Cerebrotendinous xanthomatosis caused by two new mutations of the sterol-27-hydroxylase gene that disrupt mRNA splicing

R. Garuti, N. Lelli, M. Barozzini, R. Tiozzo, M. T. Dotti,* A. Federico,* A. M. Ottomano, A. Croce, S. Bertolini,† and S. Calandra[1]

Dipartimento di Scienze Biomediche, Sezione di Patologia Generale, Università di Modena; Istituto di Scienze Neurologiche,* Università di Siena; and Centro Prevenzione Arteriosclerosi,† Dipartimento di Medicina Interna, Università di Genova, Italy

Abstract Cerebrotendinous xanthomatosis (CTX) is an inherited sterol storage disease associated with the accumulation of cholestanol and cholesterol in various tissues. CTX is caused by a deficiency of sterol-27-hydroxylase, a mitochondrial enzyme that oxidizes the side chain of cholesterol in the pathway leading to the formation of bile acids. In the present study we report two mutations of sterol-27-hydroxylase gene (*CYP27* gene) found in Italian CTX patients. Proband T.C. is homozygous for a G → A transition at the first nucleotide of intron 7. This mutation causes the formation of minute amounts of an abnormal mRNA, in which exon 6 joins directly to exon 8 with the skipping of exon 7. The exon 6–exon 8 junction results in a frame shift, downstream from the codon for Arg_{362}, which generates a string of 28 novel amino acids preceding a premature termination codon. Proband C.U. is homozygous for a G → C transversion at the last nucleotide of exon 3. This mutation, which changes the consensus sequence of the 5′ donor splice site, is associated with barely detectable levels of sterol-27-hydroxylase mRNA, of normal size, in proband fibroblasts. As both mutations change the sites for two restriction enzymes, rapid methods were devised for the identification of the healthy carriers among the probands' family members and for the screening of these mutations in other CTX patients.—**Garuti, R., N. Lelli, M. Barozzini, R. Tiozzo, M. T. Dotti, A. Federico, A. M. Ottomano, A. Croce, S. Bertolini, and S. Calandra.** Cerebrotendinous xanthomatosis caused by two new mutations of sterol-27-hydroxylase gene that disrupt mRNA splicing. *J. Lipid Res.* 1996. **37:** 1459–1467.

Supplementary key words CYP27 gene • nucleotide substitution • donor splice site • alternative splicing

Cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessive disorder of cholesterol metabolism characterized by abnormal deposition of cholestanol and cholesterol in multiple tissues (1). Deposition of these lipids in the central nervous system leads to neurological dysfunctions (dementia, behavioral abnormalities, spinal cord paresis, and cerebellar ataxia) (2, 3), whereas the deposition in other tissues causes tendon xanthomas and cataracts (1), and may promote the development of premature atherosclerosis (4). CTX is due to a defect of sterol 27-hydroxylase (EC 1.14.13.15), a mitochondrial enzyme catalyzing the initial steps in the hepatic oxidation of the side chain of sterol intermediates in the metabolism and biliary excretion of cholesterol (1, 5). This enzyme, which belongs to the cytochrome P450 family, hydroxylates a spectrum of sterol substrates including vitamin D_3 (6). In CTX the capacity to convert cholesterol to bile acids is impaired and the incomplete oxidation of the cholesterol side chain leads to the accumulation of cholesterol and abnormal tetra- and penta-hydroxylated bile alcohols (1).

An early diagnosis of CTX is crucial as the long term treatment with chenodeoxycholic acid (7, 8), with or without an inhibitor of hydroxy-methylglutaryl-CoA reductase (9), may prevent or reduce the progression of the neurological complications of the disease (8, 10). The molecular cloning of the human sterol 27-hydroxylase cDNA (11), the characterization of its gene (*CYP27* gene) (12), and the identification of some mutations of this gene in CTX patients (11–18) have opened a new field of investigation and provided new tools for the early detection of CTX patients and the identification of the healthy carriers. Several point mutations of *CYP27* gene have been identified so far in various populations

Abbreviations: CTX, cerebrotendinous xanthomatosis; *CYP27* gene, gene encoding human sterol-27-hydroxylase; PCR, polymerase chain reaction; NMR, nuclear magnetic resonance; RT-PCR, reverse transcription polymerase chain reaction; SSCP, single-strand conformation polymorphism.

[1]To whom correspondence should be addressed at Sezione di Patologia Generale, Dipartimento di Scienze Biomediche, Università di Modena, Via Campi 287, 41100 Modena, Italy.

(11–18). These mutations lead to amino acid substitutions (11, 13, 16), shifts in the reading frame (12, 14, 18), premature termination codon (17), or disruption of the mRNA splicing process (12). We have recently started the characterization of *CYP27* gene mutations in Italian patients with CTX. In the present study we report the characterization of two novel point mutations that disrupt the splicing of sterol-27-hydroxylase mRNA.

MATERIAL AND METHODS

Subjects

Proband T.C. is a 44-year-old female born from a consanguineous marriage (her parents were first cousins). Tendon xanthomas were detected at the age of 25 and cataract at the age of 41. On neurological examination the patient was found to have a normal intelligence, brisk tendon reflexes, and abnormal gait. Plasma cholestanol was 3.5 mg/dl (n.v. <1 mg/dl) and plasma cholesterol 170 mg/dl. NMR of the brain showed mild signs of cortical atrophy. Proband T.C. is at the moment under treatment with an HMG-CoA-reductase inhibitor (simvastatin); previous treatment with chenodeoxycholic acid had been discontinued because of severe gastric side effects.

