Four novel mutations of sterol 27-hydroxylase gene in Italian patients with cerebrotendinous xanthomatosis

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Abstract We report the characterization of eight mutations of sterol 27-hydroxylase gene (*CYP27*) in five Italian patients with cerebrotendinous xanthomatosis, who were found to be compound heterozygotes. Four mutations (C → T at nt 45 of exon 4, G_{+1} → A in intron 6, G_{+5} → T in intron 7, and G_{-1} → A in intron 7) are novel. The C → T at nt 45 of exon 4 converts the arginine codon into a stop codon thus generating a truncated protein of 198 amino acids. The three splice site mutations reduced the content of *CYP27* mRNA in skin fibroblasts to very low or undetectable levels and generated minute amounts of abnormal mRNAs. The G_{+1} → A transition in intron 6 produced three abnormal mRNAs. In the first, the 5′ half of exon 6 joins to exon 7, skipping 89 bp of exon 6, and in the second, exon 5 joins directly to exon 7. The predicted translation products of these mRNAs are truncated proteins. In the third abnormal mRNA, exon 5 joins to exon 8 with an in-frame deletion of 246 bp. The G_{+5} → T transversion in intron 7 generates a single abnormal mRNA in which exon 6 joins directly to exon 8, with a frameshift and a premature stop codon. In the G_{-1} → A transition in intron 7, two mRNAs are generated. In the first, the retention of the whole intron 7 causes a frameshift and a premature stop codon; in the second, the joining of exon 7 to exon 8 is associated with an in-frame deletion of the first 6 nucleotides. All these novel mutations are predicted to produce structurally abnormal enzymatic proteins with no measurable biological activity.—**Garuti, R., M.A. Croce, R. Tiozzo, M.T. Dotti, A. Federico, S. Bertolini, and S. Calandra.** Four novel mutations of sterol 27-hydroxylase gene in Italian patients with cerebrotendinous xanthomatosis. *J. Lipid Res.* 1997. **38:** 2322–2334.

Supplementary key words polymerase chain reaction • *CYP27* gene • reverse transcription-PCR

Cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessive disorder of cholesterol metabolism characterized by abnormal deposition of cholestanol and cholesterol in multiple tissues (1). The clinical manifestations of CTX include neurological dysfunctions (dementia, behavioral abnormalities, spinal cord paresis, and cerebellar ataxia), tendon xanthomas, and cataracts (1) and, at least in some patients, premature atherosclerosis (1–4). CTX is due to a defect of sterol 27-hydroxylase (EC 1.14.13.15), a mitochondrial enzyme that belongs to the cytochrome P450 family. This enzyme catalyzes the initial steps in the oxidation of the side chain of cholesterol and also hydroxylates a spectrum of sterol substrates including vitamin D (1, 5, 6). In hepatocytes, sterol 27-hydroxylase oxidizes the side chain of sterol intermediates in the metabolism and biliary excretion of cholesterol (1, 5). In CTX patients, the ability 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). In normal human macrophages, sterol 27-hydroxylase appears to be involved in the elimination of cholesterol from the cell and in the control of intracellular cholesterol content (7, 8). In human skin fibroblasts, 27-hydroxycholesterol (and other oxysterols derived from it) formed by the action of sterol 27-hydroxylase is thought to play a role in the regulation of cholesterol biosynthesis and the LDL-receptor expression (9, 10). An early diagnosis of CTX is crucial as it has been reported, although not conclusively proven, that long-term treatment with chenodeoxycholic acid (11, 12), with or

Abbreviations: CTX, cerebrotendinous xanthomatosis; CYP27 gene, gene encoding human sterol 27-hydroxylase; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

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TABLE 1. Clinical findings in CTX patients

	CTX-2 (B.M.)	CTX-3 (S.R.)	CTX-6 (C.P.)	CTX-8 (B.R.)	CTX-9 (T.M.)
Age (years)	42	46	50	21	37
Sex	F	F	M	F	F
Age of onset of CTX (years)	10	7	20	18	12
Cataract	+	+	+	+	+
Tendon xanthomas	+	+	+	+	+
Cerebellar signs	+	−	−	−	−
Spastic paraparesis	−	+	+	+	+
Mental deterioration	+	+	+	+	+
Psychiatric symptom	+	+	+	+	+
Serum cholestanol (mg/dl)	3[a]	2.8[a]	4.2[a]	5[a]	0.6[a,b]

[a]Normal value = <1 mg/dl.
[b]Under treatment with chenodeoxycholic acid.

without an inhibitor of hydroxy-methyl-glutaryl-CoA reductase (13), might reduce the progression of the neurological complications of the disease (12, 14). Since the molecular cloning of the human sterol 27-hydroxylase cDNA (15) and the characterization of its gene (*CYP27* gene) (16), mutations of this gene have been reported in CTX patients (17–26) of various racial groups. Most of these mutations are point mutations leading to amino acid substitutions (17, 18, 21), shifts in the reading frame (16, 19, 23), premature termination codon (22) or disruption of the mRNA splicing process (16, 26). Only one major rearrangement (deletion) of this gene has been reported so far in an Italian CTX patient (25). In the present study we report the characterization of four novel mutations of *CYP27* gene found in five unrelated Italian CTX patients.

MATERIALS AND METHODS

Subjects

In all probands the diagnosis of CTX was based on well-established clinical criteria (1–3), as illustrated in **Table 1.** Control subjects were chosen from 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 (27). 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 (25, 28). The full-size sterol 27-hydroxylase cDNA probe (15) was a kind gift from Dr. D. Russell (Dallas, TX).

Single strand conformation polymorphism (SSCP)

SSCP was performed according to Orita et al. (29). 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. (16). The PCR conditions for the amplification of the promoter region and the exons of *CYP27* gene were reported previously (26).

