Novel homozygous and compound heterozygous mutations of sterol 27-hydroxylase gene (*CYP*27) cause cerebrotendinous xanthomatosis in three Japanese patients from two unrelated families

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Abstract The autosomal recessively inherited cholesterol metabolic disease, cerebrotendinous xanthomatosis (CTX), is caused by mutations in the sterol 27-hydroxylase gene. Three Japanese CTX patients from two unrelated families were studied genetically. By DNA sequence analysis a novel mutation of A for G substitution at amino acid position 372 (CGG ^{372}Arg to CAG ^{372}Gln) was identified in one of the CTX families. The mutation was also found in two patients from the other family, with a compound heterozygous pattern of A for G substitution at amino acid position 441 (CGG ^{441}Arg to CAG ^{441}Gln). The latter mutation was the same as previously reported by our group (*J. Lipid Res.* 1994. **35:** 1031–1039). As the two mutations changed the restriction enzyme sites, rapid screening methods were developed for the detection of the carriers. Transfection of the two mutant cDNAs into COS cells resulted in markedly reduced sterol 27-hydroxylase activity. These results indicate that the two mutations are responsible for the deficiency of the sterol 27-hydroxylase activity in these patients. The features of mutations identified till now in Japanese CTX patients are also reviewed.—**Chen, W., S. Kubota, K-S. Kim, J. Cheng, M. Kuriyama, G. Eggertsen, I. Björkhem, and Y. Seyama.** Novel homozygous and compound heterozygous mutations of sterol 27-hydroxylase gene (*CYP*27) cause cerebrotendinous xanthomatosis in three Japanese patients from two unrelated families. *J. Lipid Res.* 1997. **38:** 870–879.

Supplementary key words cerebrotendinous xanthomatosis • sterol 27-hydroxylase gene • mutation • transfection

Cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessively inherited lipid storage disorder (1). It is characterized biochemically by abnormal deposition of cholesterol and cholestanol in multiple tissues (2, 3), and clinically by the manifestations of xanthomas (1), progressive neurological dysfunctions (4), cataracts (5), osteoporosis (6, 7) and premature atherosclerosis (8). The underlying defect of CTX is a deficiency of sterol 27-hydroxylase, a mitochondrial cytochrome P-450 enzyme (9–11). Together with two protein cofactors, adrenodoxin (12) and adrenodoxin reductase (13), the sterol 27-hydroxylase hydroxylates a variety of sterol substrates in the C_{27} position as well as vitamin D_3 in the C_1 and C_{25} positions (14, 15). In hepatic bile acid synthesis, the enzyme catalyzes the initial oxidation of side chain of sterol intermediates (16). The impairment of this capacity in CTX patients leads to excretion of large amounts of C_{27}-bile alcohols in bile, feces, and urine (17), as well as accumulation of cholestanol in different tissues. In addition, the defect of the sterol 27-hydroxylase in extrahepatic organs may be partly related to the premature atherosclerosis in CTX patients, as the enzyme seems to be involved in a defense mechanism for macrophages exposed to excess cholesterol (18).

Early diagnosis of CTX is crucial as treatment with chenodeoxycholic acid can reduce plasma cholestanol level and may prevent the disease progression (19) or even reverse some of the neurological disabilities (20). If untreated, CTX may insidiously develop serious neurological defects and may even lead to death (21). Molecular cloning of human sterol 27-hydroxylase cDNA by Cali and Russell (22) and determination of its gene

Abbreviations: CTX, cerebrotendinous xanthomatosis; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; RT-PCR, reverse transcription polymerase chain reaction; CPS, count per second.

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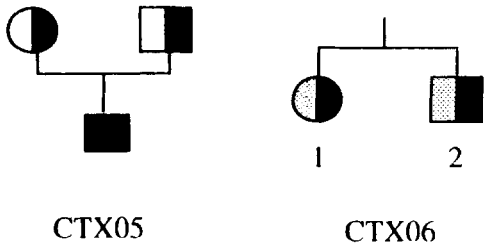

Fig. 1. Pedigree structure of CTX cases studied. ■ CTX05 (CGG ^{372}Arg to CAG ^{372}Gln homozygote); ◨ heterozygous father of CTX05; ◑ heterozygous mother of CTX05; ◑ CTX06-1 and ◨ CTX06-2 (compound heterozygotes of CGG ^{372}Arg to CAG ^{372}Gln and CGG ^{441}Arg to CAG ^{441}Gln mutations).

structure by Leitersdorf et al. (23) have made the genetic diagnosis of CTX possible and opened a new research field. So far, several mutations of the sterol 27-hydroxylase gene have been identified in CTX patients, including missense point mutation (24–31), insertion (32) and deletion (23, 33, 34), which lead to amino acid substitution, shifts in the reading frame, premature termination codon, and disruption of normal mRNA splicing process.

A relatively high prevalence of CTX has been noticed in Japan, compared to other countries (35). In the present study, we report a novel point mutation of the sterol 27-hydroxylase gene and a compound heterozygous mutation detected in three Japanese CTX patients from two families. The genetic characteristics of all the mutations identified till now in Japanese CTX patients are also reviewed.

MATERIALS AND METHODS

Subjects

Three Japanese CTX patients from two families were studied (**Fig. 1**). All the patients had typical CTX manifestations with elevated plasma cholestanol concentrations and undetectable sterol 27-hydroxylase activities. CTX05 had the manifestations of xanthomas, cataracts, and neurological dysfunctions. His parents were first cousins. The clinical symptoms of CTX06-1 and CTX06-2 were described in our previous paper (35) and the patients were designated as CTX-Y.K. and CTX-I.S., respectively. Informed consent was obtained from all the patients and their family members.

Fibroblast culture

Fibroblasts derived from the patients 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, Tokyo, Japan), penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (1.5 mM) in a 95% air and 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 (PBS), and then frozen at −80°C for DNA, RNA extraction, and the sterol 27-hydroxylase activity assay.