Proband C.U. is a 38-year-old male born from a consanguineous marriage (his parents were first cousins). Cataract was detected at the age of 8 and tendon xanthomas at the age of 15. Behavioral disorders have been present since adolescence. Neurological examination showed the presence of brisk tendon reflexes and spastic gait. NMR of the brain showed cortical and cerebellar atrophy and cerebellar xanthomas. Plasma cholestanol was 3.2 mg/dl and plasma cholesterol 156 mg/dl.

Control subjects were chosen among healthy and normolipidemic individuals working in the laboratory. Informed consent was obtained from the probands (or their relatives) and from the healthy controls.

Southern blot analysis

Genomic DNA was extracted from peripheral blood leukocytes by a standard procedure (19). DNA was digested using 5–10 U/μg DNA of several restriction enzymes (BamHI, EcoRI, SacI, HindIII, and KpnI) separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with the sterol 27-hydroxylase cDNA probe, as previously described (20). The full size sterol 27-hydroxylase cDNA probe (11) was a kind gift from Dr. D. Russell (Dallas, TX).

Cell culture

A skin biopsy was taken from the probands. Explants were cultured in 25 cm^2 flasks in DMEM (Dulbecco's modification of Eagle's medium), 100 IU/ml of penicillin and 50 μg/ml of streptomycin, 2 mM glutamine, 15% calf serum, and 95% air–5% CO_2 (20).

Northern blot analysis

Total cellular RNA was isolated by extraction in guanidine-thiocyanate (21) from cultured skin fibroblasts. RNA (15 μg) was denatured in 50 μl of 50% formamide, 2.2 M formaldehyde, and 1 × MOPS buffer [20 mM 3-(N-morpholino)-propane-sulfonic acid, 5 mM sodium acetate, and 1 mM Na_2 EDTA], separated by electrophoresis in 1.6% agarose gel and transferred to Hybond-N membranes, which were then hybridized with the full size cDNA probe. The cDNA clone pHFβ A-I of human β actin was used to normalize the RNA filters (20). Pre-hybridization and hybridization were performed as previously described (20).

Reverse transcription and PCR amplification

RNA (1 μg) from cultured fibroblasts of the probands T.C. and C.U., and of a normal subject were reverse-transcribed in a 20 μl reaction mixture containing 8 mM $MgCl_2$, 1 mM of each dNTP, 1 unit of RNAs in, 100 pmol random hexamers, and 12 units of AMV reverse transcriptase in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9). After heating the samples at 95°C for 5 min, 80 μl of 1 × PCR buffer containing 20 pmol of each primer was added as well as 2.5 units of Taq polymerase. $MgCl_2$ concentration in the reaction mixture ranged from 2 to 3 mM (22). In the case of proband T.C., the following primers were used: 5′ GAG ATC CAG GAG GCC TTG CAC GA 3′ (forward primer in exon 6 corresponding to nts 1087–1110 of the cDNA) (*6s*); 5′ CCC AGC AAG GCG GAG ACT CA 3′ (reverse primer in the 3′ untranslated region, complementary to nts 1639–1620 of the cDNA) (*9B*) (12). In the case of proband C.U. the following primers were used: 5′ TCC GGC GGC GGC AAC GGA GCT TAG A 3′ (forward primer in exon 1, corresponding to nts 170–194 of the cDNA) (*1s*); 5′ GGA GTA GCT GCA TCT CCA GCTCT 3′ (reverse primer in exon 8 complementary to nts 1489–1467 of the cDNA) (*8as*). The conditions were: 95°C for 5 min, 70°C for 1.30 min for the first time, and subsequently 95°C for 1.30 min, 70°C for 1.30 min for 28 cycles. PCR products were separated from the unincorporated primers by electrophoresis on 1.5% agarose gel. In the case of proband T.C. the bands were excised from the ethidium bromide-stained gel using Quiaex (Diagen, GmbH, Germany). The RT-PCR fragments were sequenced directly using the fmol Sequencing System (Promega Co., Madison, WI). The primer used in the sequencing reaction was primer *6s* (see above).

Single-strand conformation polymorphism (SSCP)

SSCP was performed according to Orita et al. (23). The promoter region and the exons of *CYP27* gene were amplified by PCR from genomic DNA using the primers reported previously by Leitersdorf et al. (12). The PCR conditions were the following: 95°C for 1 min, 68°C for 5 min for 30 cycles (promoter and the region encompassing exons 6–9), 95°C for 1 min and 65°C for 1 min for 28 cycles (exon 2); 95°C for 1 min and 70°C for 1 min for 28 cycles (exons 1, 3, 4, and 5). $MgCl_2$ concentrations ranged from 1 to 3 mM. PCR products were labeled by adding 5 μCi [α^{32}P]dCTP to the PCR mix. An aliquot (2 μl) of the reaction product was mixed with 20 μl of 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol in formamide. The reaction mixture was heated at 95°C for 3–5 min, placed immediately on ice, and then loaded (2 μl) onto the gel. Gels were run on a conventional sequencing apparatus (400 × 200 × 0.4 mm). The gels consisted of 5.5% polyacrylamide, 2 × TBE (0.18 M Tris-borate, 4 mM EDTA, pH 8) and 10% glycerol. The samples were electrophoresed at 250 volts for 21 h at 22–23°C. Gels were dried at 80°C for 1–2 h and then were exposed to X-ray film (Hyperfilm-MP, Amersham International, UK).