Sequencing of genomic DNA

PCR products were sequenced directly (Thermosequenase Kit, USB Amersham, U.K.). In the case of probands CTX-2 and CTX-3 (in whom SSCP analysis of exon 3 had indicated the presence of a sequence variation) the sequence of genomic DNA was performed using the following oligonucleotide: 5′ GAG CAC AAC CTC TCC CTG ACC CAT T 3′ (complementary to the 5′ end of intron 3) (3B) (16). In the case of proband CTX-8 (in whom SSCP analysis had indicated the presence of a sequence variation in exon 4) the sequence of genomic DNA was performed using the following primer: 5′ GCT GAT GCA CAG ACC TGG AGT CAC C 3′ (complementary to the 5′ end of intron 4) (4B) (16). In probands CTX-2, CTX-6, CTX-8, CTX-9 (in whom the SSCP analysis of the exon 6–exon 9 region had suggested the presence of different sequence variations in the fragment spanning from the 5′ end of exon 6 to the 3′ end of exon 8) the sequences of genomic DNA were performed using the following oligonucleotides: 5′ GAG ATC CAG GAG GCC TTG CAC GA 3′, a forward primer in exon 6 corresponding to nts 1087–1110 of the cDNA (6*s*) (25); 5′ GAT TGG GCA GCA TGA ATG CCA CTC 3′, a reverse primer complementary to nts 75–52 of intron 7 (7B) (26); 5′ GGA GTA GCT GCA

TCT CCA GCT CT 3′ (reverse primer in exon 8 complementary to nts 1489–1467 of the cDNA) (8*as*) (25); 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) (16). In the case of proband CTX-3, we sequenced the promoter and all 9 exons to find the second mutation (located at the first nucleotide of intron 6) as the SSCP analysis had failed to show abnormal migration patterns.

Rapid screening of some mutations of *CYP27* gene

We have previously shown that the G → C transversion at the last nucleotide of exon 3 eliminates a KpnI site (26). To confirm the presence of this mutation in probands CTX-2 and CTX-3, exon 3 was amplified by PCR from genomic DNA and digested with KpnI as previously reported (26). In a previous report we have also shown that the mutation of the donor splice site of intron 7 (G_{+1} → A transition) creates a recognition site for the enzyme Hsp92III (26). To confirm the presence of this mutation in proband CTX-8, (in whom SSCP analysis had revealed a sequence variation in exon 6–exon 8 region), the exon 6–exon 8 region was amplified by PCR from genomic DNA and digested with Hsp92II (26).

The C → T transition in exon 4 (R198X) of proband CTX-8 eliminates an ItaI restriction site. To screen subjects for this mutation genomic DNA was amplified by PCR using primers 4A and 4B (16). The conditions were 95°C for 1 min and 70°C for 1 min for 28 cycles. The PCR products were digested with ItaI and the DNA fragments were separated by 5–10% polyacrylamide gradient gel electrophoresis and stained with ethidium bromide. The G → A transition in exon 8 (R441Q) creates a StuI restriction site. To screen subjects for this mutation (CTX-6) genomic DNA was amplified by PCR using primers 6A and 9B (16). The conditions were: 95°C for 1 min and 68°C for 5 min for 30 cycles. PCR products were digested with StuI and the fragments were separated in a 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Cell culture and assay of sterol 27-hydroxylase in fibroblasts

A skin biopsy was taken from probands CTX-2, CTX-3, and CTX-9. Explants were cultured in 25-cm^2 flasks in DMEM (Dulbecco's modification of Eagle's medium), 100 IU/ml of penicillin and 50 mg/ml streptomycin, 2 mM glutamine, 15% calf serum, and 95% air–5% CO_2. For experimental purposes, cells were cultured in dishes. They were seeded at a density of 5,000–6,000 × cm^2. Subconfluent cells (1–2 × 10^6 per dish) were first pre-incubated for 24 h in a medium containing 10% LPDS (lipoprotein-deficient serum). The assay of sterol 27-hydroxylase was performed using a fibroblast suspension prepared according to the procedure described by Atsuda and Okuda (30), as modified by Skrede et al. (31) and used by us in a previous study (26). Fibroblasts of CTX probands (CTX-2, CTX-3, and CTX-9) and one control subject were incubated at 37°C in the presence of 2 μCi of [^{14}C]cholesterol (Amersham, UK)–albumin suspension, as previously reported (26).

Northern blot analysis

Total cellular RNA of proband CTX-2 and her mother, of proband CTX-3, and proband CTX-9 was isolated by extraction in guanidine–thiocyanate (32) 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_2EDTA], 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 loading on the filters (26). Pre-hybridization and hybridization were performed as previously described (26).

Reverse transcription and PCR amplification

Total RNA (1 μg) from cultured fibroblasts of probands CTX-2, CTX-3, and CTX-9 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 RNAsin, 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 (33). For all probands 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 (6*s*); (25); 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) (16). In the case of proband CTX-3 another reaction was performed with the following primers: 5′ TGG CAT CCA GGT GTC TGG CT 3′ (forward primer in exon 5 complementary to nts 930–949 of the cDNA) (5*s*); as reverse primer we used primer 9B (see above) (16). The conditions were: 95°C for 3 min, 65°C for 1 min for the first time, and subsequently 95°C for 1 min, 65°C for 1 min for 28 cycles. PCR products were separated from the unincorporated primers by electrophoresis on 1.5% agarose gel. Ethidium bromide-stained bands were excised from the gel using Jetsorb (Genomed). The RT-PCR fragments

TABLE 2. Mutations of sterol 27-hydroxylase in CTX patients

Proband		Mutation	Effect	Previously Reported
CTX-2	allele A	Ex 3 G → C	A183P[a]	ref. 26
	allele B	In 7 G_{+5} → T	frameshift → stop[b]	no
CTX-3	allele A	Ex 3 G → C	A183P[a]	ref. 26
	allele B	In 6 G_{+1} → A	frameshift → stop[b]	no
CTX-6	allele A	Ex 8 G → A	R441Q	ref. 21
	allele B	In 7 G_{-1} → A	frameshift → stop[b]	no
			in-frame deletion[b]	no
CTX-8	allele A	Ex 4 C → T	R198X	no
	allele B	In 7 G_{+1} → A	frameshift → stop[b]	ref. 26
CTX-9	allele A	E7-E9 deletion	no mRNA	ref. 25
	allele B	In 7 G_{-1} → A	frameshift → stop[b]	no

[a]Associated with a reduced level mRNA (ref. 26).
[b]Associated with very low or undetectable levels of mRNA in Northern blot and with aberrantly spliced mRNAs.

were sequenced directly using the Thermosequenase Kit (USB Amersham, U.K.) The primer used in the sequencing reaction was primer 6*s* for probands CTX-2 and CTX-9 and primer 5*s* for proband CTX-3.