Assay of sterol 27-hydroxylase activity

Assay of sterol 27-hydroxylase activity was performed by a procedure described by Skrede et al. (36). Labeled substrate, 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol with specific activity 150 cpm/pmol was synthesized as described previously and purified by HPLC (37). The substrate (480,000 cpm, 3.2 nmol) was dissolved in 10 μl acetone (which was then evaporated under a nitrogen stream), and solubilized in 250 μl of 5% bovine serum albumin followed by the 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 glucose-6-phosphate dehydrogenase; 1.2 mM NADPH, and 15 mM $MgCl_2$. The mitochondrial pellet isolated from the fibroblasts was suspended in 250 μl of 0.25 M sucrose and added to the incubation mixture to start 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 1 M HCl. Extraction with 5 ml ethylacetate was performed twice and the converted 27-hydroxylated product was detected by HPLC (LC-10A Shimadzu, Kyoto, Japan) using a LC-18 column (250 × 4.6 mm, Supelco, Bellefonte, PA). The radioactivity of the product was measured by a radiodetector (RLC-700, Aloka, Tokyo, Japan).

PCR and RT-PCR amplification

The 5′-flanking region and 9 exons as well as the splicing-junctions of the sterol 27-hydroxylase gene were amplified respectively using the primers listed in **Table 1**. Genomic DNA (500 ng) extracted from the cultured fibroblasts was used for each of the amplification. The PCR reaction mixtures (100 μl) contained 1 × PCR buffer, 0.2 mM of each dNTP, 0.2 μM upstream and downstream primers listed above and 2.5 U Taq DNA polymerase using the PCR kit (TaKaRa, Japan). All the PCR amplification reactions were performed for 30 cycles in a thermal cycler (Perkin Elmer, GeneAmp PCR system 9600) using the following conditions: 1 min at 95°C for denaturation and 4 min at 68°C for annealing and extension. After DNA amplification, 8 μl of the products was electrophoresed on a 2% agarose gel and subjected to ethidium bromide staining to confirm the successful amplification. In order to confirm that the

TABLE 1. Sequence and location of primers in sterol 27-hydroxylase gene

Primer	Location	Amplification	Sequence 5′ to 3′	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	exon 8	exon 7–8	TGGAAGCTTTCAGGCTCAGAGAAG	1355–1332[a]
E8up	exon 8	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]
372up	exon 6	exon 6–8	TTTGCCCACATGCCGTTGCTCAA	1159–1181[a]
372d	exon 8	exon 6–8	TGGAAGCTTTCAGGCTCAGAGAAG	1355–1332[a]
FLup	exon 1	full-cDNA	CCATGGCTGCGCTGGGCTGCG	20–40[a]
FLd	3′-flanking	full-cDNA	CCCAGCAAGGCGGAGACTCAGC	1639–1618[a]

[a]Nucleotide number at the cDNA reported by Cali (ref. 22).
[b]Minimal distance from exon.

PCR reactions were free of contamination, water and DNase I-digested genomic DNA were used as no-template control and under amplification using the same conditions.

To amplify the full length cDNA of the sterol 27-hydroxylase, 1 μg total RNA, extracted from fibroblasts by acid guanidine-phenol-chloroform (38) was first converted to cDNA in 20 μl reaction mixture containing 5 mM $MgCl_2$, 1 × PCR buffer II (10 mM Tris-HCl, pH 8.3; 50 mM KCl), 1 mM of each dNTP, 1 U RNase inhibitor, 1 μM oligonucleotide dT, and 5 U reverse transcriptase using the RT-PCR kit (TaKaRa, Japan). The reaction tube was incubated at 42°C for 60 min (annealing and extension), heated at 95°C for 5 min (inactivation of reverse transcriptase and denaturation of RNA cDNA hybrids), and then soaked at 5°C for 5 min. 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), 11.4 μl DMSO, and 2.5 U Taq DNA polymerase. The amplification reaction was performed for 30 cycles using the following conditions: 1.5 min at 95°C for denaturation, 30 sec at 68°C for annealing and 2 min (4 sec increment every successive cycle) at 72°C for extension. After amplification, 8 μl of the RT-PCR product was electrophoresed on a 0.8% agarose gel and subjected to ethidium-bromide staining to confirm the successful amplification of 1620 bp full length cDNA. Amplifications were also conducted using water and RNase-digested RNA as no-template control to confirm the RT-PCRs were free of contaminations.

Sequence analysis

PCR direct sequence analyses were performed to detect mutation in the coding region, the 5′-flanking and splicing-junction regions of the gene. In order to confirm the compound heterozygous mutation detected, the PCR products of exon 7–exon 8 region of the CTX patients and a normal subject were further subcloned using TA-cloning system (Invitrogen, San Diego, CA) and the plasmids were prepared using QIAGEN Plasmid Mini Kit (Funakoshi, Japan). Taq terminator sequencing reaction was carried out using 200 ng plasmid and 3.2 pmol unlabeled primers. After an initial denaturation at 92°C for 2 min, 8 μl of Terminator Ready Reaction Mix was added, giving a final volume of 20 μl. The sequence PCR was performed for 25 cycles in a thermal cycler (Perkin Elmer, GeneAmp PCR system 9600) using the following conditions: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. After removing the excess unincorporated terminators by the Centri-Sep Spin column according to the manufacturer's protocol, the products were dried, resuspended in 25 μl Template Suppression Reaction and heated to 92°C for 2 min. Then, the samples were loaded on the ABI PRISM™ 310

