. To quantify the relative proportion of the two mRNAs, a quantitative RT-PCR (the DNA-mimic PCR reaction) was carried out. Results show that the proportion of the inserted mRNA with respect to the deleted mRNA is only 1.2%. The father, mother, and two brothers of the proband were heterozygous in the G → C mutation in the +1 nucleotide of the intron considered, while the two alleles of another brother were free of the mutation.—**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.** 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.* 1996. **37:** 2420–2432.

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3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) lyase (HL)(1), (EC 4.1.3.4) is a mitochondrial enzyme that catalyzes the cleavage of HMG-CoA to acetyl-CoA and acetoacetic acid. This reaction is the common final step of the ketogenic pathway and the catabolic pathway of leucine (**Fig. 1**). The functional avian enzyme is a dimer of 31.4 kDa located in the mitochondrial matrix (2, 3) and the size of human HL is 31.6 kDa (3). Ketone bodies (acetoacetate and D-3-hydroxybutyrate), as oxidative fuels, can act as alternative substrates to glucose for peripheral tissues, but they also have pivotal roles as lipogenic precursors and as regulators of metabolism (1).

In humans, the deficiency of HMG-CoA lyase is a rare, clinically heterogeneous inborn error of metabolism, with a bimodal distribution, with about 30% of cases appearing between 2 and 5 days of life, 60% between 3 and 11 months. This disease usually presents with vomiting, hypotonia, and lethargy, which progresses to
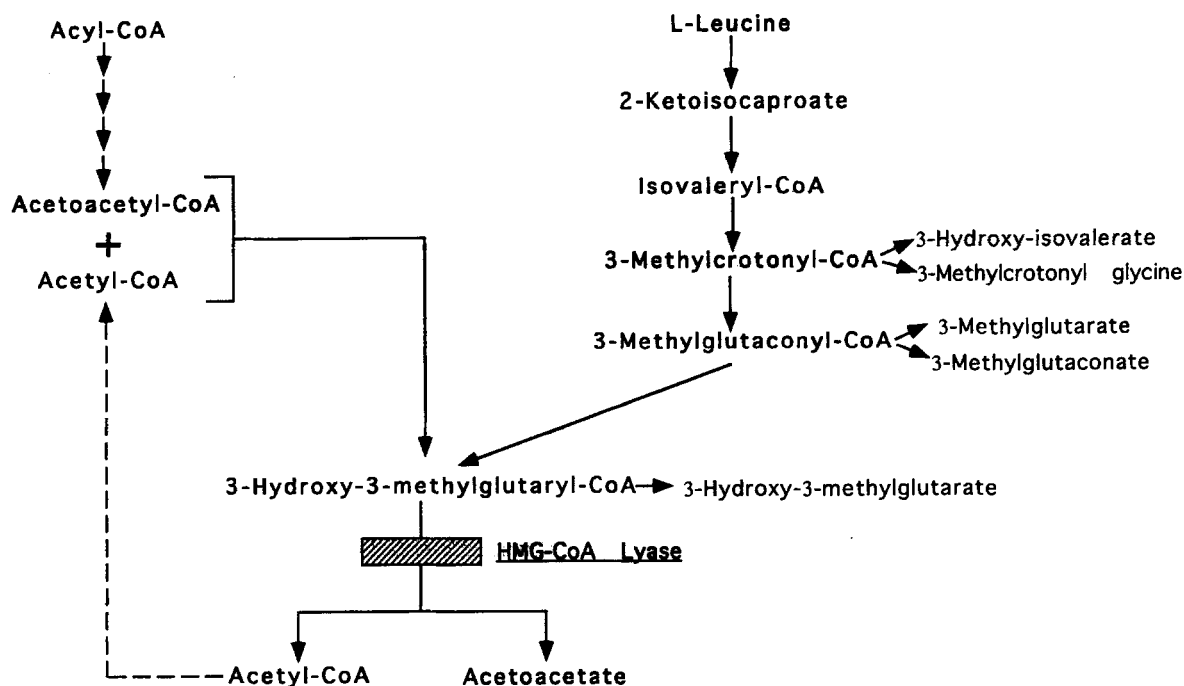

Fig. 1. Metabolic interrelationships of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The site of the defect in patients with HMG-CoA lyase deficiency is indicated by the cross-hatched box. In liver, HMG-CoA lyase, along with mitochondrial HMG-CoA synthase, plays a critical role in the production of ketone bodies. In other tissues lacking mitochondrial HMG-CoA synthase, HMG-CoA lyase is primarily involved in leucine degradation.

coma in about 10% of cases. All patients have metabolic acidosis and they are prone to severe hypoketotic hypoglycemia on fasting, hyperammonemia, hepatomegaly, and elevation of transaminases. Some patients were macrocephalic and severely mentally retarded, one was microcephalic, and many presented developmental delay (4, 5). This autosomal recessive disease has proven fatal in about 20% of cases (6–9) and, in other cases, fatal cardiomyopathy (10) and sudden infant death syndrome (11) have been associated with HL deficiency. A number of patients have either been initially diagnosed with Reye's syndrome or described as having symptoms very similar to those of Reye's syndrome (12–14).

The characteristic abnormal metabolites in urine include 3-hydroxy-3-methylglutaric acid and others derived from the metabolism of leucine (Fig. 1). The amount of residual enzymatic 3-hydroxy-3-methylglutaryl-CoA lyase activity was shown to be essentially the same in fibroblasts of the patients with a mean of 1.1% of the normal range (15).

Recently, cDNA clones for 3-hydroxy-3-methylglutaryl-CoA lyase were obtained from both chicken and human cDNA libraries (3) and the gene was assigned to the distal short arm of human chromosome 1. Human fibroblasts and liver show a single 1.7-kb mRNA on Northern blots. The genetic study in two Acadian

French-Canadian siblings (3) with the disease has shown the occurrence of a small deletion of two nucleotides determining a truncated protein in the amino terminal domain, thus unable to perform catalytic HMG-CoA lyase activity. More recently, a new frameshift mutation has been diagnosed, produced by the insertion of one nucleotide (16) determining an HL activity of the encoded protein of less than 7% of control values.

