Silent Nucleotide Substitution in the Sterol 27-Hydroxylase Gene (*CYP* 27) Leads to Alternative Pre-mRNA Splicing by Activating a Cryptic 5′ Splice Site at the Mutant Codon in Cerebrotendinous Xanthomatosis Patients†

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ABSTRACT: A functionally silent nucleotide substitution of the sterol 27-hydroxylase gene (*CYP* 27), identified in two families with cerebrotendinous xanthomatosis (CTX), was confirmed to cause alternative pre-mRNA splicing of the gene. Full-length RT-PCR analysis of the *CYP* 27 gene in a patient from one of the CTX families revealed one major and an additional faint band. Sequence analysis of the cloned RT-PCR product showed three species of cDNA: 3′ terminal 13 bp of exon 2 deleted cDNA, exon 2 skipped cDNA, and full-length cDNA with a functionally silent G to T mutation at codon 112 (GGG ^{112}Gly to GGT ^{112}Gly). Only a single base change was identified by genomic DNA sequence analysis of the *CYP* 27 gene in the patient: T replaced G at the third position of codon 112, 13 bp upstream from the 3′ terminus of exon 2. Transfection of constructed minigenes, with or without the mutation, confirmed that this silent mutation resulted in alternative pre-mRNA splicing by activating a cryptic 5′ splice site around the mutant codon. The mutation was also identified in two patients from another CTX family, with a compound heterozygous pattern of A for G substitution at codon 372, a mutation reported previously by our group. The results elucidate a novel molecular basis for the CTX and suggest the significance of a silent nucleotide substitution with regard to pre-RNA splicing.

The mitochondrial P-450 enzyme, sterol 27-hydroxylase, is involved in the metabolism and biliary excretion of cholesterol. Mutation in the sterol 27-hydroxylase gene (*CYP* 27)[1] results in cerebrotendinous xanthomatosis (CTX), a sterol metabolism disorder inherited in an autosomal recessive trait (*1*). The disease was first described in 1937 (*2*) and is clinically characterized by tendon xanthomas, cataracts, and diverse neurological dysfunctions, including pyramidal weakness, cerebellar signs, and dementia (*1*). Osteoporosis with recurrent bone fractures (*3*) and premature atherosclerosis (*4*) can also occur. The major symptoms of CTX relate to accumulation of cholesterol and cholestanol, a 5α-dihydro derivative of cholesterol in neural and other tissues (*5*, *6*). Early diagnosis of CTX is crucial as treatment with chenodeoxycholic acid can reduce the plasma cholestanol level and may prevent progression of the disease (*7*) or even reverse some of the neurological disabilities (*8*). If untreated, CTX can result in serious neurological defects and even death (*9*). Cloning of the human *CYP* 27 gene cDNA (*10*) and determination of its gene structure (*11*) have facilitated genetic investigations of CTX. Molecular studies revealed that *CYP* 27 gene mutations in CTX patients may reflect insertion (*12*), deletion (*13*, *14*), and, most frequently, point mutation which leads to amino acid substitution or premature codon termination (*15*−*21*).

The significance of a silent nucleotide substitution in a gene may not be given enough attention as the mutation does not alter the amino acid codon. In this study, a functionally silent nucleotide substitution in the *CYP* 27 gene, identified in two unrelated Japanese CTX families, was confirmed to cause alternative pre-mRNA splicing by activating a cryptic 5′ splice site around the mutant codon, and to be responsible for deficiency in the enzyme activity. This study elucidates a new molecular basis for the CTX and provides information on our understanding of alternative pre-mRNA splicing of a gene. The results also show how a functionally silent nucleotide substitution can have a marked effect on pre-mRNA splicing.

MATERIALS AND METHODS

Subjects. Two Japanese families with CTX, designated as CTX 08 and CTX 09, with three patients and five heterozygous carriers were studied (Figure 1). The diagnosis of CTX was based on defined clinical criteria and elevated

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[1] Abbreviations: *CYP* 27 gene, sterol 27-hydroxylase gene; CTX, cerebrotendinous xanthomatosis; pre-mRNA, precursor messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; HPLC, high-performance liquid chromatography.

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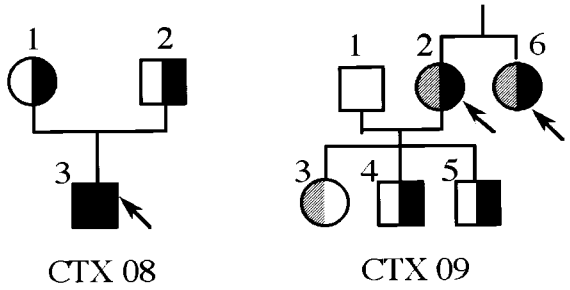

FIGURE 1: Pedigree structure of the CTX families studied. CTX 08, homozygote (3) and heterozygote (1 and 2) of the silent G to T mutation at codon 112 (GGG ^{112}Gly to GGT ^{112}Gly), respectively. CTX 09, heterozygote (4 and 5) of the mutation at codon 112, heterozygote (3) of the G to A mutation at codon 372 (CGG ^{372}Arg to CAG ^{372}Gln), compound heterozygote (2 and 6) of the mutations at codons 112 and 372, and normal subject (1). The patients are indicated by arrows.

serum cholestanol concentration as well as on undetectable sterol 27-hydroxylase activity. Informed consent was obtained from all the subjects.

Biochemical Analysis. Serum cholesterol and cholestanol levels were measured by high-performance liquid chromatography (HPLC), using fasting blood samples (*22*).

Fibroblast Culture. Fibroblasts derived from the patients, carriers, and healthy control subjects were grown and maintained as monolayer in culture dishes (100 mm) in Dulbecco's Modified Eagle's Medium (Life Technologies, Tokyo, Japan) supplemented with 10% fetal calf serum (Life Technologies) in a 95% air/5% CO_2 atmosphere at 37 °C in a humidified incubator. The cells were harvested with trypsin (0.25%), washed three times in phosphate-buffered saline, and then frozen at −80 °C prior to DNA/RNA extraction and the sterol 27-hydroxylase activity assay.