RESULTS

Southern blot and SSCP analysis

To ascertain the presence of major structural rearrangements of *CYP27* gene (25), genomic DNA from all probands and from two control subjects was digested with several restriction enzymes. After Southern blotting and hybridization with the full-size sterol 27-hydroxylase cDNA, only one subject (CTX-9) was found to be heterozygote for a 2 kb deletion at the 3′ end of the *CYP27* gene. The boundaries and the size of this deletion (data not shown) were identical to those previously reported by our group in an Italian CTX-homozygote (25). The SSCP analysis of the promoter region and all exons, performed in all probands, showed abnormal migration patterns in different regions of the *CYP27* gene, namely: exon 3 in probands CTX-2 and CTX-3; exon 4 in proband CTX-8; exon 6–exon 9 region in probands CTX-2, CTX-6, CTX-8, CTX-9 (data not shown). The nucleotide sequence of these regions (see below) revealed that all probands were compound heterozygotes (**Table 2**).

Sequence of *CYP27* gene

Proband CTX-2 and her sister (also affected by CTX) as well as proband CTX-3 and her CTX brother were carriers of a G → C transversion at the last nucleotide of exon 3 (A183P) (data not shown), a mutation previously reported by us in an Italian homozygous CTX patient (26). This mutation, which eliminates a KpnI restriction site (26) was confirmed after KpnI digestion of PCR amplified exon 3 (data not shown).

Proband CTX-2 (B.M.) was also a carrier of a G → T transversion at nucleotide +5 of intron 7 (**Fig. 1**). This mutation was also found in her mother. Proband CTX-3 was a carrier of a G → A transition at the first nucleotide of intron 6 (**Fig. 2**). These two mutations have not been reported previously.

Proband CTX-6 was a carrier of a G → A transition in exon 8 (R441Q) (data not shown), a mutation previously reported in a Japanese patient (21). This mutation, which eliminates a StuI restriction site (21), was confirmed after StuI digestion of PCR amplified of exon 6–exon 9 region. Proband CTX-6 was also a carrier of a G → A transition at the last nucleotide of intron 7 (**Fig. 3**) (novel mutation).

The fourth compound heterozygote (proband CTX-8) carried a C → T transition in exon 4 which converts the codon for arginine at position 198 into a termination codon (R198X) (**Fig. 4**). (novel mutation). She was also a carrier of a G → A transition at the first nucleotide of intron 7, a mutation we previously reported in an Italian CTX homozygote (26). The C → T transition in exon 4, which eliminates an ItaI site, was confirmed after ItaI digestion of exon 4 amplified from proband's genomic DNA (data not shown).

The fifth compound heterozygote (proband CTX-9) was found to carry a 2 kb deletion eliminating exon 7–exon 9 region (see above and ref. 25) as well as the G → A transition at the last nucleotide of intron 7 (data not shown). The latter mutation is identical to that found in proband CTX-6 (Fig. 3).

Effects of splice site mutations on sterol 27-hydroxylase mRNA

As four point mutations found in our probands (CTX-2, CTX-3, CTX-6, CTX-8, and CTX-9) (Table 2)

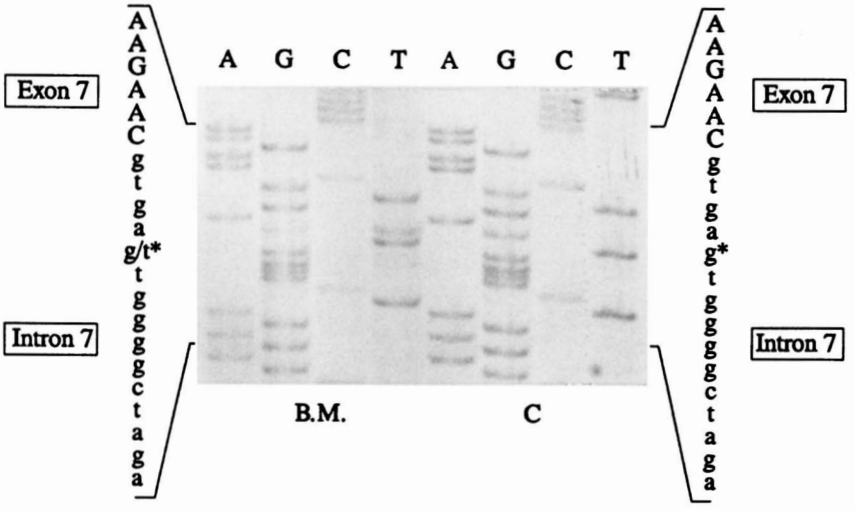

Fig. 1. Nucleotide sequence of the exon 7–intron 7 junction of *CYP27* gene in proband CTX-2 (B.M.) and in a control subject (C). Proband CTX-2 is heterozygous for a G → T transversion (indicated by a star) at the 5th nucleotide of intron 7 ($G_{+5} \rightarrow T$).

were expected to influence mRNA splicing (their location being at the intron–exon boundaries) the content and the type of sterol 27-hydroxylase mRNA were investigated in cultured skin fibroblasts. This study was performed in three probands (CTX-2, CTX-3, and CTX-9) (Table 2), as the effect of the $G_{+1} \rightarrow A$ transition in intron 7 on mRNA splicing (proband CTX-8) had been previously reported by our group in another homozygous CTX patient (26).