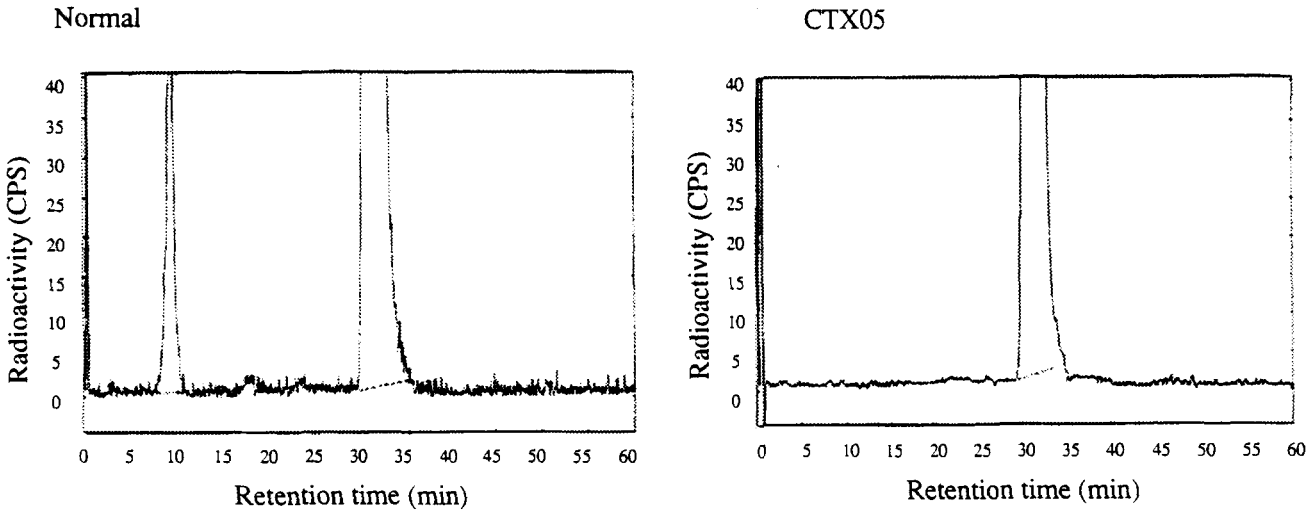

Fig. 2. Assay of sterol 27-hydroxylase activity. The 27-hydroxylated product 5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol was separated from the substrate by HPLC after incubation of 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol with fibroblasts derived from a normal subject (A), and the patient CTX05 (B). Peaks at 9 and 34 min of retention time showed the converted product and the substrate, respectively. No product peak was detected in the fibroblasts from the patient.

Genetical Analyzer for sequence analysis. All of the fluorescein sequencing reagents were purchased from Applied Biosystems (Foster, CA). All the samples were sequenced in both directions in order to confirm the identified mutations.

Screening of mutations by restriction endonuclease analysis

The mutation of CGG ^{372}Arg to CAG ^{372}Gln detected by sequence analysis abolished a normal *Hap*II restriction enzyme cleavage site of the sterol 27-hydroxylase gene. Eight μl of the PCR products amplified from genomic DNA by primers 372up and 372d (Table 1) was digested with 4 U of *Hap*II (TaKaRa, Japan) for 2 h at 37°C. Another mutation of CGG ^{441}Arg to CAG ^{441}Gln created a new *Stu*I restriction enzyme site and the PCR product of primer E8up and primer E8d was used for *Stu*I analysis. The digested products were electrophoresed on a 10% polyacrylamide gel and the fragments were confirmed by ethidium bromide staining of the gel.

Transfection analysis of CTX mutations

To determine the effects of the two identified mutations on the sterol 27-hydroxylase activity, transfection experiment was carried out. The normal and mutant cDNAs of the sterol 27-hydroxylase were ligated into pCAGGS expression vector using *Eco*R I site. The vector (a kind gift from Dr. Miyazaki, J., Osaka University, Japan) contains a ubiquitously strong promoter based on the beta-actin promoter, several unique restriction sites, a SV40 polyadenylation signal and the SV40 ori for transient expression (39). The nucleotide sequences of the inserted normal cDNA and mutant cDNAs were further confirmed by sequence analysis. Plasmids carrying normal cDNA and mutant cDNAs were prepared using QIAGEN Plasmid Kit, and further purified by CsCl ultracentrifuge method. COS-1 cells obtained from JCRB cell Bank (Tokyo, Japan) were maintained and used for transfection. Cells (1×10^{6}) were seeded in 100-mm dishes (5 dishes for each transfection) and cultured in DMEM containing 10% fetal calf serum for 24 h at 37°C before transfection. Then, the cells were transfected with 10 μg, 50 μg, and 100 μg purified pCAGGS vectors carrying normal cDNA or mutant cDNAs, using the method of calcium phosphate co-precipitation (40). Forty-eight hours after transfection, cells were harvested with 0.25% trypsin, washed with PBS, suspended in 2 ml of 0.25 M sucrose, 3 mM Tris (pH 7.4) and 0.1 mM EDTA, and subsequently sonicated for mitochondrial isolation. The isolated mitochondrial pellets were used for sterol 27-hydroxylase assay as described above.

RESULTS

Biochemical analyses

All three patients had elevated plasma cholestanol concentrations, with 37.8 μg/ml in CTX05, 27.2 μg/ml in CTX06-1, and 30.8 μg/l in CTX06-2. **Figure 2** shows detection of the sterol 27-hydroxylase activity in fibroblasts from a normal subject and patient (only the data