Assay of Sterol 27-Hydroxylase Activity. Sterol 27-hydroxylase activities in cultured fibrobalsts derived from the patients and family members were assayed using a procedure described by Skrede et al. (*23*). Labeled substrate, 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol with a specific activity

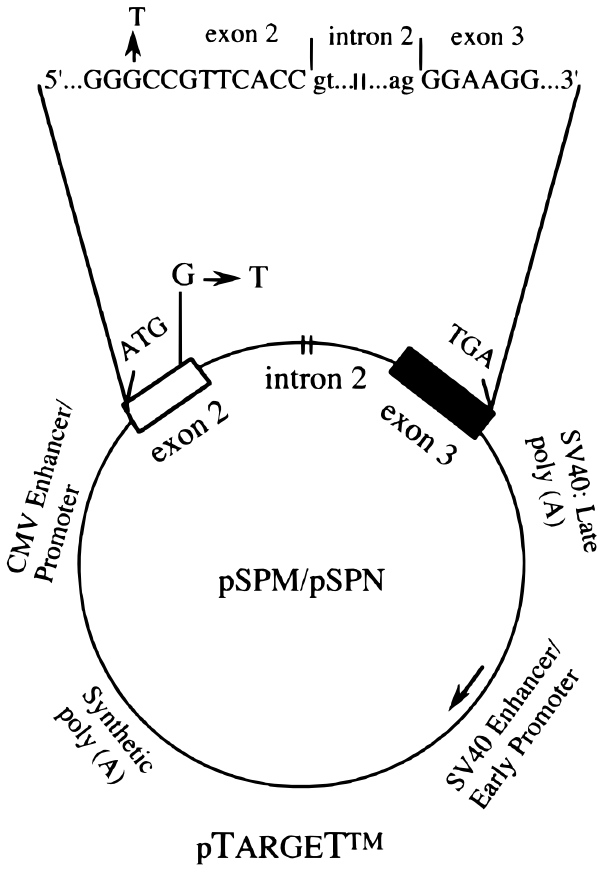

FIGURE 2: Schematic representation of mutant (pSPM) and normal (pSPN) minigene constructs. The insert containing exons 2 and 3 as well as truncated 209 bp intron 2 was cloned into the pTARGET expression vector which contains the CMV enhancer/promoter region, a chimeric intron for high-level expression of the insert, and the SV40 late polyadenylation signal. The silent G to T mutation is indicated by an arrow.

Table 1: Sequence and Location of Primers in the Sterol 27-Hydroxylase Gene

primer	location	amplification	5′ to 3′ sequence	position
FRup	5′ flanking	5′ flanking	GGTGTGGGGCTTCCCGATTT	−312 to −293[a]
FRd	exon 1	5′ flanking	CCTCAGCCTCGCGCAGCCCA	51−32[a]
E1up	5′ flanking	exon 1	ACTCAGCACTCGACCCAAAGGTGCA	−22 to 3[a]
E1d	intron 1	exon 1	CCACTCCCATCCCCAGGACGCGATG	14[b]
E2up	intron 1	exon 2	TGGCCCAGTTATTCAGTTTTGATTG	10[b]
E2d	intron 2	exon 2	GGGCCCTGTTCCAGTCCCTTCAGGC	10[b]
E3up	intron 2	exon 3	GCTTATCTTTGTGCTGTTCCTCTGC	9[b]
E3d	intron 3	exon 3	GAGCACAACCTCTCCCTGACCCATT	33[b]
E4up	intron 3	exon 4	TCTGCCTCCTGTGATGGCCTCTGTG	10[b]
E4d	intron 4	exon 4	GCTGATGCACAGACCTGGAGTCACC	39[b]
E5up	intron 4	exon 5	GCTCTTGGTCCTTGGAGATCATGAC	40[b]
E5d	intron 5	exon 5	ACTGGTTACGGTTGGGAGCTGGGGG	30[b]
E6up	intron 5	exon 6	TTCCTAGAATCGCCTCACCTGATCT	17[b]
E6d	intron 6	exon 6	TTCCCTCCCCACAAAGAGATCCTGT	27[b]
E7up	intron 6	exon 7−8	GCAGACTCCAGACATTCTTTTCCCT	4[b]
E7d	intron 7	exon 7−8	TGGAAGCTTTCAGGCTCAGAGAAG	1355−1332[a]
E8up	intron 7	exon 8	CCTTCTCTGAGCCTGAAAGCTTCC	1331−1354[a]
E8d	intron 8	exon 8	GTGGATTGTGTGTTTGCCATCCACT	28[b]
E9up	intron 8	exon 9	AGTGGATGGCAAACACACAATCCAC	28[b]
E9d	3′ flanking	exon 9	CCCAGCAAGGCGGAGACTCA	1639−1620[a]
FLup	exon 1	full cDNA	CCATGGCTGCGCTGGGCTGCG	20−40[a]
FLd	3′ flanking	full cDNA	CCCAGCAAGGCGGAGACTCAGC	1639−1618[a]
P357	exon 2	exon 2	GAGGGAAAGTACCCAGTACGG	382−402[a]
372up	exon 6	exon 6−8	TTTGCCCACATGCCGTTGCTCAA	1159−1181[a]
372d	exon 8	exon 6−8	TGGAAGCTTTCAGGCTCAGAGAAG	1355−1332[a]

[a] Nucleotide number at the cDNA reported by Cali and Russell (*10*). [b] Minimal distance from the exon.

Table 2: Clinical Manifestations in the Three CTX Patients[a]

	patient 3 in CTX 08	patient 2 in CTX 09	patient 6 in CTX 09
	Background Data		
sex	male	female	female
age (year)	47	45	42
age at onset	12	32	31
consanguinity	−	+	+
	Physical Findings		
tendon xanthomas	+++	++	++
cataracts	−	+	−
dementia	−	−	−
pyramidal signs	−	++	+
cerebellar signs	−	+	+

[a] +++ to − represents severe to absent.

of 150 cpm/pmol was synthesized as described previously and purified by HPLC (*24*). The substrate (480 000 cpm, 3.2 nmol) was dissolved in 10 μL of acetone (which was then evaporated under a stream of nitrogen) and solubilized in 250 μL of 5% bovine serum albumin, followed by addition of the following incubation mixture: 33 mM Hepes (pH 7.4), 5 mM ATP, 5 mM potassium malate, 1 mM glucose 6-phosphate, 0.5 IU of glucose-6-phosphate dehydrogenase, 1.2 mM NADPH, and 15 mM $MgCl_2$. The fibroblast pellet harvested from two dishes was suspended in 250 μL of 0.25 M sucrose and added to the incubation mixture to initiate the reaction, giving a final volume of 608 μL. After incubation at 37 °C for 1 h, the reaction was terminated by adding 0.1 mL of 1 M HCl. Extraction with 5 mL of ethyl acetate was performed twice, and the converted 27-hydroxylated product was detected by HPLC (LC-10A Shimazu, Kyoto, Japan) using a LC-18 column (250 × 4.6 mm, Supelco, Bellefonte, PA). The radioactivity of the product was measured using a radiodetector (RLC-700, Aloka, Tokyo, Japan).