We report here the third genetic study performed, in a patient of Turkish origin with a biochemically confirmed diagnosis of deficiency of HMG-CoA lyase. The donor splice site mutation in the HL gene, leads to two aberrantly spliced mRNAs, one producing the skipping of an exon of 126 bp and the other producing an insertion of 78 bp originally present in the downstream intron. The proteins encoded by these abnormal mRNAs would be unable to act as efficient enzymes as i) the truncated protein corresponding to the deletion affected most of the catalytic site, and ii) the protein corresponding to the insertion contains 17 aberrant amino acid residues preceding a stop codon, and lacks the important Cys-323 necessary to conform the physiological homodimeric enzyme. Further, the expression of the mRNA with the insertion is largely abolished. This was observed not only by Northern blot, but also by a quanti-

tative RT-PCR (the DNA-mimic PCR reaction), which showed that the amount of mRNA with the insertion was only 1.2% of that of the mRNA with the deletion.

MATERIAL AND METHODS

Subjects

The proband (E.C.) was a 4-day-old male born to Turkish parents, who on physical examination had all the clinical features (i.e., tachypnea, hepatomegaly, lethargy, and hypotonia) of classical HMG-CoA lyase deficiency. The organic acids found in blood and urine confirmed this diagnosis. He was treated at the Wilhelmina Kinderziekenhuis at Utrecht (Netherlands) with intravenous glucose and $NaHCO_3$ solutions, and he recovered within a few days. The proband's parents and brothers were asymptomatic, with serum glucose concentrations in the normal range. All subjects gave their informed consent for this study.

Biochemical analysis

All common biochemical analyses, such as blood glucose, ketone bodies, transaminases, and organic acids in blood and urine, were carried out in the laboratory of the Wilhelmina Children's Hospital at Utrecht.

Fibroblast culture

A skin biopsy was taken from the proband and every member of his family. Explants were cultured in 25-cm² flasks in minimal essential medium (MEM), 100 IU/ml of penicillin, and 50 mg/ml of 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 (17). DNA was digested using 5–10 U/mg of several restriction enzymes, separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with human HL cDNA probes as reported (3).

RT-PCR conditions

Poly(A+)-rich RNA was purified 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 PCR-RACE experiments were synthesized by Pharmacia. Radioactive compounds were obtained from Amersham. The primers used in the amplifications were:

orf-f 5′ GGCCAACATGGCAGCAATG 3′ (bases −7 to 12 from human cDNA)(15);

orf-r 5′ CCCTATTTCCACATCATCCC 3′ (bases 1022 to 1002 from human cDNA);

f1 5′ ATAGACATGCTTTCTGAAGC 3′ (bases 177 to 197 from cDNA);

f2 5′ CTGCCTCAGAGCTCTTCACC 3′ (bases 386 to 405 from cDNA);

f3 5′ AGATCTCCCTGGGGGACACC 3′ (bases 596 to 615 from cDNA);

f4 5′ CTTGGAGGCTGTCCCTACGC 3′ (bases 787 to 806 from cDNA);

r1 5′ CTTAGGAGACACAAAGCTGG 3′ (bases 240 to 221 from cDNA);

r2 5′ CTGAAAACTCTCCTCTATGG 3′ (bases 444 to 425 from cDNA);

r3 5′ ATGTCTTTCATGATCCCTGG 3′ (bases 650 to 631 from cDNA);

r4 5′ TGGCCAAGTTTCCTGATGCC 3′ (bases 832 to 813 from cDNA);

f3bis 5′ ACACCTTGATGGCCCTGCA 3′ (bases 731 to 749 from cDNA);

r4bis 5′ CATCCACTTTTGTTCTCAGC 3′ (bases 75 to 56 from the inserted sequence from the intron).

The PCR amplification conditions were as follows: 1 min at 94° C, 30 sec at 55° C, 30 sec at 72°C, then 35 cycles and a final extensions of 20 min at 72°C. Taq DNA Polymerase was purchased from Perkin-Elmer. PCR products were separated from each other and from the unincorporated primers by electrophoresis on a 2% agarose gel. After staining with ethidium bromide, the bands were excised and DNA was extracted from the gel using Quiaex (Diagen, GmbH, Germany).

DNA sequencing

Purified PCR amplification products were sequenced either by the dideoxynucleotide chain termination method (18) with modified T7 DNA polymerase (Sequenase, United States Biochemical), or automatically with an Applied Biosystems 373 DNA sequencer using the fluorescent terminator kit (Perkin-Elmer).

PCR amplification of genomic DNA

To amplify the region of HL encompassing the point mutation at the 5′ end of the intron, 1 μg of genomic DNA was amplified in a 100 μl mixture containing 0.2 mM of each dNTP, 50 pmol of each primer, 2.5 units of Taq DNA polymerase in 1 × PCR buffer and 1.5–5 mM $MgCl_2$. f4 forward primer (see above) and orf-r reverse primer were used. The conditions were: 98°C for 10 min, 55°C for 30 sec, 72°C for 1.5 min for the first time and subsequently 94°C for 1 min, 55°C for 30 sec, 72°C for 1.5 min for 30 cycles. PCR products were separated and purified (see above).

RNA blot analysis

Total RNA from the cultured fibroblast was isolated as described (19). RNA samples were fractionated in 1% agarose/formaldehyde gels, transferred to Nytran-N membranes (Schleicher & Schuell) and cross-linked by heat. Hybridizations were carried out as described (17) using either the full-length HL cDNA or the mentioned parts of it as a probe (RT-PCR conditions), 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 Molecular Dynamics 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).

Transcriptional analysis of the levels of the two mRNAs for HMG-CoA lyase by amplification of a competitive PCR

Generation of the competitive PCR fragment (DNA-mimic). DNA-mimic is a cDNA fragment used as competitive internal standard in PCR amplification, which may be used for quantitation of mRNA levels of target genes. Two DNA-mimics were prepared. The first to measure the proportion of the mRNA with the insertion, and the second to determine the amount of the two mRNAs. Both DNA-mimics were constructed by two rounds of PCR amplification of the generic DNA fragment EcoRI-BamHI (0.6 kb) of the v-erb B oncogene (20), using specific primers: the forward primer f3 was the same in the determination of both DNA-mimics. The reverse primers were different in each case. To quantify the mRNA with the insertion, the primer r4 was chosen in the exon preceding the insertion, so the deleted mRNA could not be amplified. To quantify the mixture of both mRNAs the primer (orf-r) of the next exon was chosen.