Northern blot analysis (**Fig. 5,** panel A) demonstrated that *CYP27* mRNA is hardly detectable in fibroblasts of proband CTX-2 (G → C transversion at the last nucleotide of exon 3 and $G_{+5} \rightarrow T$ transversion in intron 7). This finding confirms our previous observation that the G → C transversion at the last nucleotide of exon 3 greatly reduces mRNA level (26) and suggests that a similar effect is produced by the $G_{+5} \rightarrow T$ in intron 7. The latter suggestion is supported by the observation that the level of *CYP27* mRNA is substantially reduced in the mother of proband CTX-2, who is heterozygote

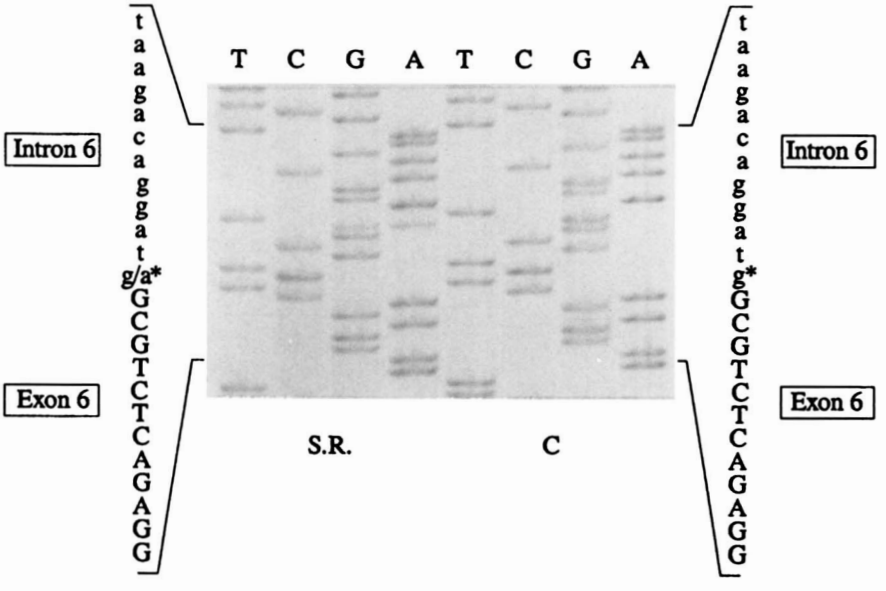

Fig. 2. Nucleotide sequence of the exon 6–intron 6 junction in proband CTX-3 (S.R.) and in a control subject (C). Proband CTX-3 is heterozygous for a G → A transition at the first nucleotide of intron 6 (indicated by a star) ($G_{+1} \rightarrow A$).

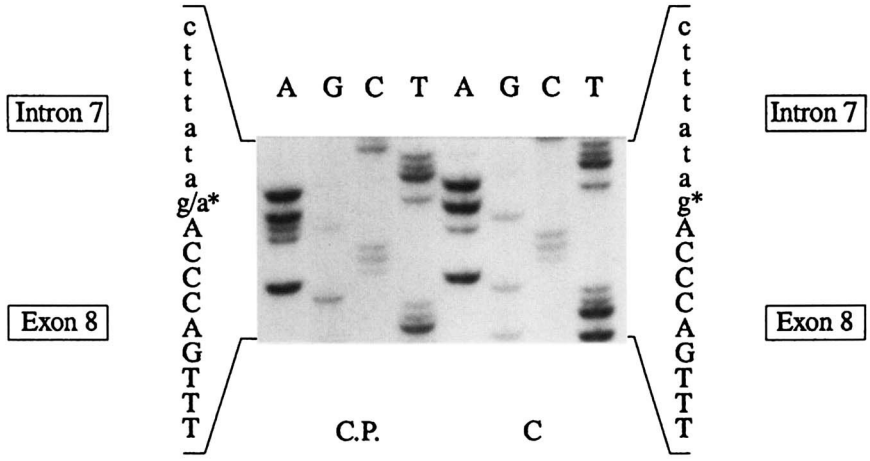

Fig. 3. Nucleotide sequence of intron 7–exon 8 junction of *CYP27* gene in proband CTX-6 (C.P.) and in a control subject (C). Proband CTX-6 is heterozygous for a G → A transition at the last nucleotide of intron 7 (indicated by a star) ($G_{-1} \rightarrow A$).

for the $G_{+5} \rightarrow T$ in intron 7 (Fig. 5, panel A). Only by using reverse transcription PCR (RT-PCR) were we able to detect the sterol 27-hydroxylase mRNA present in skin fibroblasts of proband CTX-2. RT-PCR obtained by using primers complementary to exon 6 and the 3′ untranslated region, resulted in two fragments of 553 and 474 bp, respectively (Fig. 5, panel C: lane 1), as opposed to a single 553 bp fragment in the control subject (Fig. 5, panel C: lane 3). The sequence of the abnormal fragment (474 bp) demonstrated that exon 6 joined directly to exon 8 with the complete skipping of exon 7 (**Fig. 6**).

In proband CTX-3 (G → C transversion at the last nucleotide of exon 3 and $G_{+1} \rightarrow A$ transition in intron 6) Northern blot analysis showed a barely detectable mRNA (Fig. 5, panel B). For the reasons given above (see proband CTX-2) this finding indicates that the $G_{+1} \rightarrow A$ in intron 6 disrupts the splicing process and greatly reduces mRNA level in cultured fibroblasts. RT-PCR of exon 6–3′ untranslated region showed, in addition to the normal fragment of 553 bp, an abnormal fragment of 464 bp (Fig. 5, panel C, lane 2).

The sequence of this fragment shows that the 5′ half of exon 6 is followed by exon 7, with the skipping of

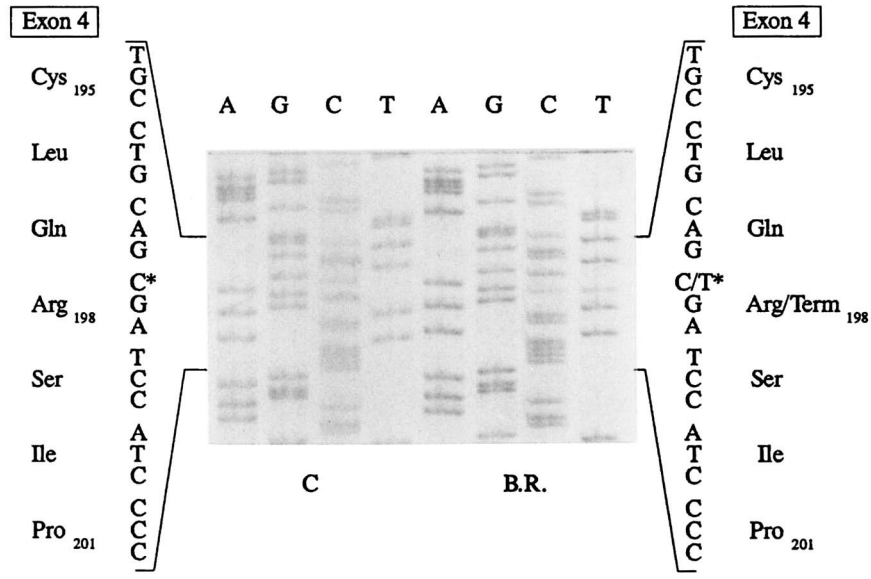

Fig. 4. Nucleotide sequence of exon 4 of *CYP27* gene in proband CTX-8 (B.R.) and in a control subject (C). Proband CTX-8 is heterozygous for a G → T transition at nucleotide 45 of exon 4 (R198X) (indicated by a star).