For the analysis of the region spanning from the 5′ end of exon 6 to exon 9 (using primers *6A* and *9B*) (12), the PCR product of 1073 bp was digested with the restriction enzyme AvaII prior to the SSCP analysis. An aliquot (5 μl) of the digestion product was diluted in 20 μl of the loading solution (0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA in formamide) and then applied (2.5 μl) to a nondenaturing 5% polyacrylamide gel in the presence of 10% glycerol and 2 × TBE. The samples were electrophoresed at a constant power of 50 watt at 22–23°C for 4 h.

Sequencing of genomic DNA

In the case of proband T.C., (in whom SSCP analysis of the exon 6–exon 9 region had indicated the presence of a putative mutation in the fragment spanning from the 5′ end of exon 7 to the 5′ end of exon 8), the sequence of genomic DNA was performed using the following oligonucleotide: 5′ GAT TGG GCA GCA TGA ATG CCA CTC 3′ (*7B*) a reverse primer complementary to nucleotides 75–52 of intron 7 (24).

In the case of proband C.U., in whom SSCP analysis had indicated the presence of a putative mutation in exon 3, the sequence of genomic DNA was performed using the following primer: 5′ GAG CAC AAC CTC TCC CTG ACC CAT T 3′ (complementary to the 5′ end of intron 3) (*3B*) (12).

Screening of the two mutations in healthy carriers

The mutation present in proband T.C. (a G → A transition at the donor splice site of intron 7) creates a recognition site for the enzyme Hsp92II. To identify the presence of this mutation in the heterozygotes, genomic DNA of the healthy sister of proband T.C. was amplified by PCR using primers *6s* and *8as* (see above). The conditions were 95°C for 5′, 70°C for 1 min for the first time and subsequently 95°C for 1 min, 70°C for 1 min for 28 cycles. The PCR product was digested with the enzyme Hsp92II (Promega, Madison WI) and the restriction fragments were separated by 12% polyacrylamide gel electrophoresis and stained with ethidium bromide.

The mutation present in proband C.U. (a G → C transversion at the last nucleotide of exon 3) eliminates a KpnI site. To identify the presence of this mutation in the heterozygotes, genomic DNA from the parents and the healthy brother was amplified by PCR using primer *3A* and *3B* (12) under the conditions specified above. PCR products were digested with KpnI (Boehringer Mannheim Italia, Milano) and the restriction fragments were separated by 10% polyacrylamide gel electrophoresis.

Assay of sterol 27-hydroxylase activity in skin fibroblasts

The assay of sterol 27-hydroxylase was performed by using a frozen and thawed fibroblast suspension according to the procedure described by Atsuta and Okuda (25) as modified by Skrede et al. (26). Frozen and thawed fibroblasts of CTX probands (1.5 mg of protein) and a control subject (1.5 mg of protein) were incubated for 2 h at 37°C in the presence of 1 μCi [^{14}C]cholesterol (Amersham, U.K.)–albumin suspension (sp act: 1 μCi/μmol) and an NADPH generating system as specified by Skrede et al. (26). At the end of the incubation, the reaction mixtures were extracted in chloroform–methanol 2:1 (v/v), evaporated under nitrogen, and resuspended in chloroform–methanol 19:1 (v/v). After the addition of 27-hydroxycholesterol (5-cholesten-3β, 27 diol) (Steraloids, Wilton, NH) as an internal standard, the lipid extracts were subjected to thin-layer chromatography (silica gel G, Merck, Darmastadt, Germany) using benzene–ethyl acetate 1:1 (v/v) as developing system (27). The gels were exposed to iodine vapors and the spots corresponding to 27-hydroxycholesterol were scraped and counted in a liquid scintillation counter. The identity of 27-hydroxycholesterol was confirmed by gas chromatography–mass spectrometry of the trimethylsilyl ether (27). All determinations were done in duplicate and the coefficient of variations was 6–7%.

