Frameshift and Splice-Junction Mutations in the Sterol 27-Hydroxylase Gene Cause Cerebrotendinous Xanthomatosis in Jews of Moroccan Origin

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Abstract

The sterol 27-hydroxylase (EC 1.14.13.15) catalyzes steps in the oxidation of sterol intermediates that form bile acids. Mutations in this gene give rise to the autosomal recessive disease cerebrotendinous xanthomatosis (CTX). CTX is characterized by tendon xanthomas, cataracts, a multitude of neurological manifestations, and premature atherosclerosis. A relatively high prevalence of the disease has been noted in Jews originating from Morocco. The major objectives of the present investigation were to determine the gene structure and characterize the common mutant alleles that cause CTX in Moroccan Jews. The gene contains nine exons and eight introns and encompasses at least 18.6 kb of DNA. The putative promoter region is rich in guanidine and cytosine residues and contains potential binding sites for the transcription factor Sp1 and the liver transcription factor, LF-B1. Blotting analysis revealed that the mutant alleles do not produce any detectable sterol 27-hydroxylase mRNA. No major gene rearrangements were found and single-strand conformational polymorphism followed by sequence analysis identified two underlying mutations: deletion of thymidine in exon 4 and a guanosine to adenosine substitution at the 3′ splice acceptor site of intron 4 of the gene. The molecular characterization of CTX in Jews of Moroccan origin provides a definitive diagnosis of this treatable disease. (*J. Clin. Invest.* 1993. 91:2488–2496.) Key words: atherosclerosis • bile salts • cholesterol metabolism • cytochrome P450 • dementia

Introduction

Cerebrotendinous xanthomatosis (CTX)[1] is a rare, autosomal recessive lipid-storage disease. The clinical hallmarks of the disease are tendon xanthomas, juvenile cataracts, and progressive neurological dysfunction (1). The latter includes behavioral abnormalities; dementia; pyramidal paresis; cerebellar, brain-stem, spinal, and peripheral nerve disorder; and epileptic seizures (2). In addition, osteoporosis with frequent bone fractures (3) and premature atherosclerosis (4) have recently been documented. Cholestanol, the 5α-dihydro derivative of cholesterol, is found in all tissues, particularly in the Achilles tendons, brain, and lungs (5, 6). The clinical diagnostic criteria include the demonstration of increased concentrations of bile alcohols in the serum and urine with low to normal plasma cholesterol concentrations (7). If untreated, CTX is a slowly progressive lethal disease (5). Early diagnosis of CTX is crucial inasmuch as long-term treatment with chenodeoxycholic acid (8) with or without an hydroxymethylglutaryl coenzyme A reductase inhibitor (9) may prevent the neurological complications of the disease.

Based on intermediary metabolism studies, Salen and coworkers (10) postulated in 1979 that a defect in a microsomal sterol 24S-hydroxylase causes CTX. In 1980, Oftebro et al. (11) reported that a defect in the mitochondrial sterol 26-hydroxylase (currently designated 27-hydroxylase) is the cause of CTX. These authors could detect no sterol 26-hydroxylase activity in a liver biopsy from a Norwegian CTX subject. Several subsequent studies employing fibroblasts from different CTX patients have confirmed a lack of mitochondrial 26-hydroxylase activity in this disease (12).

The sterol 27-hydroxylase (EC 1.14.13.15) is a mitochondrial enzyme catalyzing the initial steps in the oxidation of the side chain of sterol intermediates in the pathway for metabolism and excretion of cholesterol in mammals. The enzymatic hydroxylation of 5β-cholestane-3α,7α,12α-triol at C-27 occurs in mitochondria and is catalyzed by an enzyme complex comprising sterol 27-hydroxylase, ferredoxin, and NADPH-ferredoxin reductase (13). The sterol 27-hydroxylase was purified and characterized as mitochondrial member of the cytochrome P450 family (14, 15). This enzyme hydroxylates a spectrum of sterol substrates as well as vitamin D_3 (14, 16). In agreement with this finding, abnormal vitamin D metabolism has been recently observed in CTX patients (3).

Molecular cloning of the human sterol 27-hydroxylase cDNA has shown that the protein consists of a 498-amino acid mature enzyme and a 33-amino acid mitochondrial signal sequence (17). RNA blotting experiments demonstrated mRNAs of ~ 1.8–2.2 kb in liver and fibroblast cells. The gene encoding human sterol 27-hydroxylase (*CYP27*) has been mapped to the distal portion (q33-qter) of the long arm of chromosome 2 (18). After the molecular cloning of the human sterol 27-hydroxylase complementary DNA, two missense mutations were characterized in CTX cases (18). Identification of mutations that result in absence of any detectable mRNA required the characterization of the sterol 27-hydroxylase gene structure, a major goal of the current investigation.