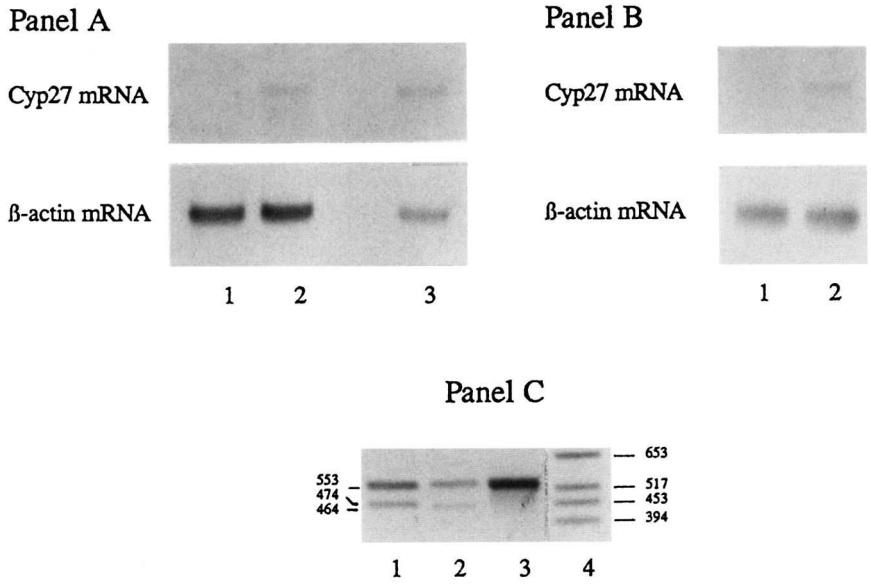

Fig. 5. Northern blot analysis of sterol 27-hydroxylase mRNA in proband CTX-2 and her mother (panel A) and in proband CTX-3 (panel B). In panel A, lane 1: proband CTX-2; lane 2: proband CTX-2's mother; lane 3: control subject. In panel B, lane 1: proband CTX-3; lane 2: control subject. Panel C shows the reverse transcription PCR (exons 6–9 of the cDNA) in probands CTX-2 (lane 1) and CTX-3 (lane 2) and in a control subject (lane 3). Lane 4: molecular weight markers.

the first 89 nt of exon 6 (**Fig. 7**). In another RT-PCR experiment where primers complementary to exon 5 (5*s*) and the 3′ untranslated region (9B) were used, we were also able to detect trace amounts of two additional fragments of 543 bp and 464 bp that were not detectable in the control subjects (**Fig. 8**). The nucleotide sequence showed that in the 543 fragment exon 5 joins to exon 7 and in the 464 fragment exon 5 joins to exon 8 (Fig. 8).

In proband CTX-9 (2 kb deletion of exon 7–exon 9 region and $G_{-1} \rightarrow A$ transition in intron 7) Northern blot analysis showed the presence of trace amounts of an apparently 'normal' mRNA. (**Fig. 9,** left panel). In RT-PCR, performed using primers complementary to exon 6–3′ untranslated region, we detected two fragments, one (of low intensity) of 639 bp and the other (of high intensity) migrating in the same position as the 553 bp fragment (corresponding to the normal mRNA)

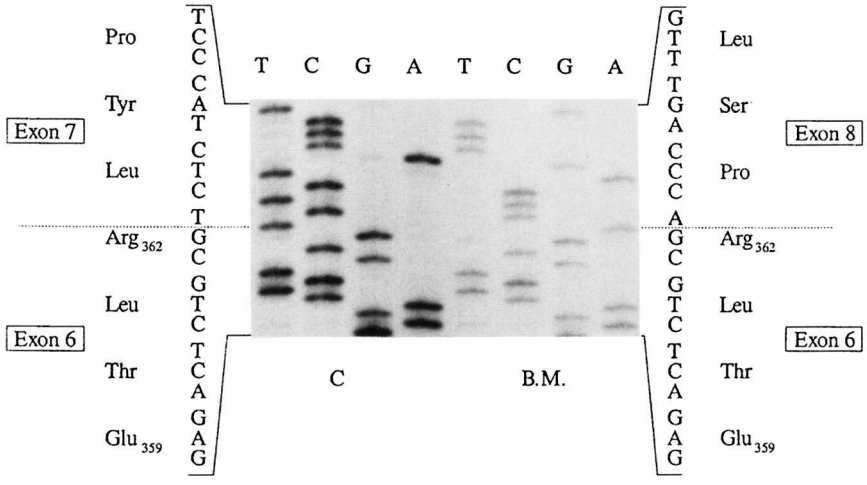

Fig. 6. Nucleotide sequence of 474 bp RT-PCR fragment (lane 1, panel C of Fig. 5) found in proband CTX-2 (B.M.) showing that exon 6 joins to exon 8 with the complete skipping of exon 7. The exon 6–exon 7 junction of the normal 553 RT-PCR fragment (lane 3, panel C of Fig. 5) is also shown (C).