In the first PCR reaction in each case, two composite primers were used. Each composite primer had the mitochondrial HMG-CoA lyase gene primer sequence attached to a short, 20-nucleotide stretch of sequence designed to hybridize to opposite strands of a heterologous DNA fragment (0.6 kb EcoRI-BamHI of the v-erb B oncogene). This ensured that all DNA–DNA-mimic molecules had the complete gene-specific primer sequences. These composite primers have the following sequences:

foward primer: 5′ AGATCTCCCTGGGGGACACCCG CAAGTGAAATCTCCTC 3′;
reverse primer-1: 5′ TGGCCAAGTTTCCTGATGCCTT GAGTCCATGGGGAGCT 3′;

reverse primer-2: 5′ CCCTATTTCCACATCATCCCTT GAGTCCATGGGGAGCT 3′.

The procedures for primary (a) and secondary amplification (b) are the following. a) The primary amplification was carried out in a final volume of 50 µl with 5 µl of PCR buffer, 0.2 mM dNTP each, 2 ng of v-erb B DNA fragment, 0.4 µM each composite primer, and 2.5U Taq DNA polymerase. The cycle parameters were: denature 45 sec, 94°C, anneal 45 sec, 60°C, and extend 90 sec, 72°C, 16 cycles. A dilution of the first PCR reaction was then amplified again using only the gene-specific primers.

b) Two µl of the primary PCR reaction was removed and diluted in 200 µl in water. Two µl of this dilution was used to carry out the secondary amplification, under the same conditions as the first, but using the specific primers depending on the mRNA to be analyzed. In the case of the mRNA with the insertion, the forward primer was taken from the previous exon of HMG-CoA lyase, 5′ AGATCTCCCTGGGGGACACC 3′ and from the deleted exon of HMG-CoA lyase (reverse) 5′ TGGCCAAGTTTCCTGATGCC 3′ to a total of 25 cycles. In the case of the quantification of both mRNAs the forward primer was the same and the reverse primer was taken from the exon that follows the deleted exon: 5′ CCCTATTTCCACATCATCCC 3′. After the second PCR amplification the PCR DNA-mimics were purified by passage through CHROMA SPIN+TE-100 columns (Clontech Laboratories). The yield of the DNA-mimics was then calculated by absorbance at 260 nm.

Analysis of the levels of the deleted and inserted mRNAs. Poly A+-rich RNA was purified and a first strand cDNA was synthesized with the reverse transcriptase (RT). The reaction was set up in 20 µl of a mixture containing 0.1 µg of DNA-free mRNA (in water), 0.4 µg of utr-r primer 5′ CGTAGCTCTCCACTTTCCAC 3′ (bases 1485 to 1505 from human HL cDNA), 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphates, 4 µl of 5× RT buffer (250 mM Tris/HCl, pH 8.3; 375 mM KCl, and 15 mM MgCl$_2$) and 200 U of mouse mammary leukemia virus reverse transcriptase (Bethesda Research Laboratories), and incubated at 37°C for 60 min, heated to 95°C for 5 min, and then quick-chilled in ice. The cDNA was amplified using the orf-f and utr-r primers. The amplification conditions were as follows: 1 min at 94°C, 30 sec at 55°C, 90 sec at 72°C, then 35 cycles and a final extension of 20 min at 72°C. The final product was used as a template to amplify the fragments used in the competitive PCR.

The HMG-CoA lyase mRNA levels were determined by PCR co-amplification as follows. Three µl of the amplified cDNA fragment (orf-f–utr-r) and increasing amounts, between 6 and 3.75 × 10^3 attomoles, of DNA mimic were added to the reaction mixture composed

of 4 µl of PCR buffer (100 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$), 0.2 mM dNTP each, 0.2 µM each composite primer, and 2 U ampliTaq DNA polymerase (5 U/µl) and 2.5 µCi (0.25 µl) of [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) to a final volume of 50 µl. As the estimated amounts of the mRNA with deletion were much higher than the mRNA with the insertion, the determination was carried out in the same manner but diluting (1/10) the amplified cDNA (orf-f–utr-r) used before, and increasing amounts, between 3.6 × 10^2 and 22.5 × 10^4 attomoles, of the DNA mimic. PCR was performed for 25 cycles. The amplification was carried out using the following cycle parameters: denature 60 sec, 94°C, anneal 75 sec, 59°C, and extend 45 sec, 72°C. The primers were from the previous exon of HMG-CoA lyase (forward), 5' AGATCTCCCTGGGGGACACC 3' and from the next exons of HMG-CoA lyase (reverse) 5' TGGCCAAGTTTCCTGATGCC 3' and 5' CCCTATTTC CACATCATCCC 3'. The reaction products (10 µl) were analyzed on 1.8% agarose gels in Tris acetate/EDTA (10 mM/1 mM) buffer pH = 8, visualized by staining with ethidium bromide, and documented on Polaroid no. 665 film. For quantitation of relative band intensities, gels were cut and counted in a liquid scintillation counter.

RESULTS

Clinical data

Routine clinical chemical analysis of the proband (E.C.) revealed the following data: glucose 1 mM (N 2.8–6.0), ALT 291 U/l (N 7–43), AST 495 U/l (N 3–47), uric acid 1.14 mM (N 0.05–0.40), pH 7.07, pCO$_2$ 3.8 kPa, standard bicarbonate 7.9 mmol/l (N 21–28). Cerebrospinal glucose was 1.2 mM (N 3.2–4.5). Selective screening for inborn errors of metabolism showed 3-hydroxyisovaleric, 3-methylglutaric, 3-methylglutaconic, and 3-hydroxy-3-methylglutaric acids to be very high in urine. Neither acetoacetic nor β-hydroxybutyric acid was found. A confirmatory enzyme diagnosis was made in isolated platelets, with an HL activity of <0.1 nmol/protein per min. (N: 27.5 ± 3.9). The activities found in the parents and two brothers were in the heterozygous range. Another brother had normal activity.

Southern blot analysis

To rule out the presence of major structural defects of HMG-CoA lyase gene, genomic DNA from the proband, from his relatives and from a normal control subject was digested with EcoRI and HindIII. After Southern blotting and hybridization with the HL cDNA as a probe, no major rearrangements of the HL gene were detected (data not shown).