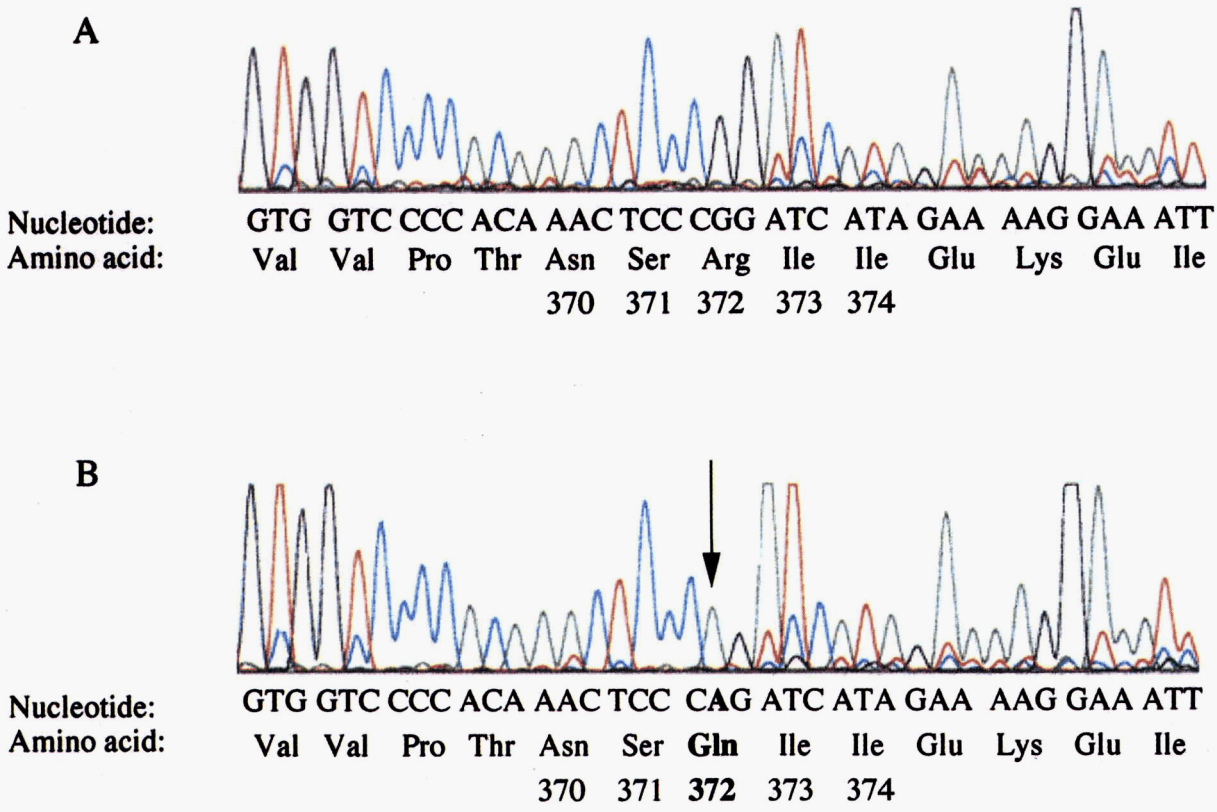

Fig. 3. Nucleotide sequence of the sterol 27-hydroxylase gene in a normal subject (A) and CTX05 (B). The normal sequences of the region across the mutation and their corresponding amino acids were shown. The A for G substitution was indicated by an arrow.

of CTX05 is shown as a typical example). Fibroblasts from the normal subject significantly converted the substrate 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (retention time, 34 min) into 27-hydroxylated product 5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol (retention time, 9 min). Fibroblasts from the patients showed undetectable product peak.

Sequence analysis

The coding region, the 5′-flanking and splicing-junction regions of the sterol 27-hydroxylase gene were sequenced completely as described in Materials and Methods. A single base pair substitution that converted arginine to glutamine at amino acid position 372 (CGG ^{372}Arg to CAG ^{372}Gln) was detected in patient CTX05 (**Fig. 3**). No additional nucleotide changes were found in other regions of the gene, including the 5′-flanking region and splicing-junctions. This mutation was also found in CTX06-1, with a compound heterozygous pattern of another Arg to Gln substitution mutation at amino acid 441, because out of the 12 clones sequenced, half had the CGG ^{372}Arg to CAG ^{372}Gln mutation with normal ^{441}Arg, and half had the CGG ^{441}Arg to CAG ^{441}Gln mutation with normal ^{372}Arg (**Fig. 4**). The same results were obtained from CTX06-2. The results suggested that CTX06-1 and CTX06-2 are compound heterozygotes of the CGG ^{372}Arg to CAG ^{372}Gln and CGG ^{441}Arg to CAG ^{441}Gln mutations. The compound heterozygotes were further confirmed by the restriction enzyme analysis as described below.

Screening for the mutations by restriction enzyme analysis

The CGG ^{372}Arg to CAG ^{372}Gln mutation eliminated a normal *Hap*II site of the sterol 27-hydroxylase gene. In a normal subject, digestion of the PCR product amplified from genomic DNA by the primers 372up and 372d (Table 1) with *Hap*II generated three fragments of 34, 167, and 270 bp (**Fig. 5,** lane 7). In CTX05, only two fragments (34 and 437 bp) were observed due to the loss of a *Hap*II site by the mutation (lane 2). The abnormal 437 bp was present in addition to the normal three fragments in the heterozygous father (lane 3) and mother (lane 4) of patient CTX05. CTX06-1 and CTX06-2 showed the same heterozygous patterns (lanes 5 and 6).