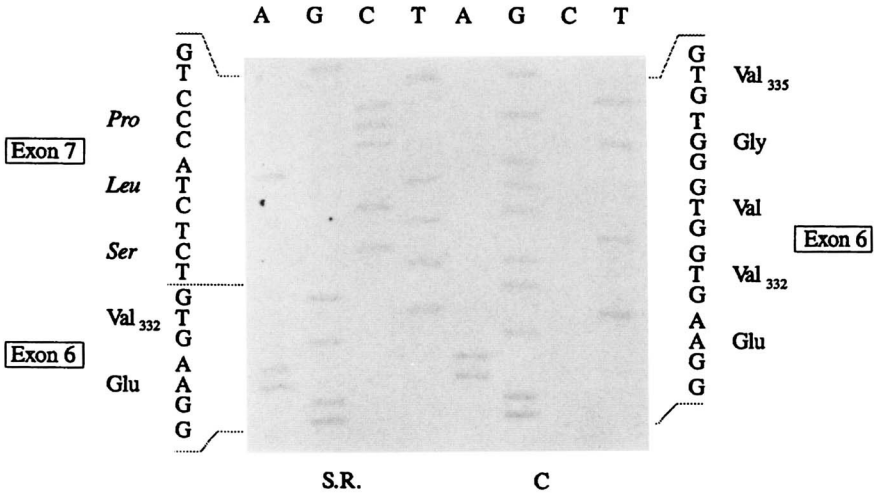

Fig. 7. Nucleotide sequence of 464 bp RT-PCR fragment (lane 2, panel C of Fig. 5) found in proband CTX-3 (S.R.) showing that the 5′ half of exon 6 joins to exon 7 with the skipping of 89 nucleotide of exon 6. The sequence of the corresponding region of normal exon 6 is also shown (C).

(Fig. 9, right panel). **Figure 10** shows that: *a*) in the 639 bp fragment, exon 7 is followed by intron 7; and *b*) in the 553 bp fragment, exon 7 joins to exon 8 at the 5′ end of which the first six nucleotides are deleted.

Assay of sterol 27-hydroxylase in skin fibroblasts

The assay of sterol 27-hydroxylase activity in fibroblasts showed no measurable 'residual activity' in CTX-2, CTX-3, and CTX-9 (84 and 106, 135 cpm respectively vs. 2830 cpm per mg of protein in control cells).

DISCUSSION

In this study we report eight mutations of the *CYP27* gene in five Italian CTX patients. All probands were determined to be compound heterozygotes (Table 2). Four mutations (C → T at nt 45 of exon 4, G_{+1} → A in intron 6, G_{+5} → T and G_{-1} → A in intron 7) were reported for the first time (Table 2). Three of the already known mutations (G → C transversion at the last nucleotide of exon 3, G_{+1} → A in intron 7, and the 2 kb deletion eliminating the exon 7–exon 9 region) were previously reported by our group in homozygous Italian CTX patients (25, 26). The other mutation found in proband CTX-8, a G → A transition at nt 158 of exon 8 (R441Q), had been previously reported by other investigators (21).

One of the novel mutations (found in proband CTX-8) converts the arginine codon at position 198 of the mature protein into a termination codon. The predicted protein encoded by this allele is devoid of the putative ferrodoxin and the heme binding domains (15). As the other allele of proband CTX-8 was found to abolish the enzyme activity (26), it is most unlikely that any residual enzymatic activity is present in CTX-8 fibroblasts. We have been unable to confirm this prediction as the patient's parents did not give their consent to skin biopsy.

The other novel mutations reported here occur at or close to the splice sites of intron 6 and intron 7. In the present study we show that these novel mutations (G_{+1} → A in intron 6, G_{+5} → T and G_{-1} → A in intron 7) cause a disruption of mRNA splicing, resulting in a very low or an undetectable content of *CYP27* mRNA in fibroblasts and in the production of minute amounts of abnormal mRNA species. The G_{+1} → A transition in intron 6 (proband CTX-3) generates three abnormal mRNAs. In the first, the 5′ half of exon 6 joins directly to exon 7, with the skipping of 89 bp of exon 6 generated by the activation of a cryptic donor splice site. The shift in the reading frame and the occurrence of a premature stop codon in this abnormal mRNA are expected to produce a truncated peptide of 348 amino acids that has a string of 16 novel amino acids at its carboxy-terminal end. This peptide is devoid of the three basic amino acids (Lys_{354}, Lys_{358}, Arg_{362}) that are thought to be involved in the binding of ferrodoxin, as inferred from the analysis of other enzymatic proteins belonging to the mitochondrial cytochrome P_{450} family (24). In addition this truncated protein is devoid of the heme binding domain (15, 24). In the second abnormal mRNA, exon 5 joins directly to exon 7 with a disruption of the reading frame and the occurrence of a premature