Full-Length RT-PCR Amplification. To amplify the full-length cDNA of the sterol 27-hydroxylase gene, 1 μg of total RNA was first converted to cDNA in a 20 μL reaction mixture containing 5 mM $MgCl_2$, 1× PCR buffer II [10 mM Tris-HCl (pH 8.3) and 50 mM KCl], each dNTP at 1 mM, 1 unit of RNase inhibitor, 1 μM oligonucleotide dT, and 5 units of reverse transcriptase, using a RT-PCR kit (TaKaRa, Otsu, Japan). The reaction tube was incubated at 42 °C for 60 min, heated at 95 °C for 5 min, and then soaked at 5 °C for 5 min in a thermal cycler (Perkin-Elmer, GeneAmp PCR system 9600). PCR amplification was immediately performed after the RT reaction by adding 80 μL of a PCR Master Mix containing 1.25 mM $MgCl_2$, 1× PCR buffer II, 0.25 μM upstream primer FLup and downstream primer FLd (Table 1), 11.4 μL of DMSO, and 2.5 units of Taq DNA polymerase. The amplification reaction was performed for 30 or 20 cycles (for quantitative analysis), under the following conditions: 1.5 min at 95 °C for denaturation, 30 s at 68 °C for annealing, and 2 min (4 s increment every successive cycle) at 72 °C for extension. Electrophoresis was performed on a 2% agarose gel.

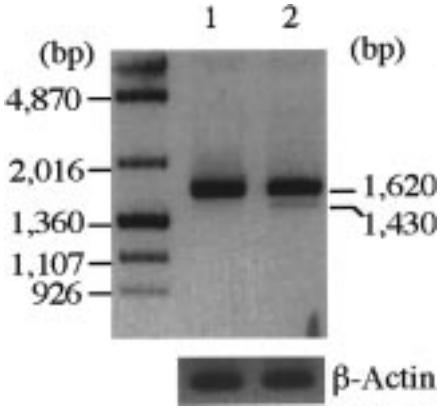

FIGURE 3: RT-PCR analysis of the sterol 27-hydroxylase mRNA in patient 3 from the CTX 08 family and a healthy subject. Full-length cDNA of the *CYP* 27 gene was amplified as described in Materials and Methods. β-Actin mRNA was simultaneously amplified and used for correction of quantitation. pHY DNA marker was used: lane 1, healthy subject; and lane 2, patient.

Quantitative Analysis of Transcript. Quantitative analysis of the mRNA was determined by Southern blotting of the full-length RT-PCR products. It has been demonstrated that mRNA quantitation by cDNA/PCR is possible in the exponential phase of amplification, usually before the 20th cycle of amplification (*25*). Four microliters of full-length RT-PCR products, amplified by 20 cycles from the patient and a healthy subject, was separated by electrophoresis on a 2% agarose gel. After alkaline denaturation, the DNA in the gel was transferred to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany). Prehybridization in standard buffer (5× SSC, 2% blocking reagent for nucleic acid hybridazation, 0.1% lauroylsarcosine, and 0.02% SDS) was carried out at 68 °C for 24 h. The membrane was then hybridized at 68 °C for 24 h with the full-length sterol 27-hydroxylase cDNA probe labeled with digoxigenin-11-dUTP by the random primed method according to the manufacturer's instructions (Boehringer Mannheim). Detection was performed with colorimetric detection reagents from Boehringer Mannheim. Four microliters of β-actin RT-PCR products amplified by 20 cycles was also separated using the same procedure, as a control. Quantita-

Table 3: Serum Cholesterol, Cholestanol Concentrations, and Fibroblast Sterol 27-Hydroxylase Activity in the CTX 08 and CTX 09 Families

	CTX 08			CTX 09						
	1	2	3	1	2	3	4	5	6	normal
cholesterol (mg/mL)	1.82	1.64	1.34	2.25	1.70	1.71	1.23	1.38	2.04	1.75 ± 0.42 (n = 17)
cholestanol (μg/mL)	3.12	3.44	27.01	1.60	9.51	4.60	1.53	1.7.1	10.90	2.71 ± 0.80 (n = 17)
ratio (%)[a]	0.17	0.21	2.02	0.07	0.56	0.27	0.12	0.12	0.53	0.16 ± 0.05 (n = 17)
enzyme activity [pmol of product (mg of protein)$^{-1}$ h]	20[b]	20[b]	UD[c]	104	UD	26	56	78	UD	110 ± 20 (n = 4)

[a] Cholestanol/cholesterol. [b] Enzyme activity in white blood cell (normal range, 96.2 ± 44.8, n = 13). [c] UD, undetectable.

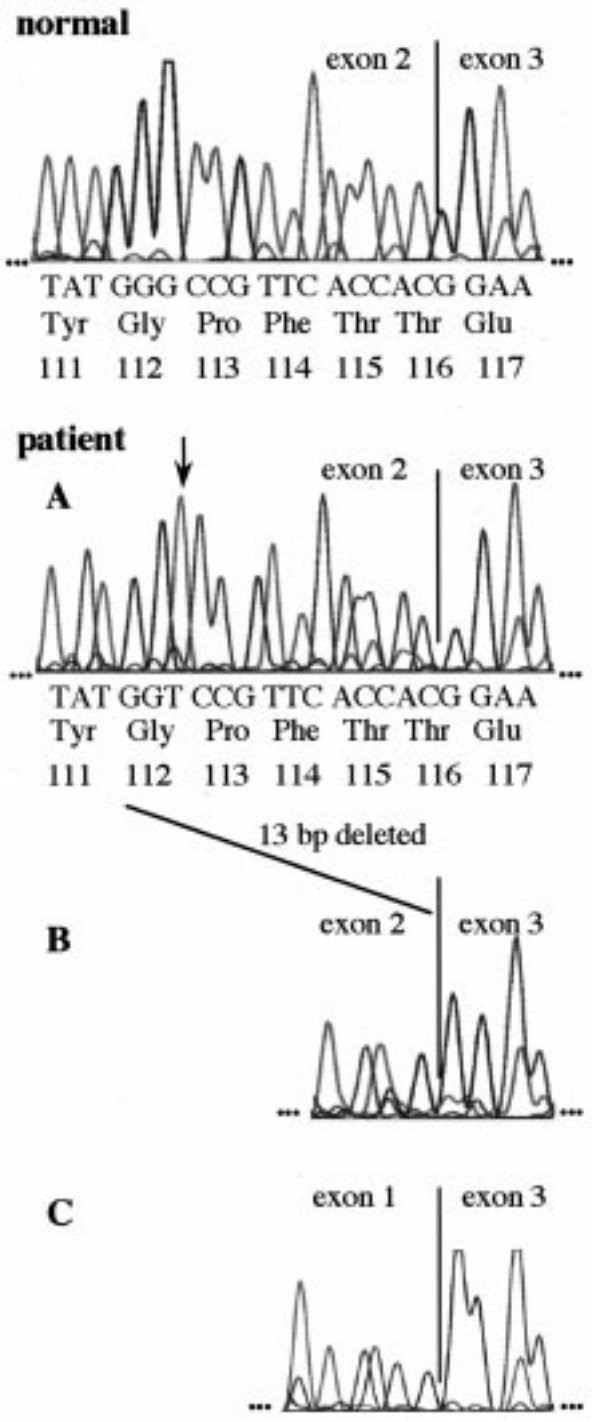

FIGURE 4: Sequence analysis of the subcloned full-length RT-PCR products. Full-length RT-PCR products amplified from the RNAs derived from patient 3 in the CTX 08 family and a healthy subject were subcloned into the pTARGET vector and sequenced. In the patient, three forms with different inserts (A−C) were detected.

tive analysis was carried out by normalizing the signals to that of the β-actin control using NIH Image, 1.61.