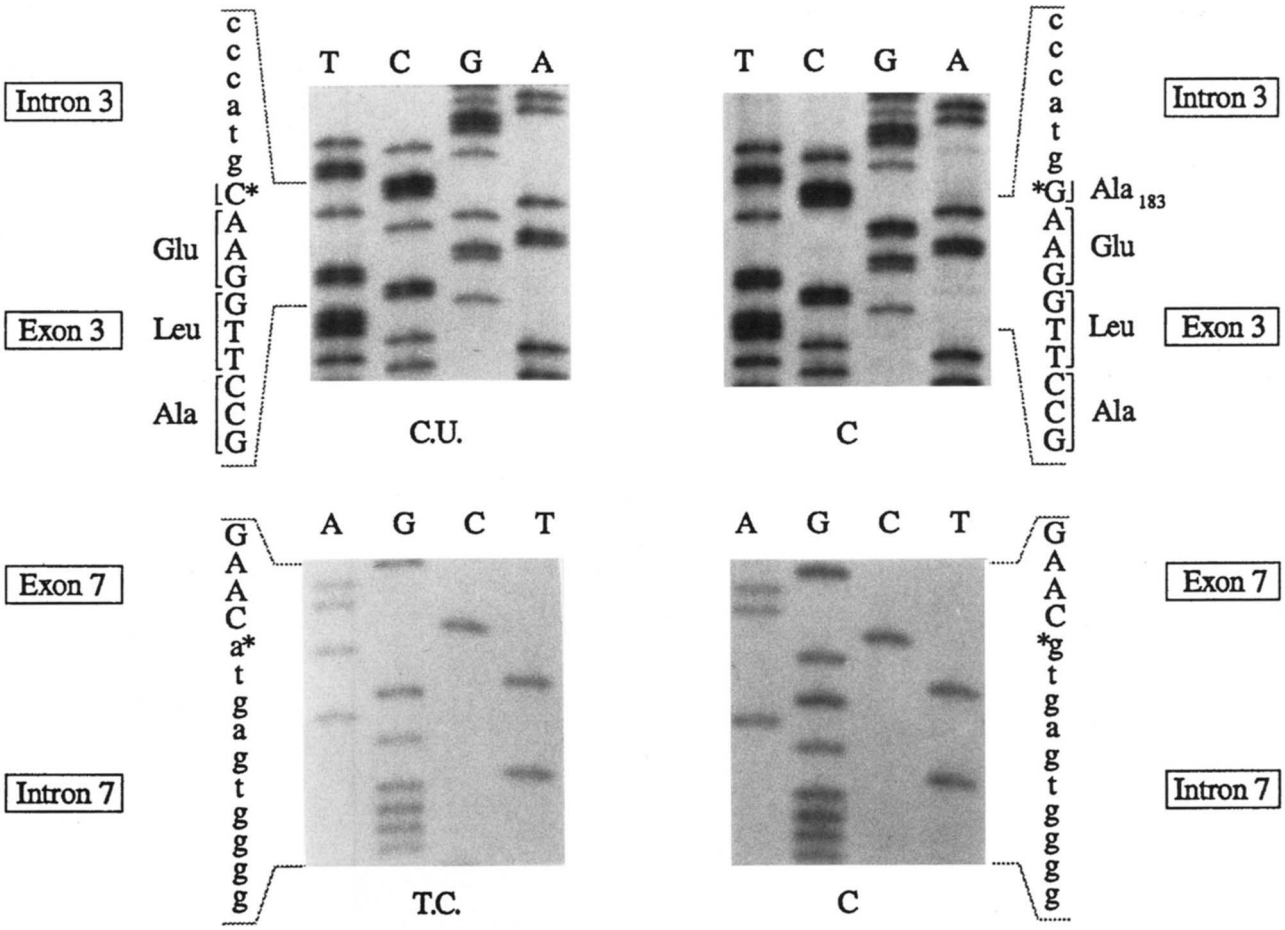

Fig. 1. Nucleotide sequence of the exon 3–intron 3 and exon 7–intron 7 boundaries of *CYP27* gene in CTX probands C.U. and T.C. and in a control subject (C). Proband C.U. is homozygous for a G → C transversion (indicated by a star) at the last nucleotide of exon 3 (at position -1 with respect to the 5′ donor splice site of intron 3). Proband T.C. is homozygous for a G → A transition at the first nucleotide of intron 7 (indicated by a star).

RESULTS

Southern blot and SSCP analysis

To exclude the presence of a major structural rearrangement of *CYP27* gene, genomic DNA from the probands, from some of their first degree relatives, and from control subjects was digested with several restriction enzymes. After Southern blotting and hybridization with the full-size sterol 27-hydroxylase cDNA, no major rearrangements of the *CYP27* gene were detected. However, the SSCP analysis of the promoter region and all exons showed an abnormal migration pattern in two different regions of *CYP27* gene in our probands. In proband T.C. the abnormal SSCP pattern was detected in a region spanning from exon 7 to exon 9, obtained after the AvaII digestion of a PCR fragment spanning from exon 6 to the 5′ half of exon 9 (data not shown). Proband C.U. was found to have an abnormal SSCP in exon 3 (data not shown).

Sequence of the *CYP27* gene in the probands

The nucleotide sequence of the exon 7–intron 7 boundary (**Fig. 1**) showed that proband T.C. is homozygote for a G → A transition at the first nucleotide of intron 7. The same mutation, in the heterozygote state, was found in the proband's healthy sister (data not shown). Proband C.U. was found to be homozygote for a G → C transversion at the last nucleotide of exon 3 (Fig. 1), whereas his father was heterozygote for the same mutation (data not shown). In both probands no additional nucleotide change was found in the other exons or in the promoter region of the *CYP27* gene.