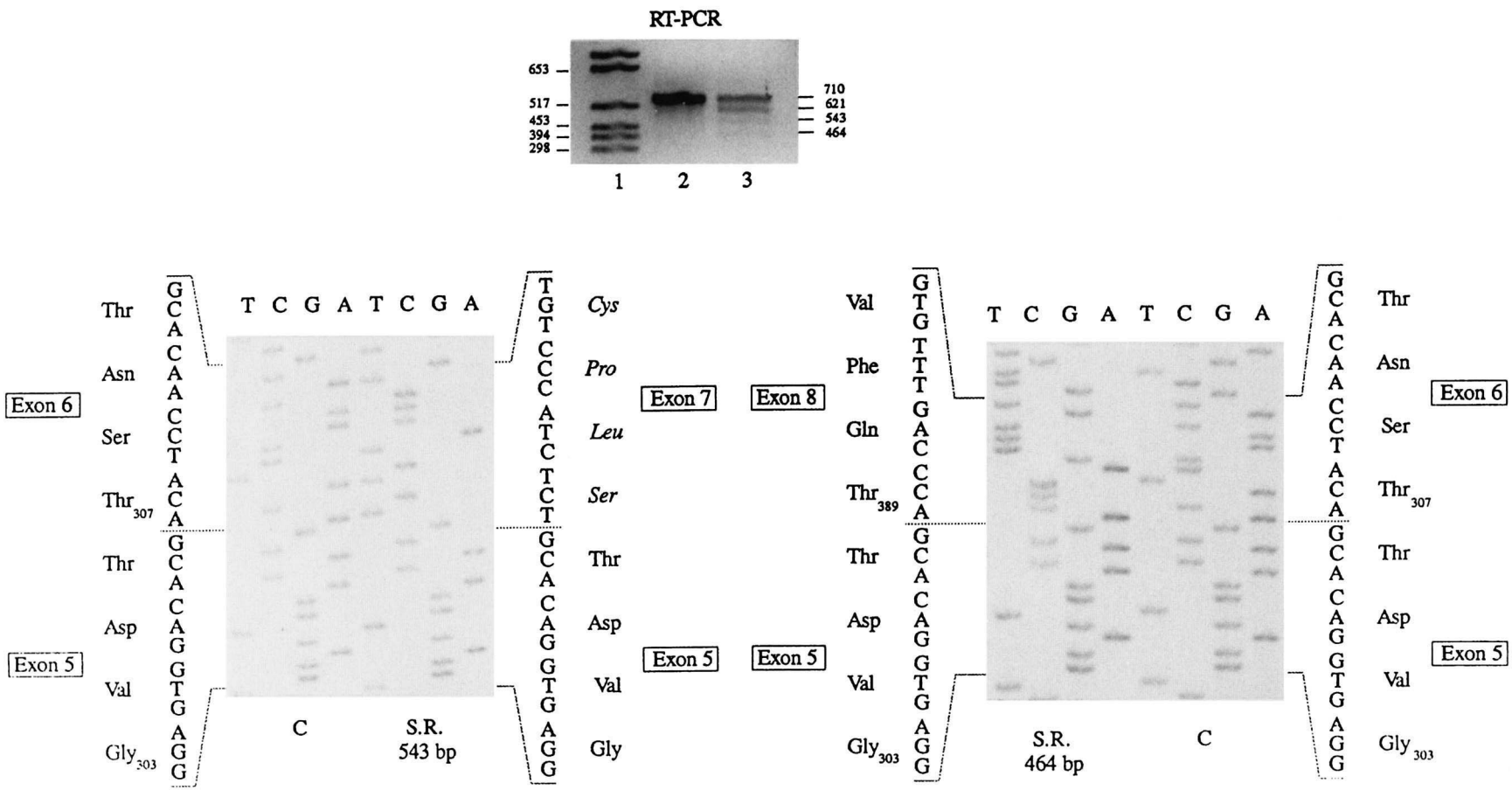

Fig. 8. Reverse transcription PCR (RT-PCR) (exons 5–9 of the cDNA) of a control subject (lane 2) and proband CTX-3 (lane 3) (upper panel). The nucleotide sequence of RT-PCR products (lower panels) shows that in the 543 bp fragment exon 5 joins to exon 7, and in 464 fragment exon 5 joins to exon 8. The sequence of the corresponding region of normal exon 5–exon 6 junction is also shown (C). The nucleotide sequence of the 621 RT-PCR fragment of proband CTX-3 is identical to that shown in Fig. 7 (data not shown).

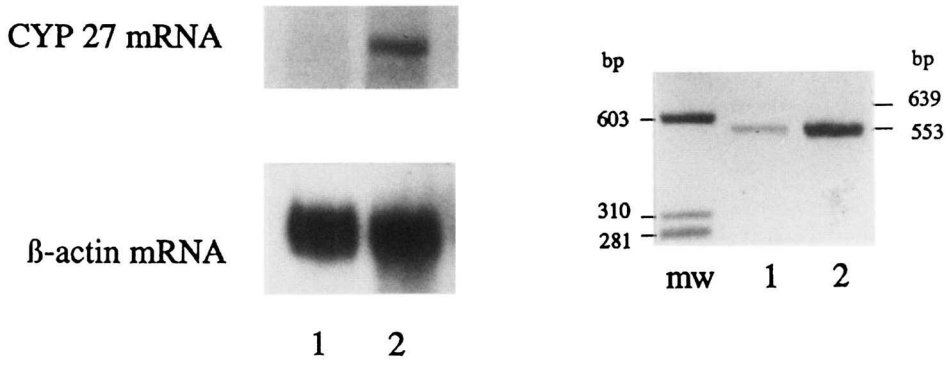

Fig. 9. Left panel: Northern blot analysis of sterol 27-hydroxylase mRNA in proband CTX-9, who is a compound heterozygote for a 2 kb deletion eliminating exon 7–exon 9 region (ref. 25) and a $G_{-1} \rightarrow A$ transition in intron 7. Lane 1: proband CTX-9; lane 2: control subject. Right panel: RT-PCR of sterol 27-hydroxylase mRNA in proband CTX-9 (lane 1) and in a control subject (lane 2).

stop codon. This mRNA is predicted to encode a truncated protein of 322 amino acids (with a sequence of 16 novel amino acids at the carboxy-terminal end) that is also devoid of the heme and ferrodoxin binding domains. In the third abnormal mRNA, exon 5 joins directly to exon 8 with no disruption of the reading frame. The predicted translation product is a protein of 416 amino acids with an internal deletion of 82 amino acids (from Thr_{307} to Asn_{388}). As this deletion eliminates the putative ferrodoxin binding domain, it is most unlikely that the mutant protein (which is expected to be present in trace amounts) has any residual enzymatic activity. The assay of sterol 27-hydroxylase of proband CTX-3 fibroblasts showed no measurable activity, in good agreement with the predicted effect of the $G_{+1} \rightarrow A$ mutation (first allele) and the $G \rightarrow C$ mutation in the last nucleotide of exon 3 (second allele) (26).

The $G_{+5} \rightarrow T$ transversion in intron 7 generates a single abnormal mRNA in which exon 6 joins directly to exon 8 with the complete skipping of exon 7. The effect of this mutation is identical to that caused by $G_{+1} \rightarrow A$ in intron 7 that we have reported previously in an Italian CTX homozygote (26). The translation product of this abnormal mRNA is a truncated protein of 390 amino acids that has a tail of 28 novel amino acids. This protein is expected to have no biological activity, being devoid of the heme binding domain (15, 24).