Genomic DNA PCR Amplification. To search for mutation in genomic DNA, the 5′-flanking region and all nine exons as well as the splicing junctions of the sterol 27-hydroxylase gene were amplified using primers listed in Table 1, with a PCR kit from TaKaRa. All PCR amplification reactions were performed for 30 cycles, under the following conditions: 1 min at 95 °C for denaturation and 4 min at 68 °C for annealing and extension.

Sequence Analysis. DNA sequence analysis was performed using the ABI PRISM 310 Genetical Analyzer, as described (*26*). Briefly, either the PCR product or the plasmid was first labeled by a Taq terminator sequencing reaction in a thermal cycler, under the following conditions: 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min for a total 25 cycles. After the excess unincorporated terminators were removed, using a Centri-Sep Spin column (ABI, Foster, CA), according to the manufacturer's protocol, the labeled products were dried, resuspended in 25 μL of Template Suppression Reaction, and heated to 92 °C for 2 min for denaturation. Then, the samples were loaded on an ABI PRISM 310 Genetical Analyzer for sequence analysis. All of the fluorescein sequencing reagents were purchased from ABI. All the samples were sequenced in both directions. The primers used for sequencing are listed in Table 1.

Construction of the Expression Vector. To observe effects of the identified silent G to T mutation on the pre-mRNA splicing, two minigene constructs designated pSPM (mutant) and pSPN (normal), consisting of exons 2 and 3 as well as truncated 209 bp intron 2, were generated from genomic DNA of a healthy subject and the patient (Figure 2). Due to large intron 2 (2.7 kb), the minigenes were set up by ligating two PCR fragments, exon 2−5′ intron 2 and 3′ intron 2−exon 3, using the *Eco*RI site. The exon 2−5′ intron 2 was amplified using primer pairs SPE2up1 (5′GCAATGTGGATGTCCTACTTAG3′) (nucleotides 300−322 in exon 2) and SPE2d1 (5′GAATTCTGCCGGTTATCCACCTG3′) (72−95 bp from the 5′ end of intron 2). The 3′ intron 2−exon 3 was amplified using primer pairs SPE2up2 (5′GAATTCCCTTGACTCTGGATGGAG3′) (94−114 bp from the 3′ end of intron 2) and SPE2d2 (5′CTAGTAGAAGAGTTGAGCCATGTC3′) (nucleotides 630−654 in exon 3). The PCR amplification reactions were performed for 30 cycles, under the following conditions: 1 min at 95 °C for denaturation and 4 min at 68 °C for annealing and extension. The ligated fragment contained a translation initiation codon ATG located at the primer SPE2up1, a stop codon TAG at the primer SPE2d2, the natural splice sites of exon 2−intron 2 and intron 2−exon 3, and a naturally conserved A residue usually located 30−40 nucleotides upstream of the 3′ splice site. The conserved A is necessary for the cleaved 5′ intron to form a branched lariat by the 5′−2′ phosphodiester bond (*27*). After confirmation of the sequences, the ligated PCR products were cloned into the pTARGET expression vector (Promega, Madison, WI) which contains the CMV immediate-early enhancer/promoter region, a chimeric intron for high-level expression of the insert, and the SV40 late polyadenylation signal. Plasmids of the minigene constructs were prepared using JETSTAR Plasmid Kit (Genomed, Research Triangle Park, NC). The two minigenes have the same structure, except for the G to T mutation, as confirmed by sequence analysis.

Transfection Analysis. COS-1 cells obtained from the JCRB Cell Bank (Tokyo, Japan) were maintained in DMEM containing 10% fetal calf serum and used for transfection. Twenty micrograms of plasmids of the normal and mutant minigene constructs was transfected in triplicate into 1×10^6 COS-1 cells by calcium phosphate coprecipitation. Forty-eight hours after transfection, total RNA was extracted by the acid guanidine/phenol/chloroform method and used

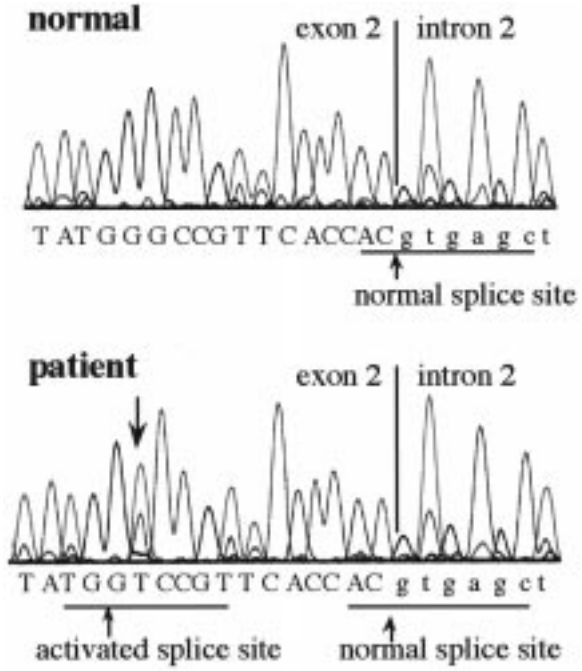

FIGURE 5: Genomic DNA sequence analysis of the sterol 27-hydroxylase gene in patient 3 from the CTX 08 family. The G to T mutation is indicated by an arrow. The normal splice site and the activated cryptic 5′ splice site are underlined.

for RT-PCR analysis using primers SPE2up1 and SPE2d2 to confirm the splicing patterns. Electrophoresis was performed using a 5% acrylamide gel.

Screening of Mutations by Restriction Endonuclease Analysis. The identified silent mutation of GGG ^{112}Gly to GGT ^{112}Gly abolished a normal *Hae*III restriction enzyme cleavage site of the sterol 27-hydroxylase gene. To screen the mutation, 8 μL of the PCR product amplified from genomic DNA using primers P357 and E2d (Table 1) was digested with 4 units of *Hae*III (TaKaRa) for 2 h at 37 °C. Another mutation of CGG ^{372}Arg to CAG ^{372}Gln reported by our group previously (*26*) eliminated a normal *Hap*II restriction site, and the mutation was screened by *Hap*II (TaKaRa) digestion of PCR products amplified from the genomic DNA using primers 372up and 372d (Table 1). The digested products were electrophoresed on a 12% polyacrylamide gel, and the fragments were confirmed by ethidium bromide staining of the gel.