Reverse transcription PCR (RT-PCR)

Fibroblast cultures obtained from skin biopsies on E.C. were used for the extraction of RNA, which was then used as a substrate for the first strand cDNA synthesis. This cDNA was used as a template for the amplification by PCR of different overlapping fragments, as summarized in **Fig. 2A.** Figure 2B shows the amplified fragments obtained from control human placenta mRNA and also the fragments amplified from the patient cDNA. The size of the fragments amplified from the patient was different from the control in some cases. First, the amplified fragment orf-f/orf-r from the patient was about 900 bp in comparison with the 1024 bp found in control with this set of primers; second, fragment 15 obtained with primers f4 and orf-r showed a clearly altered electrophoretic mobility (310 bp in the patient in contrast to 236 bp in the control); and third, amplification 16 obtained with primers f3 and orf-r also showed a significant difference in size between the patient and control (about 300 bp in the patient instead of 429 bp obtained from human placenta control mRNA). These findings thus indicated that two HL mRNA species were present in proband fibroblasts.

Sequence of the RT-PCR fragments

PCR fragments 15 and 16 from the patient were purified and sequenced. Fragment 16 revealed a 126 bp deletion from nucleotide 751 to 876 in the human lyase cDNA. In contrast, fragment 15 revealed a 78 bp insertion starting at the nucleotide 876 (**Fig. 3A**). We had thus found a deletion in one mRNA and an insertion in the other mRNA, located close to the left and to the right of the same nucleotide, respectively. After both the insertion and the deletion, the nucleotide sequence was identical to the cDNA from normal (control) human HMG-CoA lyase.

PCR amplification and sequence of the exon deleted and the downstream boundary intron

To explore the origin of the insertion and deletion, we performed PCR amplifications to establish the genomic organization of the HL gene at the boundaries of nucleotides 751 and 876 respectively. Using f4 and orf-r as primers, a fragment of about 2.1 kb was amplified by PCR using genomic DNA as a template. This amplification always yielded the same fragment whether control or patient genomic DNA was used. After purification, this fragment was sequenced using the oligonucleotide f4 as a primer. Nucleotide 876 was identified as the 3' boundary of an exon. Control genomic DNA revealed a canonical splicing donor sequence
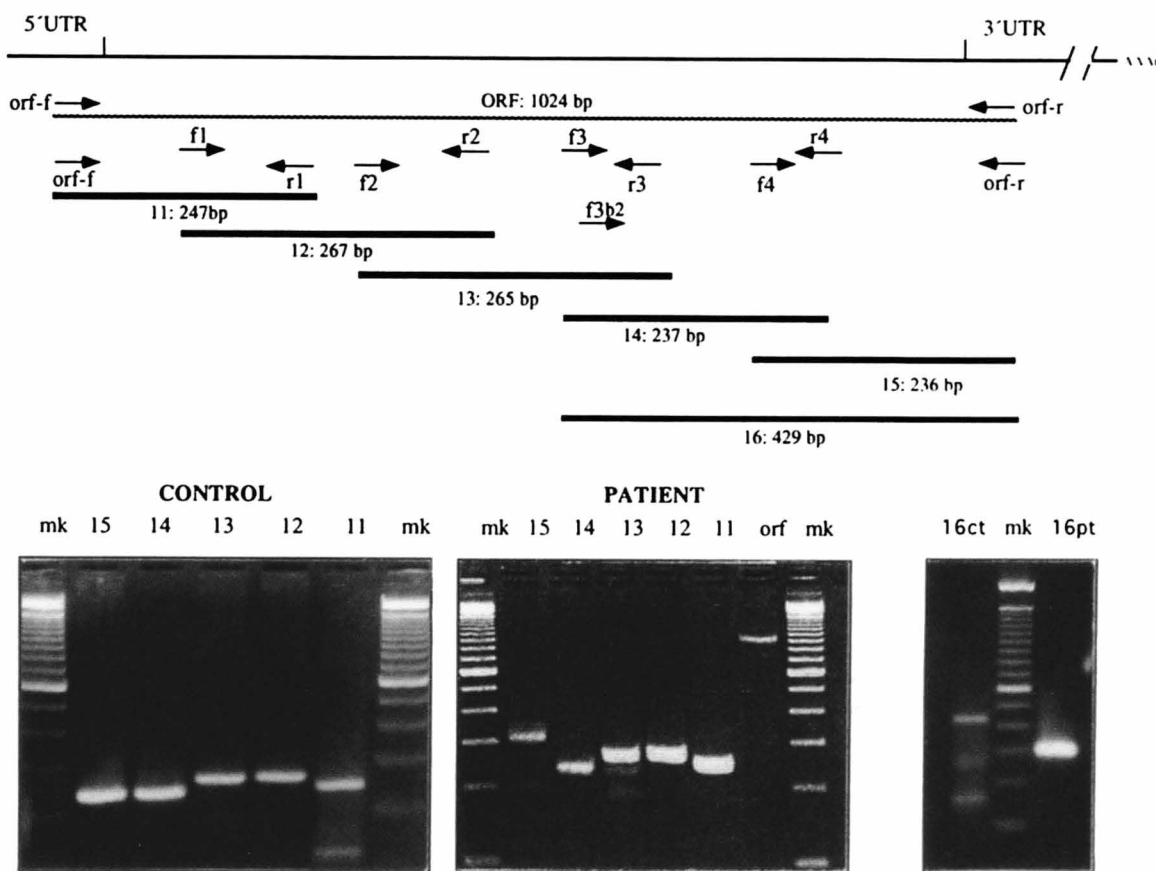
Fig. 2. Top: The pattern of PCR amplifications using different sets of specific oligonucleotides is shown. Bottom: The different amplified fragments of the control and of the patient were separated by electrophoresis in 1% agarose.

(*gtaag*) following nucleotide 876. This sequence revealed a G → C transversion in the patient DNA, which gave the non-donor-splicing consensus *ctaag*. Thus the 78 bp inserted in the mRNA was the 5′ region of the downstream flanking intron. A cryptic splicing donor sequence (*gtatt*) was found in nucleotides 79–83 of this intron (**Fig. 4**), suggesting that this cryptic donor sequence was used by the spliceosome to produce a mature mRNA with the 78 bp sequence added to the previous exon.

Figure 4 summarizes the results of the nucleotide sequences obtained. The G → C transversion, which converts the invariant *gt* of the donor splice site into *ct*, suggested that the disruption of the normal splicing of HL mRNA in proband E.C. was due to the elimination of the 5′ donor splice site of the intron. Figure 4 also shows that the presence of a cryptic donor splice site in the intron (at nucleotides 79–83) allows an alternative abnormal splicing to occur. This leads to the incorporation of 78 bp of intron at the 3′ boundary of the exon. The second mRNA species found in the proband derives from the activation of an alternative splicing that

occurs at the authentic splice sites and leads to the skipping of the exon mentioned.