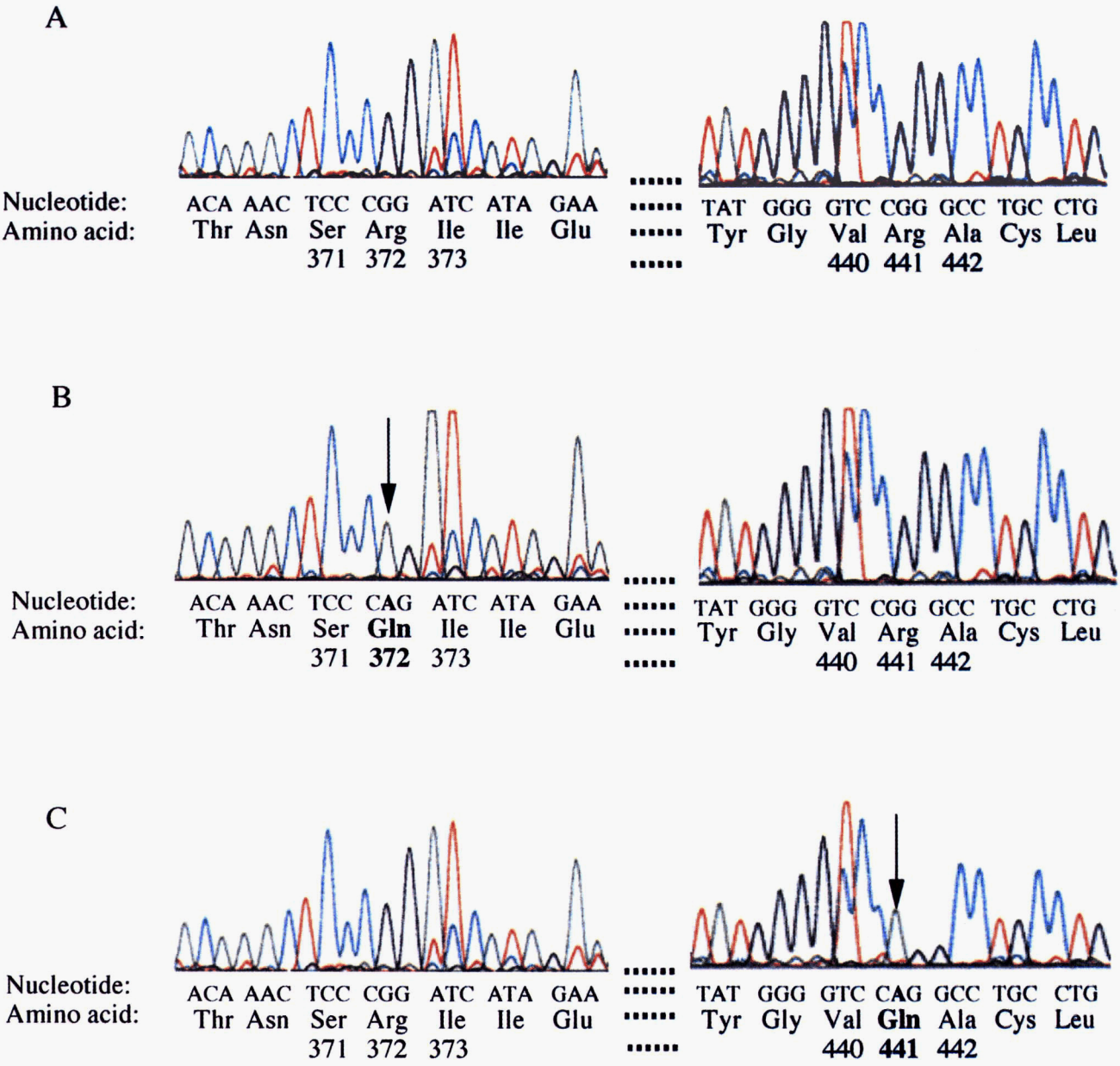

Fig. 4. Nucleotide sequence of the sterol 27-hydroxylase gene in a normal subject (A) and CTX06-1 (B, C). The A for G substitutions at amino acid positions 372 and 441 were indicated by arrows, respectively. B: sequence of a clone that carried mutation at amino acid 372 with normal 441 sequence; C: sequence of a clone that carried mutation at 441 with normal 372 sequence.

Digestion of the PCR product amplified by primers E8up and E8d with *Stu*I did not change the length (293 bp) in normal subject (**Fig. 6,** lane 8). In the homozygous patient with CGG ^{441}Arg to CAG ^{441}Gln mutation (our previously reported case), the 293 bp fragment was replaced by two fragments of 110 and 183 bp (lane 7) resulted from the created *Stu*I site by the mutation. Patient CTX05 (lane 2) and his parents (lanes 3 and 4) showed normal patterns while patients CTX06-1 and CTX06-2 showed 110 and 183 bp bands in addition to the 293 bp band (lanes 5 and 6), which, combined with the results of *Hap*II analysis, indicated that both of the mutations existed in the two patients.

Transfection analysis

In order to check the effects of the observed amino acid substitutions on the sterol 27-hydroxylase activity, each of the mutations was analyzed by transfection of the mutant cDNAs into COS cells. As shown in **Fig. 7,** transfection with a vector carrying the normal cDNA

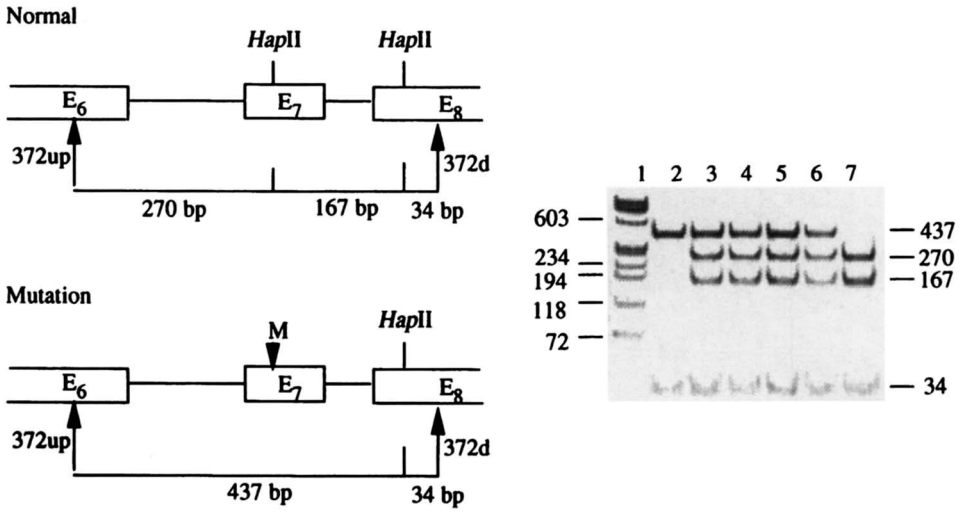

Fig. 5. Screening of the CGG [372]Arg to CAG[372] Gln mutation. The PCR product amplified from genomic DNA using primers 372up and 372d was digested with restriction enzyme *Hap*II. Lane 1: ØX174 *Hae*III DNA marker, lane 2: CTX05, lane 3: father of CTX05, lane 4: mother of CTX05, lane 5: CTX06-1, lane 6: CTX06-2, lane 7: normal sample.

(10 μg, 50 μg, and 100 μg) led to 3.03%, 8.10%, and 8.40% conversion of substrate 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol into 27-hydroxylated product 5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol, respectively (refer to Fig. 2). In contrast, transfection with the cDNA (10 μg, 50 μg, and 100 μg) carrying CGG [372]Arg to CAG [372]Gln mutation led to 0.26%, 0.48%, and 1.38% conversion, respectively. Transfection with CGG [441]Arg to CAG [441]Gln mutant cDNA (10 μg, 50 μg, and 100 μg) resulted in 0.49%, 1.30%, and 1.35% conversion, respectively. Mock transfection with the vector alone (10 μg and 50 μg) resulted in 1.27% and 1.78% conversion, respectively. The results suggested that both of the mutations are responsible for the reduced enzyme activity.