RESULTS

Clinical Manifestations and Biochemical Analysis. Table 2 shows the clinical manifestations in the patients. Serum cholesterol and cholestanol concentrations and fibroblast sterol 27-hydroxylase activities of the patients and other family members are listed in Table 3. All three patients had elevated levels of serum cholestanol concentrations. Heterozygous subject 3 in the CTX 09 family also had an elevated serum cholestanol concentration of 4.06 μg/mL. No sterol 27-hydroxylase activities could be detected in fibroblasts from the three patients. The heterozygous members in the two families showed decreased enzyme activities. Subject 1 in the CTX 09 family was normal without mutation in either allele. He had a normal serum cholestanol concentration and fibroblast sterol 27-hydroxylase activity.

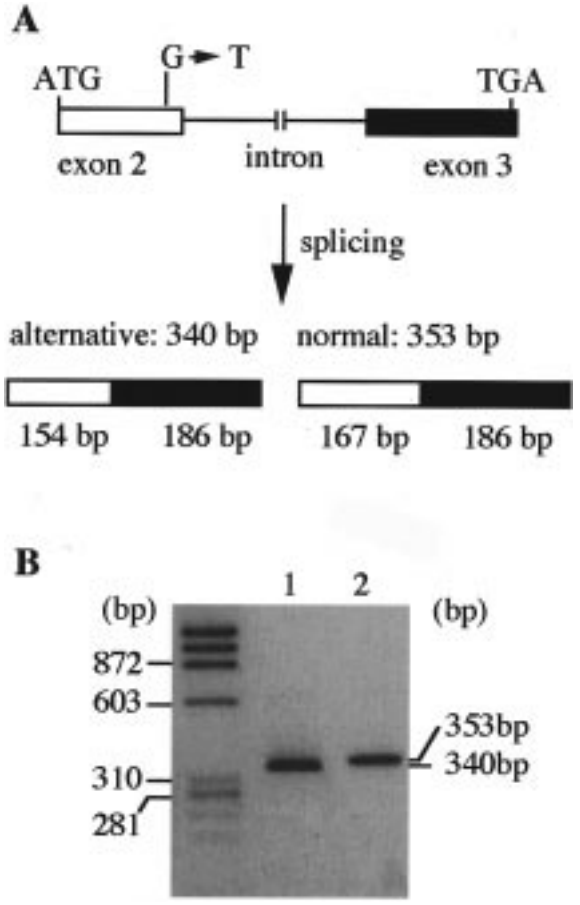

FIGURE 6: Transcription analysis of the minigene constructs in COS-1 cells. Minigenes, with or without the mutation, were transfected into COS-1 cells. RNA was extracted from the transfected cells, and RT-PCR was performed to detect the splicing pattern. (A) Scheme of the minigene constructs and the different splicing patterns. (B) RT-PCR analysis of RNAs extracted from the transfected COS cells. The øx174-*Hae*III DNA marker was used: lane 1, mutant minigene; and lane 2, normal minigene.

Full-Length RT-PCR Analysis. Full-length RT-PCR analysis of the *CYP* 27 gene in patient 3 from the CTX 08 family showed a major band in the normal 1620 bp region and a faint additional 1430 bp band (Figure 3). The total mRNA level in the patient, standardized with respect to the β-actin, represented 80.1 $\pm$ 3.1% of that in the healthy subject, as determined by Southern blotting of the RT-PCR products amplified by 20 cycles.

Sequence Analysis of the Full-Length RT-PCR Products. The full-length RT-PCR products of the patient and a healthy subject were subcloned into the pTARGET vector. Sequence analysis revealed three species of cloned cDNA present in the patient (Figure 4): (A) full-length cDNA with a functionally silent G to T mutation at codon 112 (GGG ^{112}Gly to GGT ^{112}Gly), (B) 13 bp from nucleotide 455 to 467 (3′ terminal 13 bp of exon 2) deleted cDNA, and (C) exon 2 skipped cDNA (190 bp from nucleotide 278 to 467). Out of the 36 colonies analyzed, species A accounted for 4 (4/36 = 11.1%), species B for 25 (25/36 = 69.5%), and species C for 7 (7/36 = 19.4%), giving a ratio of species A:B:C of 1:6.25:1.75. Thus, compared to that in the healthy subject, there was 8.9% (11.1% × 80.1%) of full-length cDNA with the silent mutation in the patient, which could be translated into a normal enzyme with activity. Species A with B corresponded to the major band observed in electrophoresis