Sterol 27-hydroxylase mRNA in skin fibroblasts

As both mutations were expected to interfere with mRNA splicing, their location being at the intron–exon boundaries, the content and the type of sterol 27-hydroxylase mRNA were investigated in probands' fibroblasts. In both probands a very faint sterol-27-hydroxylase mRNA band was observed in Northern

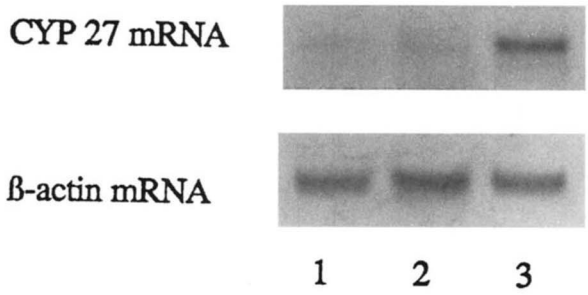

Fig. 2. Northern blot analysis of sterol-27-hydroxylase mRNA (CYP27 mRNA) in CTX probands. Lane 1: proband C.U., lane 2: proband T.C. and lane 3: control subject. Filters were hybridized with ^{32}P-labeled full size sterol-27-hydroxylase cDNA and re-hybridized with ^{32}P-labeled human β-actin cDNA (20).

blotting (**Fig. 2**). RT-PCR carried out in the presence of primers complementary to exon 6 (forward) and exon 9 (reverse) revealed that the amplification product obtained from the mRNA of proband T.C. was shorter (478 bp) than that obtained from control mRNA (557 bp) (**Fig. 3**). The size of the 478 bp fragment was consistent with the occurrence of an alternative splicing joining exon 6 to exon 8 with the complete skipping of exon 7. The nucleotide sequence of this fragment confirmed this hypothesis (**Fig. 4**).

In proband C.U. RT-PCR carried out in the presence of primers complementary to exon 1 (forward) and exon 8 (reverse) revealed the presence of a faint fragment of 1321 bp that co-migrated with the one observed in the control subject (**Fig. 5**).

Screening of the mutations

As the G → A transition in intron 7 and the G → C transversion in exon 3 alter the sites of two restriction enzymes, a rapid method was designed for the large scale screening of these mutations at the DNA level. The PCR amplification of the exon 6–exon 8 region, followed by the digestion with Hsp92II, results in the formation of four fragments (226, 190, 182, 84 bp) in the control subjects. In proband T.C. the 182 bp fragment is replaced by two fragments of 62 and 120 bp generated by the insertion of a Hsp92II restriction site (**Fig. 6**). The 120 and 62 bp fragments were also present, in addition to the normal fragments, in proband's healthy sister T.M., who is heterozygote for the G → A transition in intron 7.

The PCR amplification of exon 3 using primers complementary to intron 2 and intron 3 (see Methods for details) followed by the digestion with KpnI results in three fragments (53, 185, and 54 bp) in the controls but in only two fragments (53 and 239 bp) in proband C.U. because of the loss of a KpnI site. The abnormal 239 bp fragment is present besides the normal ones in proband's parents and healthy brother (**Fig. 7**).

Assay of sterol 27-hydroxylase activity in skin fibroblasts

The assay of sterol 27-hydroxylase activity, performed in frozen-thawed fibroblast suspension, showed a negligible or a very low "residual activity" in the two CTX probands (2045, 50, and 395 dpm/mg of protein in the control subject, proband T.C., and proband C.U., respectively).

DISCUSSION

In this study we report the characterization of two new point mutations of the *CYP27* gene in Italian patients with CTX. Proband T.C. was found to be homozygous for a G → A transition at the first nucleotide of intron 7, whereas proband C.U. was found to be homozygous for a G → C transversion at the last nucleotide of exon

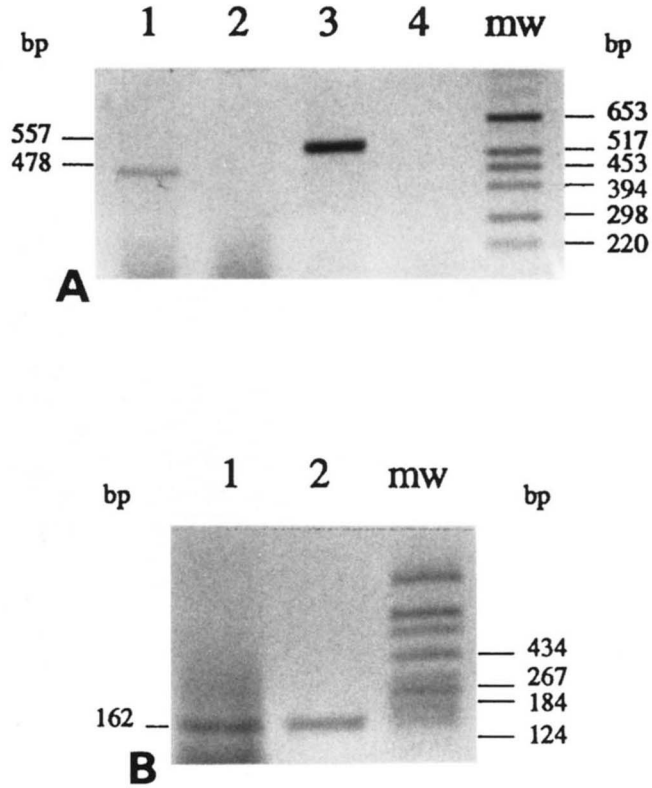

Fig. 3. Reverse transcription PCR (RT-PCR) of sterol-27-hydroxylase mRNA in proband T.C. Panel A shows the RT-PCR amplification of a region spanning from exon 6 to exon 9 (see Methods for details). Lane 1, proband T.C.; lane 3, control subject; lanes 2 and 4, mock PCR. Panel B shows the RT-PCR amplification of β_2 microglobulin mRNA used as an internal control. Lane 1, proband T.C.; lane 2, control subject.