PCR amplifications of the relatives of the proband

mRNAs were also isolated from cultured fibroblasts of the father, the mother, and three brothers of the proband. After conversion to the corresponding cDNAs, they were amplified by PCR (RT-PCR) using the primers f3 and orf-r to detect the deletion. Two amplification bands corresponding to the nonmutated and mutated mRNAs are seen in the father, the mother, and two of the brothers (GC and UC) (**Fig. 5A**). The bigger band corresponds to the nonmutated mRNA and the smaller to the mutated mRNA (in this case the mRNA is the one deleted). However, only one band is seen in the third brother (KC), suggesting that this boy has not inherited the mutated allele, and is not a carrier of the disease. The rest of the family are carriers (Fig. 5B). The consanguinity of the couple was confirmed by this experiment.

In addition, we performed PCR amplifications using the genomic DNA as a template in both parents. With
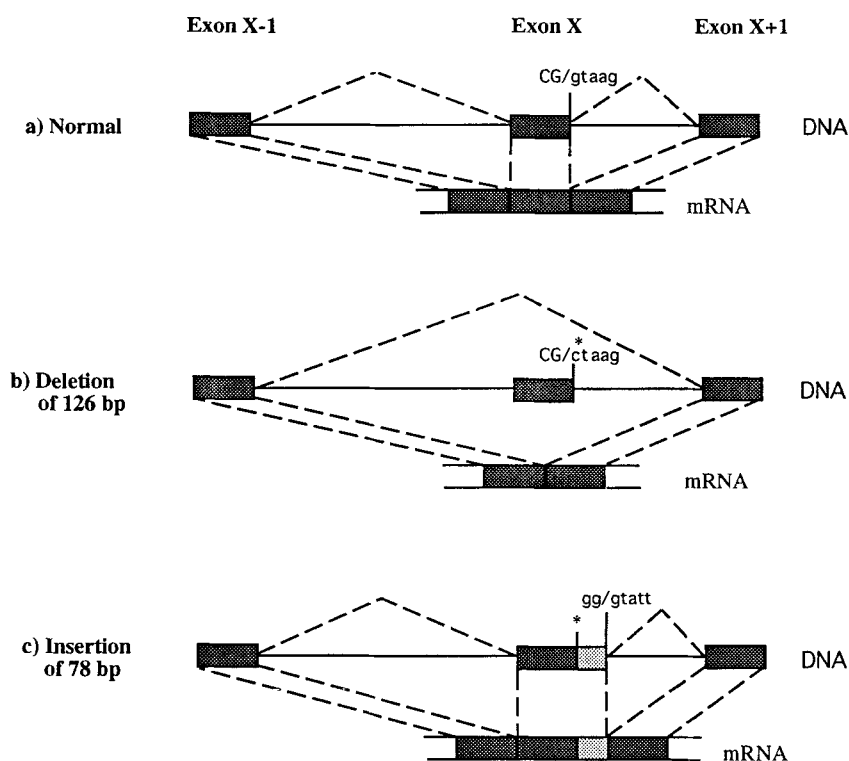
A

Deleted mRNA

```
750                                          877
 |                                            |
GGCCCTGCAG ──────── 126 bp deleted ──────── GGTG TGAATCTCCA
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Inserted mRNA

```
876
 |
CACACG CTAAGCCCAC CCACCCCCTG GTGGCGACAG CCCCGAACTG
```

```
                                877
                                 |
ACCAGGGGGC CTGAAGCTGA GAACAAAAGT GGATGGGG GGTG TGAATCTCCA
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Fig. 3. A: The sequence of the boundaries of the deleted 126 bp in the bp mRNA, and the intronic sequence of 78 bp corresponding to the insertion in the mRNA (underlined), are shown. The figures correspond to the coordinates of nucleotides of the cDNA of human HMG-CoA lyase (15). The TGA stop codon from the intron sequence is located at position 78. B: The sequence of amino acid residues corresponding to the control HMG-CoA lyase, and that of the two mutated mRNAs are shown. The 17 amino acid residues inserted, which precede a stop codon, are shown in black. The catalytic Cys-266 is marked with an asterisk.