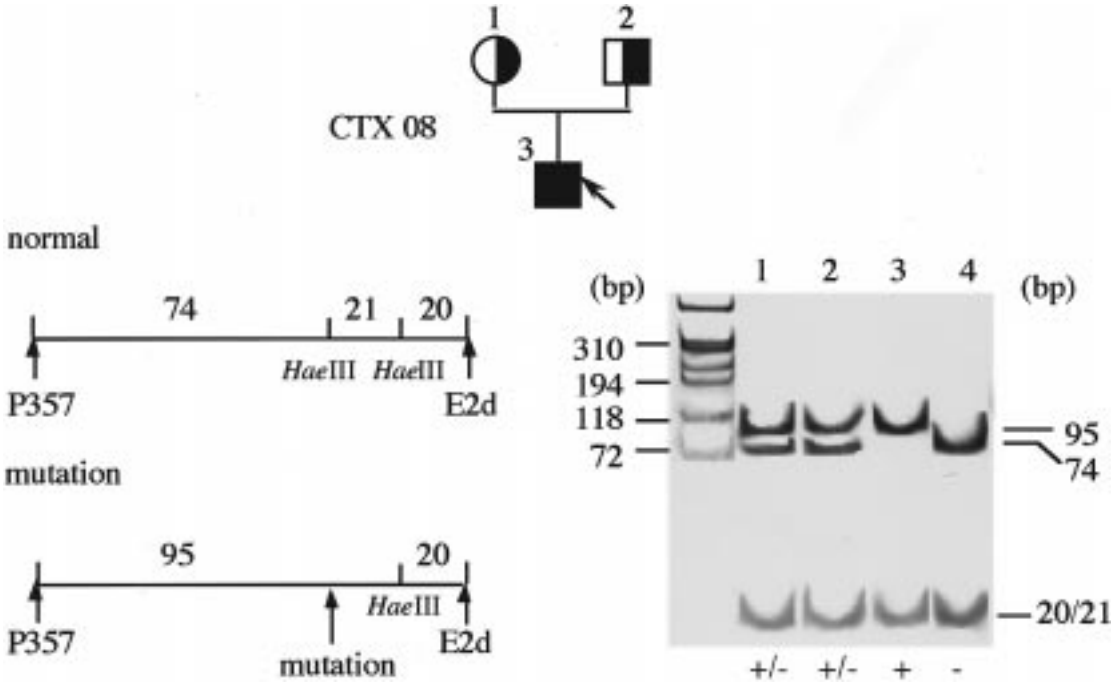

FIGURE 7: Screening of the GGG ^{112}Gly to GGT ^{112}Gln mutation in the CTX 08 family. The PCR product amplified from genomic DNA using primers P357 and E2d was digested with restriction enzyme *Hae*III followed by electrophoresis on a 12% acrylamide gel: lane 1, mother in the CTX 08 family; lane 2, father in the CTX 08 family; lane 3, patient in the CTX 08 family; and lane 4, normal sample. The øx174-*Hae*III DNA marker was used: +, homozygote; ±, heterozygote; and −, normal.

of the full-length RT-PCR product, and species C corresponded to the faint band (Figure 3). No abnormal cDNA clone was evident in the healthy subject (data not shown).

Sequence Analysis of Genomic DNA. To search for genomic mutation accounting for the mutant cDNAs observed in the patient, the 5′-flanking region and all nine exons as well as the splice junction regions of the gene were amplified using the primers listed in Table 1. Direct sequence analysis showed that, other than a single G to T mutation in codon 112 (nucleotide 457, in exon 2), no other nucleotide difference was detected between the patient and a healthy subject (Figure 5). The G to T mutation was functionally normal as both the mutant codon (GGT) and normal codon (GGG) encoded glycine. Using a comparison with the consensus splice site sequences AGGTAAGT, the nucleotides around the mutant codon 112 (TGGTCCGT) were assumed to be a cryptic 5′ splice site. Splicing at this activated cryptic site would produce 13 bp from nucleotide 455 to 467 (3′ terminal 13 bp of exon 2) deleted cDNA. To support this hypothesis, two minigenes were constructed and the following transfection experiment was conducted.

Tanscription Analysis of the Mutation in COS-1 Cells. To determine the role of the silent G to T mutation in the *CYP* 27 gene, two minigenes, with or without the mutation, were transfected into COS-1 cells and transcriptions were tested by RT-PCR analysis of the RNAs extracted from the transfected COS cells. In the normal minigene, a correctly spliced 353 bp species was observed. On the other hand, only a 340 bp abnormal splicing product was present in the mutant minigene without the normal 353 bp species (Figure 6). Direct sequence analysis confirmed that the abnormal 340 bp band lacked 13 bp of the 3′ terminal nucleotide of exon 2 (data not shown, see Figure 4B), the product of alternative splicing at the activated cryptic 5′ splice site around the mutant codon. Thus, the silent G to T mutation was apparently responsible for the alternative splicing by activating the cryptic 5′ splice site around the mutant 112 codon.

Screening of Mutations by Restriction Endonuclease Analysis. The identified silent mutation of GGG ^{112}Gly to GGT ^{112}Gly abolished a normal *Hae*III restriction site of the sterol 27-hydroxylase gene. By *Hae*III restriction endonuclease analysis, the patient in the CTX 08 family was confirmed to be homozygous with the silent G to T mutation. His parents were heterozygous with the mutation (Figure 7). This mutation was also detected in patients 2 and 6 from the CTX 09 family, in a heterozygous pattern (Figure 8A). The two patients also harbored another mutation of A for G substitution at codon 372 (CGG ^{372}Arg to CAG ^{372}Gln) (Figure 8B), a mutation reported previously by our group (*26*), which could be detected by *Hap*II restriction endonuclease analysis. The daughter (subject 3) in the CTX 09 family was a heterozygote with the G to A mutation at codon 372, and the two sons (subjects 4 and 5) were heterozygotes with the G to T silent mutation at codon 112 (Figure 8).

DISCUSSION

Most CTX cases reported heretofore were diagnosed on the basis of clinical manifestations and findings of increased serum cholestanol concentrations. The latter seemed more pertinent for the diagnosis. Here we found that heterozygous patient 3 in the CTX 09 family had an elevated serum cholestanol concentration of 4.6 μg/mL (Table 3). In the absence of a definite molecular analysis, she may have been diagnosed as a CTX patient. It is possible that the elevated cholestanol concentration may be related to her relatively low sterol 27-hydroxylase activity [26 pmol of product/(mg of protein)/h, Table 3]. It remains unknown why she had only about one-fourth of the normal enzyme activity level,