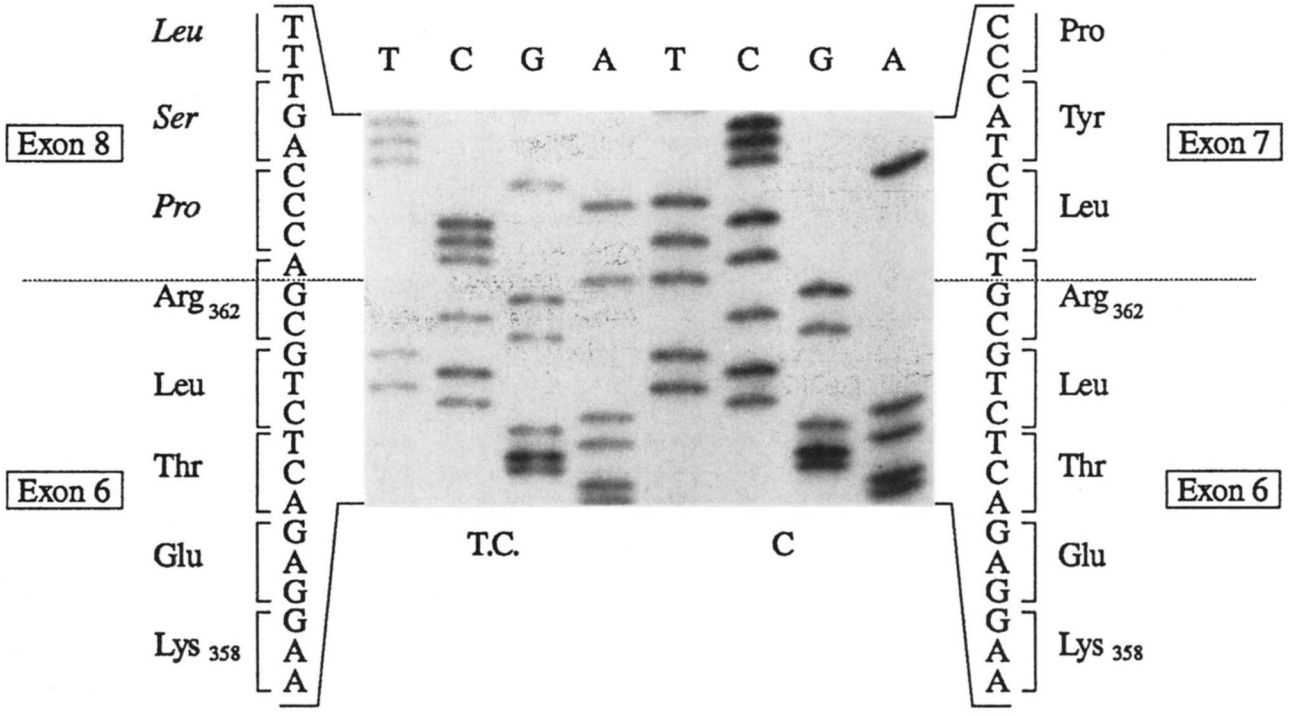

Fig. 4. Nucleotide sequence of the RT-PCR fragments of sterol-27-hydroxylase cDNA shown in Fig. 3. In proband T.C. (478 bp RT-PCR fragment) exon 6 joins directly to exon 8 with the skipping of exon 7.

3. Both mutations involve nucleotides belonging to the consensus sequence of the donor splice sites and affect the content and/or the type of sterol 27-hydroxylase mRNA in probands' skin fibroblasts.

It is firmly established that the GT dinucleotide is highly conserved at the 5′ end of introns of most eukaryotic genes (28–30). More specifically, the first nucleotide of a normal intron is never an A (29), and mutations of the strictly conserved G at this position prevent normal splicing (31). The guanine at position +1 is involved in binding U1 small ribonucleoprotein particles (U1 snRPNs) and is the residue that undergoes cleavage in the spliceosome and lariat formation (32, 33). The G → A mutation in the conserved donor splice site, reported previously in many other genetic diseases, has variable effects on the sequence and the content and of the mRNA (34, 35 and ref. 36 for review). In the present study we demonstrated that in proband T.C. the G → A transition at the 5′ donor splice site is associated with the activation of an alternative splicing whereby exon 6 is joined directly to exon 8 with the complete skipping of exon 7. We were unable to detect other species of mRNAs that might be generated by activation of cryptic splice sites (in exon 7 or intron 7) close to the mutation site. The content of the abnormally spliced mRNA in proband T.C. fibroblasts was greatly reduced as compared to that of its normal counterpart in control fibroblasts (Figs. 2, 3). This may be due to a reduced efficiency of the alternative splicing as well as to the accelerated catabolism of a mRNA carrying a premature stop codon (37) (see below). The abnormally spliced mRNA found in proband T.C. is predicted to encode a protein, downstream from the codon for Arg_{362}, that contains a string of 28 novel amino acids preceding a