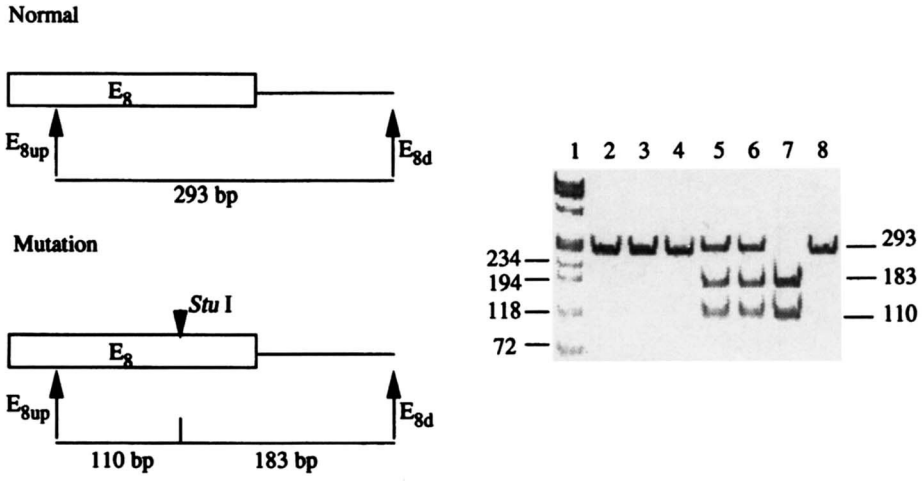

Fig. 6. Screening of the CGG [441]Arg to CAG[441] Gln mutation. The PCR product amplified from genomic DNA using primers E8up and E8d was digested with restriction enzyme *Stu*I. Lane 1: ØX174 *Hae*III DNA marker, Lane 2: CTX05, lane 3: father of CTX05, lane 4: mother of CTX05, lane 5: CTX06-1, lane 6: CTX06-2, lane 7: homozygous mutation sample, lane 8: normal sample.

DISCUSSION

By DNA sequence analysis a novel missense point mutation of A for G substitution at amino acid position 372 (CGG ^{372}Arg to CAG ^{372}Gln) was identified in the CTX05 family, with a homozygous pattern in the patient and heterozygous pattern in his parents, which strongly indicated that CTX is inherited in an autosomal recessive trait. This type of mutation was also found in CTX06-1 and CTX06-2 from the other family, with a compound heterozygous pattern of A for G substitution at amino acid position 441 (CGG ^{441}Arg to CAG ^{441}Gln). The latter was the same mutation reported by our group previously (26). The CGG ^{372}Arg to CAG ^{372}Gln mutation was 10 amino acids away from a highly conserved ^{362}Arg of cytochrome P-450s, which serves as a ligand for the adrenodoxin cofactor (41). Another Arg to Gln mutation occurred at codon 441, only 2 amino acids ahead of ^{443}Cys residue, the putative heme binding region of the enzyme (42). Thus, both the mutations were located near the two functional domains of the sterol 27-hydroxylase enzyme and would be expected to affect the activity of the enzyme. This expectation was confirmed by the transfection analysis as both of the mutant cDNAs synthesized sterol 27-hydroxylases with markedly reduced enzyme activity as shown in Fig. 7.

Japanese CTX patients accounts for nearly one third of all the CTX cases reported in the world till now (35).

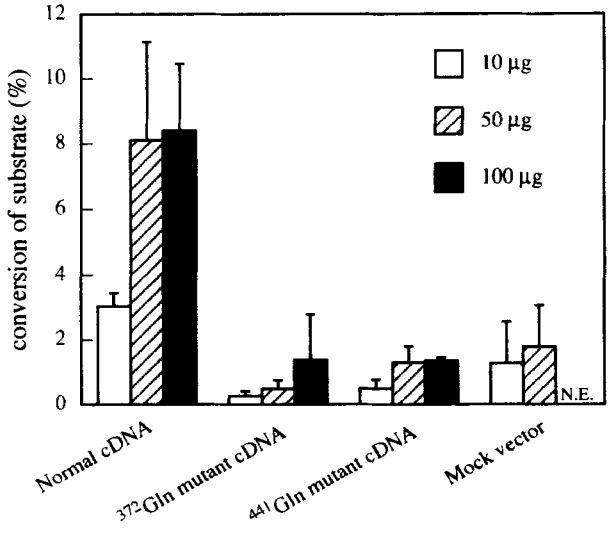

Fig. 7. Expression of normal and CTX sterol 27-hydroxylase cDNAs. COS cells were transfected with the indicated cDNA in the pCAGGS vector by calcium phosphate co-precipitation method. Forty-eight hours after transfection, the cells were harvested and the isolated mitochondria were used for the assay of sterol 27-hydroxylase activity by the method as outlined under Materials and Methods. The results shown are representative of two separate transfection experiments. N.E.: not examined.

So far, six types of mutation have been reported in 14 Japanese CTX patients from eight unrelated families (including those reported here). Within the six types of mutation (four were reported by our group), the CGG ^{441}Arg to CAG ^{441}Gln mutation was detected in six individuals. Two out of six were homozygotes and four were compound heterozygotes with other types of mutation, giving a case frequency of 42.9% (6/14) and an allele frequency of 28.6% (8/28). Another ^{441}Arg mutation (CGG ^{441}Arg to TGG ^{441}Trp), previously reported by our group (26), has recently also been found in Japanese triplets with CTX (43), making four cases harboring this mutation and giving a case frequency of 28.6% (4/14), and an allele frequency of 28.6% (8/28). The CGG ^{372}Arg to CAG ^{372}Gln mutation reported here appeared in three cases (one homozygote and two compound heterozygotes with CGG ^{441}Arg to CAG ^{441}Gln; case frequency 21.4%; allele frequency 14.3%). The remaining three types of mutation were CGG ^{104}Arg to TGG ^{104}Gln (two homozygous cases) (25), CGT ^{362}Arg to CAG ^{362}His (one homozygous case) (31) and CCC ^{368}Pro to CGC ^{368}Arg (two compound heterozygous cases with CGG ^{441}Arg to CAG ^{441}Gln mutation) (30). The ^{441}Arg codon mutations (CGG ^{441}Arg to CAG ^{441}Gln and CGG ^{441}Arg to TGG ^{441}Trp) seemed to be prominent in Japanese CTX patients. Whether there existed a selective advantage of these mutations in Japan remains unknown.

Of the six types of mutation, five were located at or near the two cofactor binding regions. The disruption effect of these mutations on the enzyme activity is likely to be due to disability to bind the cofactors, although the hypothesis was not confirmed directly. The one remaining mutation was located at amino acid position 104 (25), a codon rather apart from the cofactor binding regions. It seems possible that this naturally occurring mutation might provide insights into the active center of the sterol 27-hydroxylase. Of the six types of mutation, five occurred in dinucleotides considered to be hypermutable in the human genome (44). It is interesting to note that all of the six types of mutation identified in Japanese patients are missense point mutations, which do not affect the splicing of mRNA and are able to synthesize enzyme without activity, just like the cases reported here. Other types of mutation, such as splice-junction mutation (23, 29), deletion (23, 33, 34), and insertion (32) have been reported in other ethnic groups such as Jews or Italian. These types of mutation disrupted normal mRNA splicing and synthesis of normal structure protein.