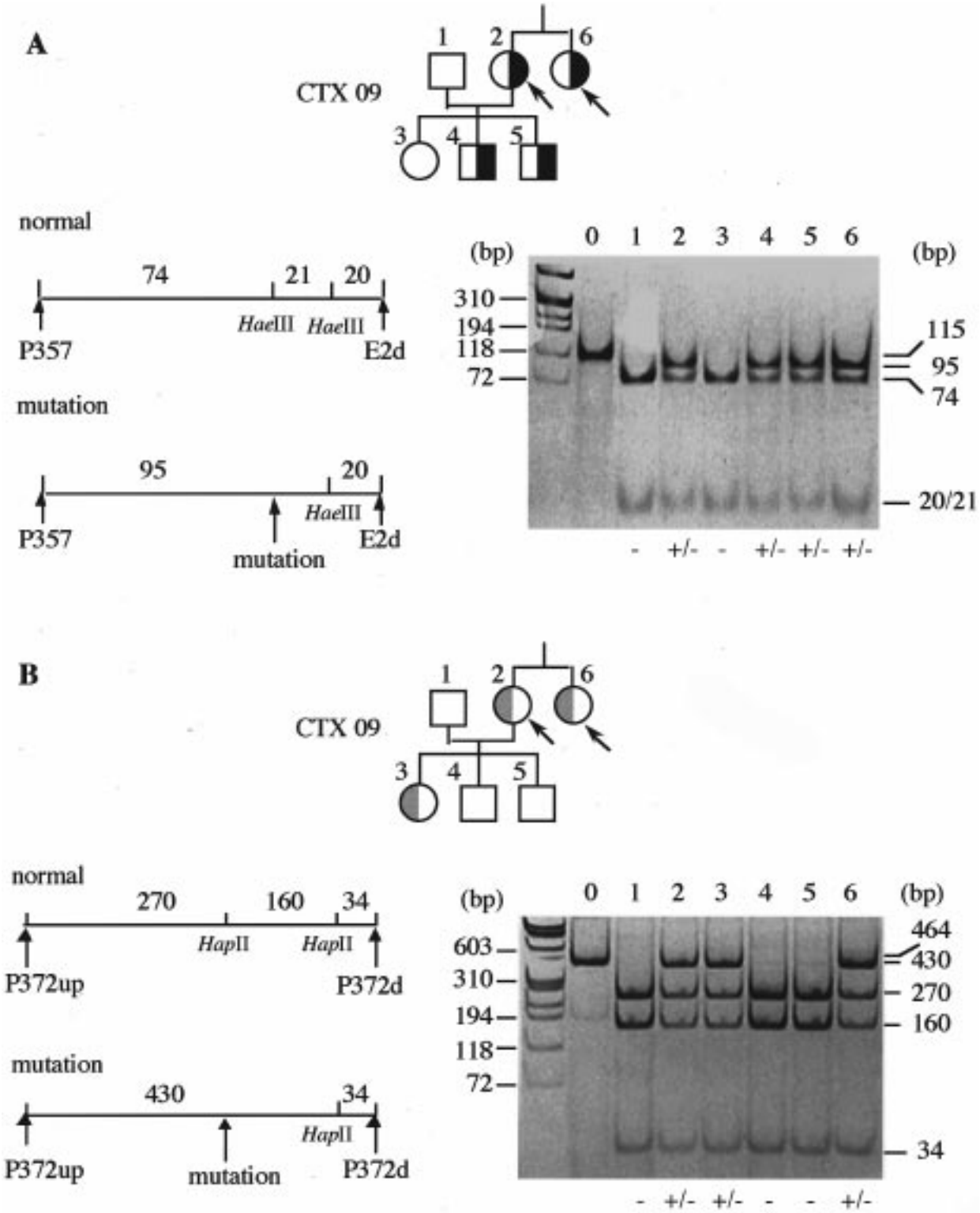

FIGURE 8: Screening of mutations in the CTX 09 family. (A) Screening of the GGG ^{112}Gly to GGT ^{112}Gly mutation. The PCR product amplified from genomic DNA using primers P357 and E2d was digested with restriction enzyme *Hae*III followed by electrophoresis on a 12% acrylamide gel: lane 0, normal sample without the *Hae*III digestion; and lanes 1−6, subjects 1−6 in the family as indicated in the pedigree structure. (B) Screening of the CGG ^{372}Arg to CAG ^{372}Gln mutation. The PCR product amplified from genomic DNA using primers 372up and 372d was digested with restriction enzyme *Hap*II followed by electrophoresis on a 12% acrylamide gel: lane 0, normal sample without the *Hap*II digestion; and lanes 1−6, subjects 1−6 in the family as indicated in the pedigree structure. The øx174-*Hae*III DNA marker was used: ±, heterozygote; and −, normal.

although it can be assumed that a heterozygote would have half the normal activity. The possibility that she may carry another mutation in another allele was ruled out by sequence analysis of genomic DNA (data not shown).

Of the three spliced mRNA species identified in the patient, the normal size one with the silent G to T mutation at codon 112 (Figure 4A), which does not change the glycine codon, accounted for 8.9% of the total transcript. This type

of mRNA species could be expected to be translated into normal sterol 27-hydroxylase with activity. Although this portion of activity could not be detected in fibroblasts of the patient, probably due to the sensitivity of the method or other factors, it indicates that the 8.9% of enzyme activity remaining in the tissue is not sufficient to prevent the accumulation of cholestanol in the serum and the appearance of CTX manifestations. Both the exon 2 skipped and 13 bp of exon 2 deleted species resulted in frame shift and created premature stop codons 79 and 34 bp downstream from the 5′ exon 3, respectively. This may explain the decrease of total *CYP* 27 gene mRNA (80.1% of the normal level) observed in the patient, as it is thought that the RNA molecule with a premature stop codon was unstable and degraded rapidly (*28*).

Four types of splice site mutations of the *CYP* 27 gene have heretofore been identified in CTX patients: two at the absolutely conserved intronic regions (*11*, *19*) and the other two at the exonic region, a G to C mutation at the last nucleotide of exon 3 (*19*) and a G to A mutation at the last nucleotide of exon 6, as we reported (*20*). The current mutation differs from those cases as it occurs at the coding region and does not alter the normal structure of the splice site, but does have a marked effect on splicing.

The mutation not only led to the 3′ terminal 13 bp of exon 2 deleted mRNA species (Figure 4B), by activating a cryptic 5′ splice site around the mutant codon in exon 2, but also caused exon 2 skipped species (Figure 4C), by interfering with the normal splicing at the end of exon 2. The splicing efficiency at the normal exon 2−intron 2 site was also influenced by the mutation, as out of the 36 colonies from the subcloned full-length RT-PCR product, only 4 were found to contain the full-length cDNA insert with the functionally silent G to T mutation at codon 112 (4/36 = 11.1%). It is unknown why the normal exon 2−intron 2 splice site was 88.9% blocked (11.1% was cleaved) while the cryptic splice site activated by the mutation was mostly used by the splicesome, although the structure of the exon 2−intron 2 splice site was not altered. Complementarity between the 5′ splice site and U1 snRNA has been shown to play an essential role in splicing processes (*29*, *30*). The normal 5′ splice site at the exon 2−intron 2 boundary of the *CYP* 27 gene (ACgtgagc) matches the U1 snRNA in 5 of 8 total positions and gives a Shapiro and Senapathy's score of 77.4 (*31*). The cryptic 5′ splice site activated by the mutation (TGGTCCGT) also matches the U1 snRNA in 5 of 8 total bases but shows a score of 70.6, a little lower than that of the normal exon 2−intron 2 site. It was reported that G is present at the −1 position of a 5′ splice site (corresponding to the last nucleotide of an exon) in 78% of the cases while C is present in only 4% (*31*). The −1 position (last nucleotide of exon 2) of the normal exon 2−intron 2 splice site is a C, while that position of the activated cryptic site is a G. Does this mean that G at the last nucleotide of exon in a 5′ splice site plays a more important role in splicing processes, by stabilizing the complementarity between the U1 snRNA and splice site, as compared to other bases in a splice site? Recently, we confirmed that a G to A mutation at the last nucleotide of exon 6 (−1 position of the normal exon 6−intron 6 splice site) of the *CYP* 27 gene resulted in alternative pre-mRNA splicing (*32*). On the other hand, a C to T mutation occurred at the penultimate nucleotide of the same exon (−2 position of the normal exon 6−intron 6 splice site) of the *CYP* 27 gene formerly reported by Cali et al. (*15*) showed no effect on splicing.