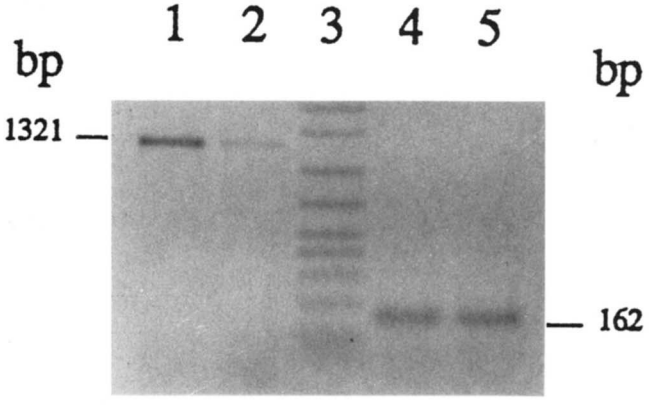

Fig. 5. RT-PCR of sterol-27-hydroxylase mRNA in proband C.U. The fragments shown in the first two lanes correspond to a region spanning from exon 1 to exon 8 of sterol 27-hydroxylase mRNA in a control subject (lane 1) and in proband C.U. (lane 2). The last two lanes correspond to the RT-PCR fragments of β_2 microglobulin mRNA in a control subject (lane 4) and in proband C.U. (lane 5).

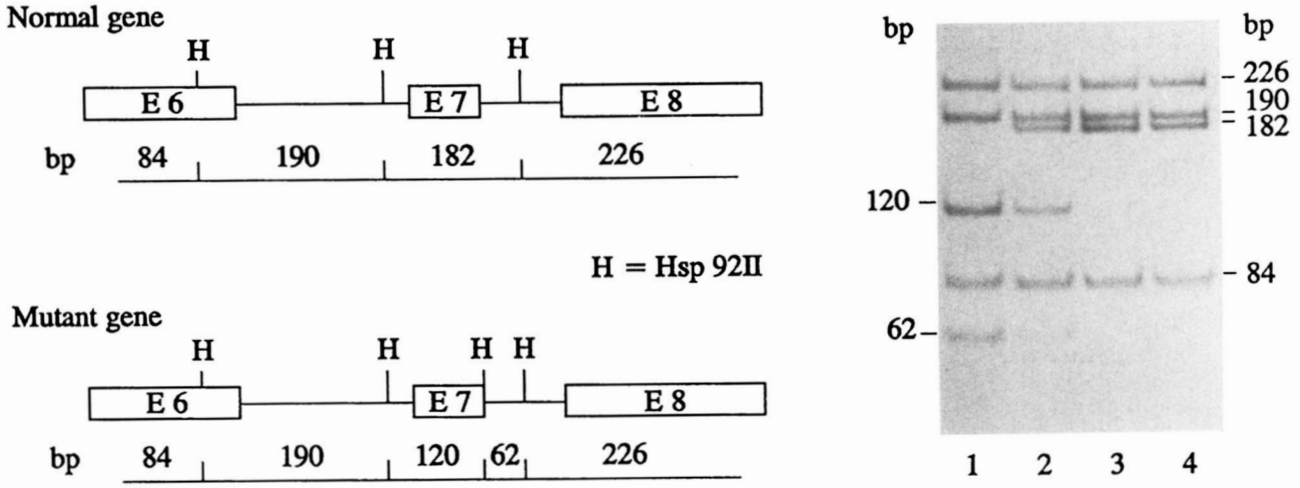

Fig. 6. Screening of the G → A transition at the first nucleotide of intron 7. Genomic DNA was amplified by PCR using primers complementary to exon 6 (*6s*) and exon 8 (*8as*) (arrows) and digested with the restriction enzyme Hsp92II (H). The size of the Hsp92II fragments (bp) is indicated above the horizontal lines and at the sides of the polyacrylamide gel. Lane 1, Proband T.C.; lane 2, proband T.C. sister (T.M.); lanes 3 and 4, control subjects.

stop codon (**Fig. 8**). This truncated protein that is lacking the heme binding domain has no enzymatic activity as demonstrated by the assay of sterol 27-hydroxylase activity in cultured fibroblasts.

The G → C transversion at the last nucleotide of exon 3 found in proband C.U. belongs to those groups of mutations located at the exon–intron boundaries that may affect mRNA splicing as reported in a number of genetic diseases (ref. 36 for review, 38, 39). From a survey of the RNA splice junctions of different classes of eukaryotic genes, Shapiro and Senapathy (30) generated the following consensus sequence at the 5′ splice site: AGGT $\frac{A}{G}$ AGT. More specifically, they found that guanine is present at position -1 of the donor splice site (corresponding to the last nucleotide of an exon) in 80% of the cases, whereas a cytosine at this position is present only in 3% of the cases. These data are confirmed by the features of the exon–intron junctions of *CYP27* gene where "G" is present at position -1 in six out of eight exon–intron boundaries (12). Within this context it is most likely that in proband C.U. the G → C mutation at position -1 of the donor splice site of intron 3 greatly reduces the normal splicing, leading to the very low mRNA level observed in proband's fibroblasts (Figs. 2