B   HMG-CoA Lyase wild type

```
250            270            290            310
 |              |              |              |
ALTNTLMALQMGVSVVDSSVAGLGGCPYAQGASGNLATEDLVYMLEGLGIHTGVNLQKLLEAGNFICQALNRKTSSKVAQATCKL
                  *
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HMG-CoA Lyase inserted

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ALTNTLMALQMGVSVVDSSVAGLGGCPYAQGASGNLATEDLVYMLEGLGIHTLSPPTPWWRQPRTDQGA
                  *
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HMG-CoA Lyase deleted

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ALTNTLMALQ----------------------------------------------GVNLQKLLEAGNFICQALNRKTSSKVAQATCKL
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the same primers as those used in the proband, an identical 2.1 kb fragment was amplified. The fragment was sequenced using the oligonucleotide f4 as a primer. We found that in the position of the nucleotide +1 of the intron there was a mixture of c (corresponding to the mutated allele, like the proband) and g (corresponding to the wild-type allele). This result indicated that both parents were carriers of the same point mutation, corroborating their consanguinity.

Predicted translation products of HL mRNAs

The 126-bp in-frame deletion produces the loss of 42 amino acids (Met-251 to Thr-292) in the mature protein. This deleted region includes the important Cys-266 of the catalytic site (21, 22). The 78-bp insertion does not affect the catalytic cysteine but produces missense codons starting at Thr-292, which leads to a stop codon 18 triplets downstream (Fig. 3B). In this case, the mature protein has 17 missense amino acids that follow Thr-292, and lacks the last 16 sense amino acid residues,

among them the Cys-323, which is responsible for the formation of the homodimer protein found in eukaryotes (21, 23). Neither predicted proteins would be catalytically active, producing the observed absence of enzymatic activity in fibroblasts in the homozygous proband.

Quantification of the inserted and deleted mRNA levels

As the amplification of the cDNA using the primers orf-f and orf-r and the cDNA obtained from the mRNA from the patient as a template yielded only one 900-bp fragment instead of the two fragments expected (900 bp of the deleted mRNA and 1100 bp of the other mRNA), we determined the levels of each mutated mRNA, (that with deletion and that with the insertion) by Northern blot. As a first probe, fragment 11 of 247 bp, which was amplified from orf-f and r1, was used. This probe recognized the two overlapping mRNAs. Then a specific probe was used to determine whether the mRNA with the insertion was produced. This probe

Fig. 4. Schematic representation of the abnormal splicing of HMG-CoA lyase mRNA in proband E.C. The G → C transversion (marked with an asterisk (*)) eliminates the 5' donor splice site in the intron and allows the formation of another mRNA species as a result of the skipping of the whole exon; in addition, the activation of one cryptic donor splice site in the same intron determines the formation of another abnormal mRNA.

was amplified by using two specific primers: f3b, which is immediately before the 5' flanking region of the deleted sequence, and a reverse primer, r4b located in position 76-56 of the inserted intron sequence. These primers amplified a 223-bp fragment from the cDNA of the patient, which could specifically recognize the inserted mRNA. The mRNA with the deletion could not be hybridized with this probe as its first 126 bp are absent due to the exon skipping, and the last 76 bp are also absent because they are a part of the intron sequence.

mRNAs from the patient and the healthy unrelated control were thus analyzed in Northern blot experiments. The membrane was then divided into two halves and processed. In one half a similar expression was observed in the proband and in the control when hybridized with the probe of 247 bp corresponding to amplification 11. In the other half of the membrane, hardly any expression of the inserted mRNA was detected in the proband when it was hybridized with the specific probe prepared for the inserted mRNA (see above), even when the film was overexposed for more than 3 weeks (**Fig. 6**). These results were interpreted to mean that the mRNA with insertion was present in much

lower amounts than the mRNA with deletion, thus scarcely contributing to the production of active HMG-CoA lyase.

As the Northern blot determinations did not afford quantitative evidence of the inserted or deleted mRNA levels, we performed quantitative determinations of the two mRNAs taking advantage of the quantitative RT-PCR determination (the DNA-mimic PCR reaction). To this end, we used two DNA-mimic templates as internal standards (one for the inserted mRNA, the other for the two mRNAs), as the amplification takes place in a competitive fashion because the DNA-mimic and mitochondrial HMG-CoA lyase sequences compete for the same primers.