Three spliced mRNA species were observed in vivo in the patient (Figure 4). With the transfection of the mutant minigene in COS-1 cells, only the 13 bp deleted species was detected without the normally spliced one (Figure 6). The exon 2 skipped species could not be observed in the transfected COS-1 cells, as the minegene does not contain exon 1 and intron 1. The disappearance of normally spliced species in transfected COS-1 cells may be due to the different splicing conditions in COS-1 cells. It has been suggested that alternative splicing could be modulated by subtle cell-specific variations. Reed and Maniatis (*33*) observed that splice site selection can sometimes be affected by diluting the splicing extract. Similar findings were obtained by varying ionic conditions in the in vitro splicing reaction (*34*). Weil et al. (*35*) reported that temperature could effectively and specifically suppress the expression of a splicing defect.

By analyzing a silent nucleotide substitution of the *CYP* 27 gene, which does not change the amino acid codon, we show here that a functionally silent mutation can have a marked effect on pre-RNA splicing and lead to deficiency in enzyme activity. A rapid screening method has been developed to detect the mutation. The data elucidate a novel molecular mechanism for the CTX and suggest that the significance of a silent nucleotide mutation should not be ignored, especially when it occurs around a putative splice site.

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REFERENCES

1. Björkhem, I., and Boberg, K. M. (1995) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Ed.) pp 2073−2099, McGraw-Hill, New York.
2. Van Bogaert, L., Scherer, H. J., and Epstein, E. (1937) in *Une Forme Cerebrale De La Cholesterinose Generalisee*, pp 1−159, Masson, Paris.
3. Berginer, V. M., Shany, S., Alkalay, D., Berginer, J., Dekel, S., Salen, G., Tint, G. S., and Gazit, D. (1993) *Metabolism 42*, 69−74.
4. Fujiyama, J., Kuriyama, M., Arima, S., Shibata, Y., Nagata, K., Takenaga, S., Tanaka, H., and Osame, M. (1991) *Clin. Chim. Acta 200*, 1−11.
5. Philippart, M., and Van Bogaert, L. (1969) A follow-up study on the original family, *Arch. Neurol. 21*, 603−610.
6. Salen, G. (1971) *Ann. Intern. Med. 75*, 843−851.
7. Nakamura, T., Matsuzawa, Y., Takemura, K., Kubo, M., Miki, H., and Tarui, S. (1991) *Metabolism 40*, 741−746.
8. Berginer, V. M., Salen, G., and Shefer, S. (1984) *N. Engl. J. Med. 311*, 1649−1652.
9. Menkes, J. H., Schimschock, J. R., and Swanson, P. D. (1968) *Arch. Neurol. 19*, 47−53.
10. Cali, J. J., and Russell, D. W. (1991) *J. Biol. Chem. 266*, 7774−7778.
11. Leitersdorf, E., Reshef, A., Meiner, V., Levitzki, R., Schwartz, S. P., Dann, E. J., Berkman, N., Cali, J. J., Klapholz, L., and Berginer, V. M. (1993) *J. Clin. Invest. 91*, 2488−2496.
12. Segev, H., Reshef, A., Clavey, V., Delbart, C., Routier, G., and Leitersdorf, E. (1995) *Hum. Genet. 95*, 238−240.

13. Leitersdorf, E., Safadi, R., Meiner, V., Reshef, A., Björkhem, I., Friedlander, Y., Morkos, S., and Berginer, V. M. (1994) *Am. J. Hum. Genet. 55*, 907−915.
14. Garuti, R., Lelli, N., Barozzini, M., Dotti, M. T., Federico, A., Bertolini, S., and Calandra, S. (1996) *J. Lipid Res. 37*, 662−672.
15. Cali, J. J., Hsieh, C.-L., Francke, U., and Russell, D. W. (1991) *J. Biol. Chem. 266*, 7779−7783.
16. Kim, K.-S., Kubota, S., Kuriyama, M., Fujiyama, J., Björkhem, I., Eggertsen, G., and Seyama, Y. (1994) *J. Lipid Res. 35*, 1031−1039.
17. Reshef, A., Meiner, V., Berginer, V. M., and Leitersdorf, E. (1994) *J. Lipid Res. 35*, 478−483.
18. Meiner, V., Marais, D. A., Reshef, A., Björkhem, I., and Leitersdorf, E. (1994) *Hum. Mol. Genet. 3*, 193−194.
19. Garuti, R., Lelli, N., Barozzini, M., Tiozzo, R., Dotti, M. T., Federico, A., Ottomano, A. M., Croce, A., Bertolini, S., and Calandra, S. (1996) *J. Lipid Res. 37*, 1459−1467.
20. Chen, W., Kubota, S., Nishimura, Y., Nozaki, S., Amashita, S., Nakagawa, T., Kameda-Takemura, K., Menju, M., Matsuzawa, Y., Björkhem, I., Eggertsen, G., and Seyama, Y. (1996) *Biochim. Biophys. Acta 1317*, 119−126.
21. Watts, G. F., Mitchell, W. D., Bending, J. J., Reshef, A., and Leitersdorf, E. (1996) *Q. J. Med. 89*, 55−63.
22. Kasama, T., Byun, D. S., and Seyama, Y. (1987) *J. Chromatogr. 400*, 241−246.
23. Skrede, S., Björkhem, I., Kvittingen, E. A., Buchmann, M. S., Lie, S. O., East, C., and Grundy, S. (1986) *J. Clin. Invest. 78*, 729−735.
24. Björkhem, I., and Gustafsson, J. (1973) *Eur. J. Biochem. 36*, 201−212.
25. Chelly, J., Montarras, D., Pinset, C., Berwald-Netter, Y., Kaplan, J. C., and Kahn, A. (1990) *Eur. J. Biochem. 187*, 691−698.
26. Chen, W., Kubota, S., Kim, K.-S., Cheng, J., Kuriyama, M., Eggertsen, G., Björkhem, I., and Seyama, Y. (1997) *J. Lipid Res. 38*, 870−879.
27. Ruskin, B., Krainer, A. R., Maniatis, T., and Green, M. R. (1984) *Cell 38*, 317−331.
28. Maquat, L. E. (1995) *RNA 1*, 453−465.
29. Rio, D. C. (1993) *Curr. Opin. Genet. Dev. 3*, 574−584.
30. Mount, S. M., Pettersson, I., Hinter-berger, M., Karmas, A., and Steitz, J. A. (1983) *Cell 33*, 509−518.
31. Shapiro, M. B., and Senapathy, P. (1987) *Nucleic Acids Res. 15*, 7155−7175.
32. Chen, W., Kubota, S., and Seyama. Y. (1998) *J. Lipid Res.* (in press).
33. Reed, R., and Maniatis, T. (1986) *Cell 46*, 681−690.
34. Schmitt, P., Gattoni, R., Keohavong, P., and Stevenin, J. (1987) *Cell 50*, 31−39.
35. Weil, D., D'Alessio, M., Ramirez, F., Steinmann, B., Wirtz, M. K., Glanville, R. W., and Hollister, D. W. (1989) *J. Biol. Chem. 264*, 16804−16809.

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