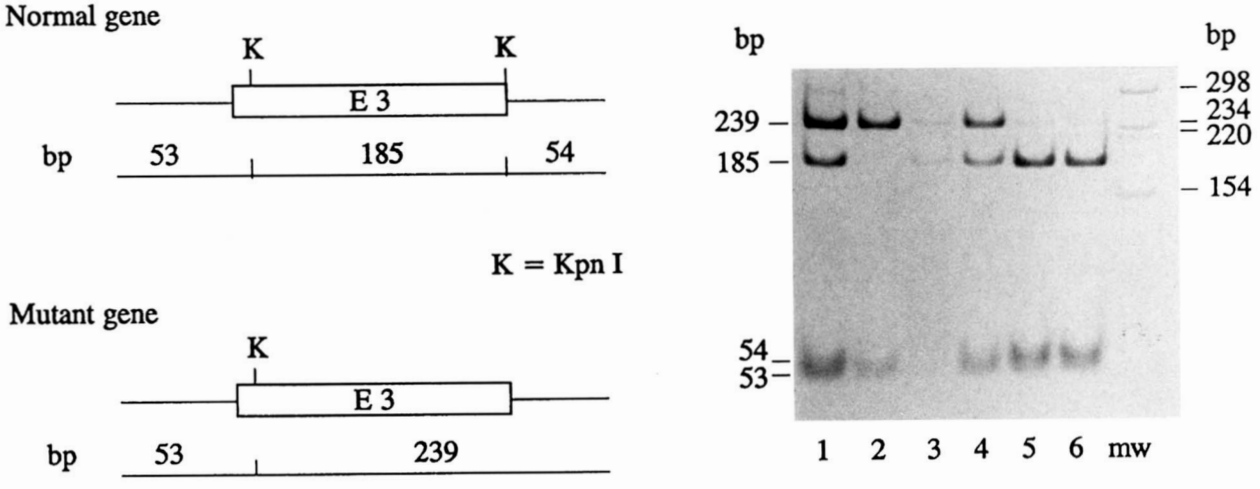

Fig. 7. Screening of the G → C transversion at the last nucleotide of exon 3. Genomic DNA was amplified by PCR using primers complementary to intron 2 (*3A*) and intron 3 (*3B*) (Ref. 12), and digested with the restriction enzyme KpnI (K). The size of the KpnI fragments (bp) is indicated above the horizontal lines and at the sides of the polyacrylamide gel. Lanes 1, 3, and 4, father, mother and brother of proband C.U.; lane 2, proband C.U.; lanes 5 and 6, control subjects.

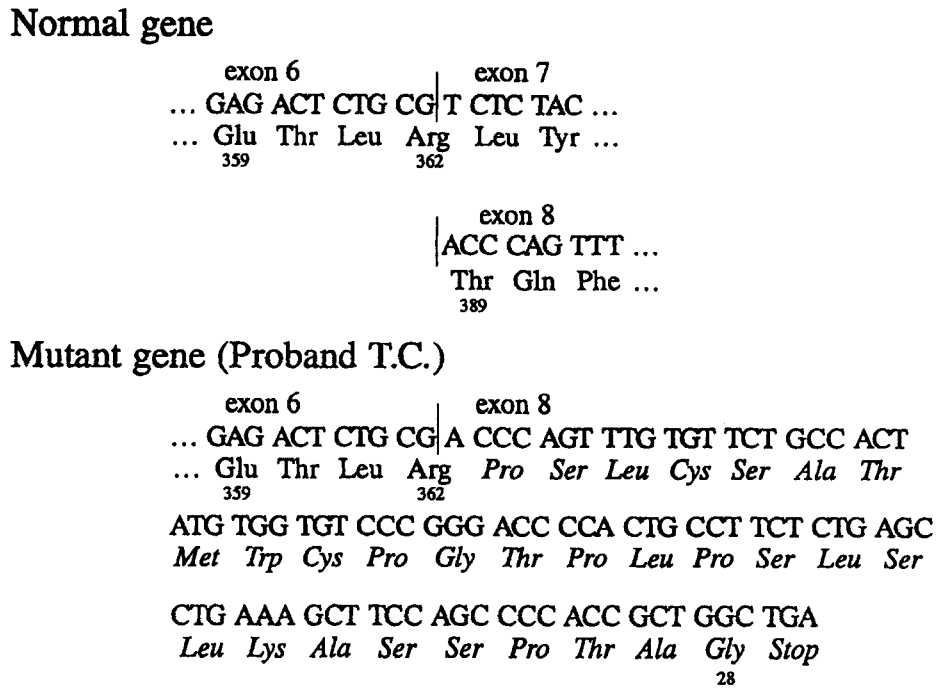

Fig. 8. Nucleotide sequence at the exon 6–exon 7 junction (normal mRNA) and at the exon 6–exon 8 junction in proband T.C. mRNA. The joining of exon 6 to exon 8, with the skipping of exon 7, causes a shift in the reading frame, resulting in a sequence of 28 novel amino acids (indicated in italics) preceding a termination codon.

and 5). This finding is consistent with the very low sterol 27-hydroxylase activity found in the fibroblasts of proband C.U.

The mutation found in proband C.U. is the second mutation of *CYP27* gene that involves the last nucleotides of an exon. The first mutation of this kind was reported by Cali et al. (11) in 1991. One of their patients (designated proband CTX2) was found to be homozygous for an $Arg_{362} \rightarrow Cys$ substitution due to C → T transition at the first nucleotide of the arginine codon. However, this mutation, which occurs at the penultimate nucleotide of exon 6 (i.e., at position -2 with respect to the 5′ donor splice site of intron 6) (see Fig. 8) did not reduce the content of sterol-27 mRNA in cultured fibroblasts (11).

Both mutations reported in this study alter a restriction enzyme recognition site, thus making the rapid screening for these mutations a relatively easy task. These procedures are currently being used not only for the screening of the family members of the two probands investigated in this study, but also for that of the other CTX patients of our series.

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