We first prepared a DNA-mimic for the DNA fragment from v-*erb* B oncogene, as described in Material and Methods. Primer templates were chosen so as to span a fragment containing part of an exon and part of the next exon from HMG-CoA lyase. The competitive PCR experiments for both mRNAs are shown in **Fig. 7**. Serial dilutions of the DNA-mimic were co-amplified with a constant amount of the HMG-CoA lyase cDNA fragment. Under competitive conditions, the absolute amount of DNA-mimic added to the reaction is equal to
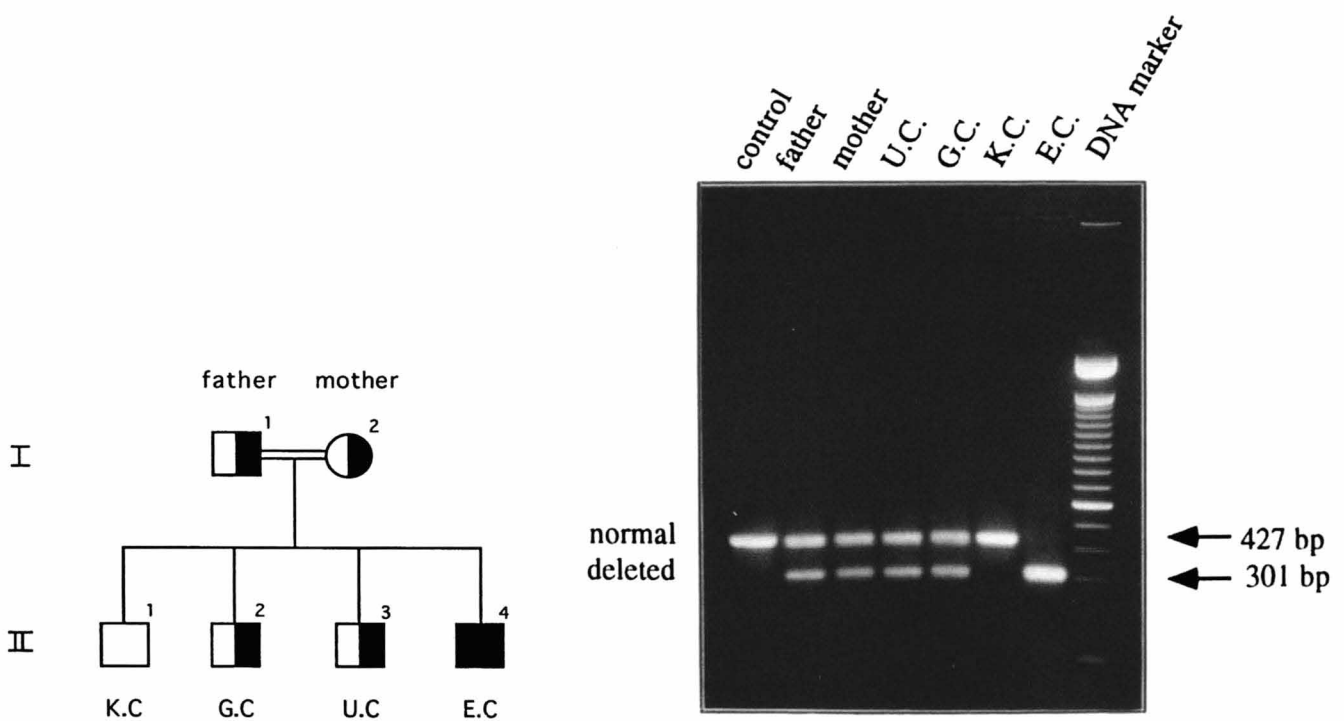
Fig. 5. PCR amplifications of the cDNAs of the proband, his parents, his brothers, and a human control. The oligonucleotides used were f3/ orf-r. The appearance of two bands corresponding to the two mRNAs (one the wild type and the other the deleted mRNA) is clearly seen in the parents and two brothers (U. C. and G. C.). The proband (E. C.) has only the deleted band. The third brother (K. C.) shows only the wild-type band in the two alleles, and is not a carrier of the disease.
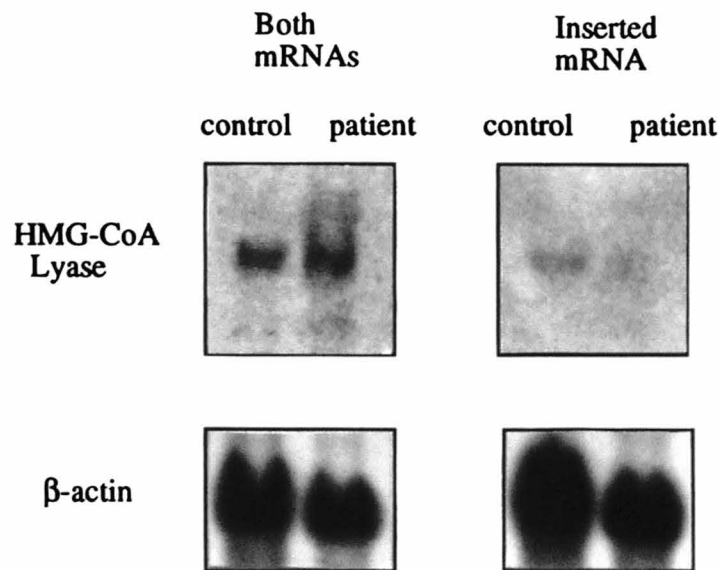

Fig. 6. Top: Northern blot analysis of the HMG-CoA lyase mRNAs of a human control and the patient. The first two lanes (left) were hybridized with the PCR cDNA-amplified probe located far upstream from the locus of insertion and deletion (fragment comprised between oligonucleotides orf-f and r1). The lanes of the right were hybridized with a PCR genomic DNA-amplified specific probe containing the 126 nucleotides corresponding to the deletion and 72 nucleotides of the inserted intron. Bottom: The amounts of the RNA samples applied to the gel were compared by determining the mRNA levels of β-actin.
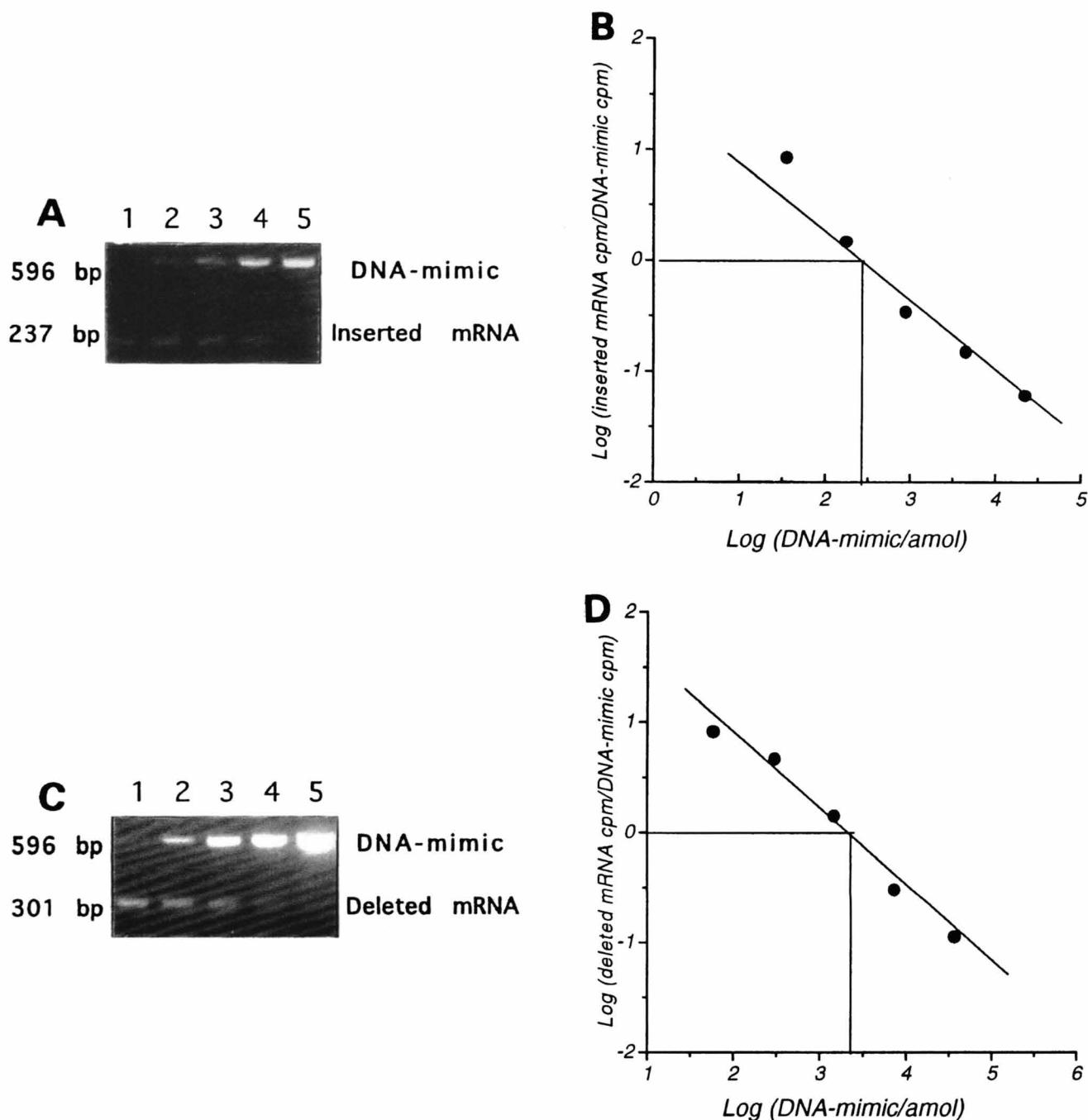
Fig. 7. Titration of a DNA-mimic with a constant amount of two different types of the cDNA fragment. Three μl (A) or 0.3 μl (C) of HL cDNA from cultured fibroblasts was added to a PCR containing up to 625 serial dilutions of a DNA mimic. After 25 cycles of amplification, aliquots of about 30% of the volume were resolved on a 2% ethidium bromide/agarose gel. Lanes 1–5 (A) correspond to amounts of 3.75×10^3, 7.5×10^2, 1.5×10^2, 30, and 6 attomoles of DNA mimic, respectively, whereas lanes 1–5 (C) correspond to amounts of 22.5×10^4, 4.5×10^4, 9×10^3, 1.8×10^3, and 3.6×10^2 attomoles of DNA mimic, respectively. The positions of the 237 bp (A) of the HL mRNA with the insertion, 301 bp (C) of the HL mRNA with the deletion, and 596 bp of the DNA mimic products are indicated. (B) and (D). The relative amounts of HL mRNA and DNA mimic products were calculated after correcting for the difference in size between them. The log of the ratios of HL mRNA to DNA mimic products were plotted as a function of the initial amount of DNA mimic added to PCR.

the amount of mRNA when the molar ratio of products becomes equal for target and DNA-mimic pairs. Molar equivalence occurs at approximately the point where equivalent amounts of radioactivity are incorporated into the mRNA and DNA-mimic. The relative amounts of target and DNA-mimic products were calculated after correcting for the difference in size between them. In the experiment shown in Fig. 7, molar equivalence was reached at 240 attomoles for the mRNA with the insertion and 2,004 attomoles for the mRNA with the deletion. Taking into consideration that the mass of the aliquot taken for the determination of the mRNA with the deletion was 1/10 of that of the mRNA with the insertion, it became clear that the proportion of the two mRNAs is 1/83 that is, 1.20% of the total mRNA contains the insertion.

DISCUSSION

The characterization of a first mutation causing HL deficiency was recently reported (3). In the present study, which is the third performed at the molecular level in the HMG-CoA lyase gene, we report a novel point mutation of this gene in a Turkish patient with homozygous HMG-CoA lyase deficiency. By using a combination of methods (RT-PCR amplification, sequencing of the amplified cDNAs that were different from control, amplification and sequencing of an intron, and comparison of the results of the proband with those of his parents and brothers) we reached the conclusion that the proband was homozygous for a G → C transversion at position +1 of the intron downstream from the exon containing the catalytic site in the encoded protein.

There is no doubt that the GT dinucleotide is highly conserved at the 5′ end of introns of most eukaryotic genes (24, 25). The first nucleotide of a normal intron is never C (24) and mutations of the strictly conserved G at this position prevent normal splicing (26). This guanine at position +1 is involved in binding U1 small ribonucleoprotein particles (U1 snRPNs) and this is the residue that undergoes cleavage in the spliceosome and lariat formation (27, 28). The G → A mutation in the conserved splice donor site has been reported in several other human genetic diseases, and represents 64% of total G mutations reported in the splice donor site (29). G → C transversions as reported in this article are less common. According to Cooper and Krawczak (30, 31), G → C transversion represents 12% of all possibilities of change starting with GT and modifying the 5′ nucleotide.