No obvious relationship seems to exist between the genotype and phenotype among the 14 cases. Other factors in addition to genetics, such as individual differences and environmental factors, may contribute to the clinical manifestations of CTX. More general conclu-

sions must await accumulation of a larger number of cases with defined mutations.

In conclusion, a novel mutation of CGG ^{372}Arg to CAG ^{372}Gln was identified in Japanese CTX patients, either existing as a homozygous pattern or a compound heterozygous pattern with CGG ^{441}Arg to CAG ^{441}Gln mutation. Transfection of the two mutant cDNA into COS cells confirmed that both of the mutations are responsible for the disruption of the sterol 27-hydroxylase activity.

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REFERENCES

1. Björkhem, I., and K. Muri-Boberg. 1994. Inborn error in bile acid biosynthesis and storage of sterols other than cholesterol. *In* The Metabolic Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, New York. 2027–2100.
2. Philippart, M., and L. Van Bogaert. 1969. Cholestanolosis (cerebrotendinous xanthomatosis). A follow-up study on the original family. *Arch. Neurol.* **21:** 603–610.
3. Salen, G. 1971. Cholestanol deposition in cerebrotendinous xanthomatosis. A possible mechanism. *Ann. Intern. Med.* **75:** 843–851.
4. Dotti, M. T., G. Salen, and A. Federico. 1991. Cerebrotendinous xanthomatosis as a multisystem disease mimicking premature aging. *Dev. Neurosci.* **13:** 371–376.
5. Morgan, S. J., P. Mckenna, and R. C. Bosanquet. 1989. Case of cerebrotendinous xanthomatosis. I: Unusual ophthalmic features. *Br. J. Ophthalmol.* **73:** 1011–1014.
6. Federico, A., M. T. Dotti, F. Lore, and R. Nuti. 1993. Cerebrotendinous xanthomatosis: pathophysiological study on bone metabolism. *J. Neurol. Sci.* **115:** 67–70.
7. Berginer, V. M., S. Shany, D. Alkalay, J. Berginer, S. Dekel, G. Salen, G. S. Tint, and D. Gazit. 1993. Osteoporosis and increased bone fractures in cerebrotendinous xanthomatosis. *Metabolism.* **42:** 69–74.
8. Fujiyama, J., M. Kuriyama, S. Arima, Y. Shibata, K. Nagata, S. Takenaga, H. Tanaka, and M. Osame. 1991. Atherogenic risk factors in cerebrotendinous xanthomatosis. *Clin. Chim. Acta.* **200:** 1–11.
9. Oftebro, H., I. Björkhem, S. Skrede, A. Schreiner, and J. I. Pedersen. 1980. Cerebrotendinous xanthomatosis: a defect in mitochondrial 26-hydroxylation required for normal biosynthesis of cholic acid. *J. Clin. Invest.* **65:** 1418–1430.
10. Björkhem, I., O. Fausa, G. Hopen, H. Oftebro, J. I. Pedersen, and S. Skrede. 1983. Role of the 26-hydroxylase in the biosynthesis of bile acids in the normal state and in cerebrotendinous xanthomatosis. An in vivo study. *J. Clin. Invest.* **71:** 142–148.
11. Björkhem, I., H. Oftebro, S. Skrede, and J. I. Pedersen. 1981. Assay of intermediates in bile acid biosynthesis using isotope dilution mass spectrometry; hepatic levels in the normal state and in CTX. *J. Lipid Res.* **22:** 191–200.
12. Okamura, T., M. E. Jone, M. X. Zuber, E. R. Simpson, and M. R. Waterman. 1985. Molecular cloning and amino acid sequence of the precursor form of bovine adrenodoxin: evidence for a previously unidentified COOH-terminal peptide. *Proc. Natl. Acad. Sci. USA.* **82:** 5705–5709.
13. Hanukoglu, I., and T. Gutfinger. 1989. cDNA sequence of adrenodoxin reductase. Identification of NADP-binding sites in oxidoreductase. *Eur. J. Biochem.* **180:** 479–484.
14. Wikvall, K. 1984. Hydroxylations in biosynthesis of bile acids. Isolation of a cytochrome P-450 from rabbit liver mitochondria catalyzing 26-hydroxylation of C27-steroids. *J. Biol. Chem.* **259:** 3800–3804.
15. Dahlback, H., and K. Wikvall. 1988. 25-Hydroxylation of vitamin-D_3 by a cytochrome P-450 from rabbit liver mitochondria. *Biochem. J.* **252:** 207–213.
16. Russell, D. W., and K. D. Setchell. 1992. Bile acid biosynthesis. *Biochemistry.* **31:** 4737–4749.
17. Setoguchi, T., G. Salen, G. S. Tint, and E. H. Mosbach. 1974. A biochemical abnormality in cerebrotendinous xanthomatosis: impairment of bile acid biosynthesis associated with incomplete degradation of the cholesterol side chain. *J. Clin. Invest.* **53:** 1393–1401.
18. Björkhem, I., O. Anderson, U. Diczfalusy, B. Sevastik, R. J. Xiu, C. Duan, and E. Lund. 1994. Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proc. Natl. Acad. Sci. USA.* **91:** 8592–8596.
19. Nakamura, T., Y. Matsuzawa, K. Takemura, M. Kubo, H. Miki, and S. Tarui. 1991. Combined treatment with chenodeoxycholic acid and pravastatin improves plasma cholestanol levels associated with marked regression of tendon xanthomas in cerebrotendinous xanthomatosis. *Metabolism.* **40:** 741–746.
20. Berginer, V. M., G. Salen, and S. Shefer. 1984. Long-term treatment of cerebrotendinous xanthomatosis with chenodeoxycholic acid. *N. Engl. J. Med.* **311:** 1649–1652.
21. Menkes, J. H., J. R. Schimschock, and P. D. Swanson. 1968. Cerebrotendinous xanthomatosis: the storage of cholestanol within the nervous system. *Arch. Neurol.* **19:** 47–53.
22. Cali, J. J., and D. W. Russell. 1991. Characterization of human sterol 27-hydroxylase: a mitochondrial cytochrome P-450 that catalyzes multiple oxidation reactions in bile acid biosynthesis. *J. Biol. Chem.* **266:** 7774–7778.
23. Leitersdorf, E., A. Reshef, V. Meiner, R. Levitzki, S. P. Schwartz, E. J. Dann, N. Berkman, J. J. Cali, L. Klapholz, and V. M. Berginer. 1993. Frameshift and splice-junction mutations in the sterol 27-hydroxylase gene cause cerebrotendinous xanthomatosis in Jews of Moroccan origin. *J. Clin. Invest.* **91:** 2488–2496.
24. Cali, J. J., C-L. Hsieh, U. Francke, and D. W. Russell. 1991. Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. *J. Biol. Chem.* **266:** 7779–7783.
25. Nakashima, N., Y. Sakai, H. Sakai, T. Yanase, M. Haji, F. Umeda, S. Koga, T. Hoshita, and H. Nawata. 1994. A point mutation in the bile acid biosynthetic enzyme sterol 27-hydroxylase in a family with cerebrotendinous xanthomatosis. *J. Lipid Res.* **35:** 663–668.
26. Kim, K-S., S. Kubota, M. Kuriyama, J. Fujiyama, I. Björkhem, G. Eggertsen, and Y. Seyama. 1994. Identification of new mutations in sterol 27-hydroxylase gene in Japanese patients with cerebrotendinous xanthomatosis (CTX). *J. Lipid Res.* **35:** 1031–1039.