The very low content of the abnormal mRNA species

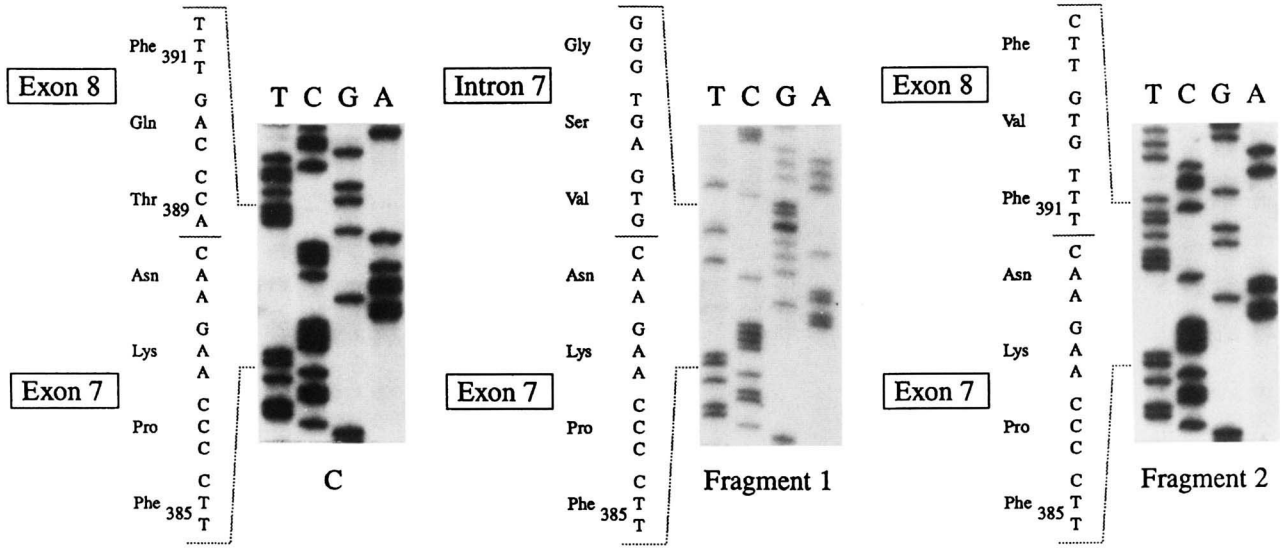

Fig. 10. Nucleotide sequence of RT-PCR fragments found in proband CTX-9 and shown in the right panel of Fig. 9. From the left: exon 7–exon 8 junction in control mRNA (C), corresponding to the 553 bp RT-PCR fragment. Fragment 1: sequence of the abnormal 639 bp fragment of proband CTX-9 showing that exon 7 is followed by intron 7. Fragment 2: sequence of the 553 bp fragment of proband CTX-9 showing that exon 7 joins to exon 8 with the skipping of the first six nucleotides of exon 8.

found in fibroblasts of probands CTX-3 and CTX-2 is not surprising. It is firmly established that the GT dinucleotide is highly conserved at the 5′ end of introns of most eukaryotic genes (34–36). More specifically, the first nucleotide of a normal intron is never an 'A' (36), and mutations of the strictly conserved 'G' at this position, as found in CTX-3, prevent normal splicing (37). 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 (38, 39). 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 of the mRNA (40–42). The content of the abnormally spliced mRNA is often much lower than that of its normal counterpart, as observed in our patients. This may be due to a reduced efficiency of the abnormal splicing process (alternative splicing or activation of cryptic splice sites) as well as to the accelerated catabolism of an mRNA carrying a premature stop codon (43).

It has also been reported (36) that at position +5 of introns of mammalian genes 'G' is present in 84% of cases, whereas 'A' is present in 5% of cases and the consensus sequence at the 5′ end of introns (from −2 to +6) is the following: AGGT(A/G)AGT (36). In proband CTX-2 the $G_{+5} \rightarrow T$ transversion in intron 7 abolishes the canonical donor splice site and causes the activation of an alternative splicing.

The effect of the $G_{-1} \rightarrow A$ transition in intron 7 (found in probands CTX-6 and CTX-9) on *CYP27* mRNA processing was investigated in fibroblasts of proband CTX-9, a patient who is also a carrier of the deletion eliminating the exon 7–exon 9 region (25). As this deletion is associated with a complete absence of *CYP27* mRNA (25), we assumed that any mRNA species present in the proband's cell was the result of the $G_{-1} \rightarrow A$ mutation in intron 7. We found that the mutation of the acceptor splice site of intron 7 causes a substantial reduction of the mRNA content and the formation of two minor mRNA species generated by alternative splicing and activation of cryptic splice sites. The more abundant abnormal mRNA, the size of which is indistinguishable from that of its normal counterpart in Northern blot and RT-PCR, contains an in-frame deletion of the first six nucleotides of exon 8. This minute deletion, which results from the activation of a cryptic splice site (AGTT) at the 5′ end of exon 8, eliminates two amino acids (Thr_{389} and Gln_{390}) in a sequence of 16 amino acids that is identical in human and rabbit sterol 27-hydroxylase (44). We do not know whether the elimination of these two amino acids reduces the enzyme activity. It is reasonable to assume, however, that in view of the extremely low content of this abnormal mRNA (Fig. 9, left panel) any residual enzymatic activity, if present, is negligible (see below). The minor mRNA species present in proband CTX-9 is larger than its normal counterpart as it contains the whole intron 7. The joining of exon 6 to intron 7 causes a shift in the reading frame with the occurrence of a premature stop codon. The predicted translation product is a truncated protein of 445 amino acids with a stretch of 57 novel amino acids at the carboxy-terminal end. This peptide is devoid of the heme binding domain (16). We found that the combination of the exon 7–exon 9 deletion (first allele) and $G_{-1} \rightarrow A$ in intron 7 (second allele) completely abolishes the enzymatic activity in CTX-9 fibroblasts.

In conclusion, the present study, together with our previous reports (25, 26), shows that: *a*) cerebrotendinous xanthomatosis is produced by a variety of different mutations of the *CYP27* gene (allelic heterogeneity), at least in the Italian population; and *b*) several point mutations involve the splice sites and disrupt mRNA processing.

Although many mutations found in our CTX patients abolish the expression of the *CYP27* gene (i.e., no sterol 27-hydroxylase mRNA and/or no detectable enzyme activity in cultured skin fibroblasts) there appears to be no simple and straightforward correlation between the biological effect of the mutation (null allele) and the severity of neurological disorders in our CTX patients. This suggests that other unknown factors play a role in the multiform phenotypic expression of cerebrotendinous xanthomatosis.

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