In the present study we have demonstrated that the G → C transversion at the 5′ donor slice site of the intron

studied of the HMG-CoA lyase gene disrupts the normal splice site in that it causes: a) the use of an alternative normal splice site with the skipping of the exon upstream of the mutation; and b) the activation of one cryptic splice site, downstream (in the intron considered) from the normal splice site. The genomic organization of the HMG-CoA lyase gene has not yet been published. However, after this study we can affirm that two introns are present at nucleotides 750 and 876 of the cDNA of HL. The intron located downstream of the 876 bp of the cDNA (called intron X) is about 1.9 kb in length. The intron (X-1) is of unknown size. Our results also indicate that the use of an alternative normal splice site (exon skipping) was a preferential mechanism of splicing as compared to the activation of a cryptic site, as the amount of the HMG-CoA lyase mRNA devoid of the exon deleted was much higher than that of the mRNA with the insertion. This is the opposite to what happens in most mutations at this G at the donor splice site. One explanation is that the cryptic splice site contains a *t* rather than a *g* (usually found in 84% proportion) at the fifth position, and a *t* rather than an *a* (usually 64%) at the fourth position.

To obtain convincing evidence that the mutated protein lacks enzyme activity, it would be necessary to express the cDNA in a suitable cell culture such as *E. coli* or COS cells. However, this experiment is not required here because of the consequences of the mutation studied. The mRNA species that derives from the use of an alternative normal splice site produces a deleted protein that obviously leads to a nonfunctional enzyme. This is because the encoded protein derived from the deleted mRNA has lost the whole catalytic site, including the catalytic cysteine-266, which, by itself, explains the occurrence of the disease. The consequences of the absence of enzymatic properties in the protein encoded by this mutated mRNA are exacerbated because this mRNA is practically the only one found.

The insertion of 78 nucleotides described herein in one of the two mRNAs of this proband constitutes a good example of an aberrant splicing produced by a single point mutation. The length of this insertion is due to a cryptic 5′ splice site in nucleotides 79 to 83 of this intron, which is activated once the nearby functional wild-type site is crippled by this mutation. The translation product of the mRNA derived from the activation of the cryptic site in the intron contains 17 amino acid residues preceding a premature stop codon. Although we have not studied the catalytic efficiency of the abnormally inserted and prematurely truncated protein, it is reasonable to assume that such great changes in the expected protein, that is, the inclusion of 17 aberrant amino acid residues, 22 amino acids from the catalytic cysteine, are enough to explain the null catalytic activity seen in the proband fibroblasts. An ad-

ditional reason for the inability of the inserted protein to catalyze the enzyme reaction normally is that the substitution of the sequence in the carboxyl terminus by the nonsense amino acids eliminates the cysteine-323. The Cys-323 is involved in regulation and the inter-subunit interaction in the homodimer of the eukaryotic HMG-CoA lyase as judged by covalent modification and site-directed mutagenesis (18, 22). HMG-CoA lyase in *Pseudomonas mevalonii* is present as a monomer, instead of a homodimer seen in eukaryotes (32). It is not unusual that the Cys-323 is lacking in HMG-CoA lyase from *P. mevalonii* as its function is not needed.

Irrespective of whether this truncated protein is functional, in this individual, the expression of the mRNA with insertion is largely abolished. mRNA in Northern blot experiments was almost undetectable, and RT-PCR worked only in the cases in which the other mRNA was not primed, i.e., when we used primers located in the deleted area. By using the competitive PCR (the DNA-mimic PCR reaction) we determined that only 1.2% of the total mRNA contained the insertion. In other words, for the amplification of this mRNA we found a situation similar to the illegitimate transcription previously described (33). Alternatively, the mRNA transcribed could be highly unstable.

The studies performed on the parents confirm the transversion $G \rightarrow C$ at the nucleotide +1 observed in the proband. In addition, the occurrence of a double nucleotide at this position, the mutated C as in the proband and the wild type G as in the human control, shows that the parents were heterozygous for the same mutation.█

Note added in proof: According to the genomic organization of HL reported recently by Wang et al. (*Genomics*. 1966. **33**: 99–104), we conclude that the exon deleted is exon 8.

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