27. Reshef, A., V. Meiner, V. M. Berginer, and E. Leitersdorf. 1994. Molecular genetics of cerebrotendinous xanthomatosis in Jews of North African origin. *J. Lipid Res.* **35:** 478–483.
28. Meiner, V., D. A. Marais, A. Reshef, I. Björkhem, and E. Leitersdorf. 1994. Premature termination codon at the sterol 27-hydroxylase gene causes cerebrotendinous xanthomatosis in an Afrikaner family. *Hum. Mol. Genet.* **3:** 193–194.
29. Garuti, R., N. Lelli, M. Barozzini, R. Tiozzo, M. T. Dotti, A. Federico, A. M. Ottomano, A. Croce, S. Bertolini, and S. Calandra. 1996. Cerebrotendinous xanthomatosis caused by new mutations of the sterol 27-hydroxylase gene that disrupt mRNA splicing. *J. Lipid Res.* **37:** 1459–1467.
30. Okuyama, E., S. Tomita, H. Takeuchi, and Y. Ichikawa. 1996. A novel mutation in the cytochrome $P450_{27}$ (*CYP*27) gene caused cerebrotendinous xanthomatosis in a Japanese family. *J. Lipid Res.* **37:** 631–639.
31. Chen, W., S. Kubota, Y. Nishimura, S. Nozaki, S. Amashita, T. Nakagawa, K, Takamura, M. Menju, Y. Matsuzawa, I. Björkhem, G. Eggertsen, and Y. Seyama. 1996. Genetic analysis of a Japanese cerebrotendinous xanthomatosis family: identification of a novel mutation in the adrenodoxin-binding region of the CYP27 gene. *Biochim. Biophys. Acta.* **1317:** 119–126.
32. Segev, H., A. Reshef, V. Clavey, C. Delbart, G. Routier, and E. Leitersdorf. 1995. Premature termination codon at the sterol 27-hydroxylase gene causes cerebrotendinous xanthomatosis in a French family. *Hum. Genet.* **95:** 238–240.
33. Leitersdorf, E., R. Safadi, V. Meiner, A. Reshef, I. Björkhem, Y. Friedlander, S. Morkos, and V. M. Berginer. 1994. Cerebrotendinous xanthomatosis in the Israeli Druze: molecular genetics and phenotypic characteristics. *Am. J. Hum. Genet.* **55:** 907–915.
34. Garuti, R., N. Lelli, M. Barozzini, M. T. Dotti, A. Federico, S. Bertolini, and S. Calandra. 1996. Partial deletion of the gene encoding sterol 27-hydroxylase in a subject with cerebrotendinous xanthomatosis. *J. Lipid Res.* **37:** 662–672.
35. Kuriyama, M., J. Fujiyama, H. Yoshidome, S. Takenaga, K. Matsumuro, T. Kasama, K. Fukuda, T. Kuramoto, Y. Hoshita, Y. Seyama, Y. Okuda, and M. Osame. 1991. Cerebrotendinous xanthomatosis: clinical and biochemical evaluation of eight patients and review of the literature. *J. Neurol. Sci.* **102:** 225–232.
36. Skrede, S., I. Björkhem, E. A. Kvittingen, M. S. Buchmann, S. O. Lie, C. East, and S. Grundy. 1986. Demonstration of 26-hydroxylation of C27-steroids in human skin fibroblasts, and a deficiency of this activity in cerebrotendinous xanthomatosis. *J. Clin. Invest.* **78:** 729–735.
37. Björkhem, I., and J. Gustafsson. 1973. ω-Hydroxylation of steroid side-chain in biosynthesis of bile acids. *Eur. J. Biochem.* **36:** 201–212.
38. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
39. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene.* **108:** 193–199.
40. Andersson, S., D. L. Davis, H. Dahlback, H. Jornvall, and D. W. Russell. 1989. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264:** 8222–8229.
41. Tuls, J., L. Geren, and F. Millett. 1989. Fluorescein isothiocyanate specifically modifies lysine 338 of cytochrome P-450scc and inhibits adrenodoxin binding. *J. Biol. Chem.* **26:** 16421–16425.
42. Gonzales, F. J. 1989. The molecular biology of cytochrome P450s. *Pharmacol. Rev.* **40:** 243–288.
43. Nagai, Y., M. Hirano, T. Mori, Y. Takakura, S. Tamai, and S. Ueno. 1996. Japanese triplets with cerebrotendinous xanthomatosis are homozygous for a mutant gene coding for the sterol 27-hydroxylase (Arg441Trp). *Neurology.* **46:** 571–574.
44. Cooper, D. N., and H. Youssoufian. 1988. The CpG dinucleotide and human genetic disease. *Hum. Genet.* **78:** 151–155.