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Received for publication 20 October 1992 and in revised form 23 December 1992.

1. *Abbreviations used in this paper:* CTX, cerebrotendinous xanthomatosis; SSCP, single-strand conformational polymorphism.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/93/06/2488/09 $2.00
Volume 91, June 1993, 2488–2496

Although rare in most populations, an interesting and experimentally approachable instance of abnormally high frequency of CTX has been reported in Israel (19). This finding mandates introduction of direct detection methods to diagnose the heterozygote state. The molecular characterization of mutations causing CTX in Moroccan Jews was an additional objective of the present study.

Methods

Determination of the sterol 27-hydroxylase gene structure

Genomic cloning. A human lymphocyte genomic library (in Lambda Dash) was obtained from Stratagene, Inc. (La Jolla, CA) and screened with ^{32}P-labeled oligonucleotides complementary to cDNA sequences of the human sterol 27-hydroxylase cDNA. One clone (HG27-4) hybridized with oligonucleotide JC21 5′-TCCGGCGGCGGCAACGGAGCTTAGA-3′ derived from 5′ sequences of the cDNA. Three clones (HG27-1,2,3) hybridized with oligonucleotides JC25 5′-AAGCAGCGCTCTATACGGATGCTTT-3′ and JC33 5′-TGGTCCCCACAAACTCCCGGATCATA-3′ derived from other coding sequences including the 3′ region of the cDNA. An EcoRI fragment of clone HG27-1 was subcloned into a pBS vector (Stratagene, Inc.) and designated p27OHGE. The BamHI-SphI fragment of p27OHGE was subcloned into bacteriophage M13 mp18, mp19 vectors (New England Biolabs, Beverly, MA) and designated 27OHBS. A SacI fragment from HG27-4 was subcloned into a pBS vector and designated p27OHGS. The SacI-SmaI fragment of this plasmid and a PstI fragment of HG27-4 were subcloned into bacteriophage M13 and were designated 27OHSS and 27OHPS, respectively (Fig. 1 *A*).

PCR amplification. We used PCR amplification (20) to verify the size of introns 2 and 5. Oligonucleotides were devised according to the cDNA sequence (17). PCR-oligonucleotide JC52 (nucleotides 428–448) (5′-ACCGGGACCAGCACGACCTGA-3′) and JC24 (nucleotides 585–560) (5′-TCGAGTCATAAAGTCATCAATCACCT-3′) were used for the amplification of intron 2. Oligonucleotides RL7 (nucleotides 1012–1031) (5′-GAGCTGCTCATGGCTGGAGT-3′) and RL8 (nucleotides 1092–1073) (5′-GATCTCAGGGTCCTTTGAGA-3′) were used to amplify intron 5 of the gene. Plasmid DNA (p27OHGE) was used as a DNA template. The PCR protocol included 1 min of denaturation at 95°C and 5 min of annealing and extension at 68°C using *Thermus aquaticus* DNA polymerase enzyme (Perkin Elmer Cetus, Norwalk, CT) for 35 cycles. After DNA amplification, products were size fractionated on a 6% polyacrylamide gel and subjected to ethidium staining. Band sizes were determined according to ΦX-HaeIII size standards.

DNA sequence analysis. DNA sequence analysis was performed using a Sequenase kit (United States Biochemicals, La Jolla, CA) either on single-stranded DNA templates (bacteriophage M13 clones) or on double-stranded plasmid DNA (p27OHGE) using oligonucleotide primers derived from the sterol 27-hydroxylase cDNA sequence.

Southern blotting analysis of genomic DNA. High molecular weight human genomic DNA was prepared from peripheral blood leukocytes as described (21). The DNA was quantified and diluted to a final concentration of 0.1 mg/ml. 8 μg of genomic DNA were digested with each restriction endonuclease using the buffer suggested by the manufacturer. Subsequently, the DNA was subjected to electrophoresis on a 0.8% (wt/vol) agarose and transferred to a nylon membrane (Biotrans, ICN Biomedicals, Irvine, CA). A single-stranded [α-^{32}P]dCTP-labeled probe derived from the human sterol 27-hydroxylase cDNA (exons 3–8) was prepared by the method of Church and Gilbert (22). Hybridization and washing conditions were as described (23).

Characterization of sterol 27-hydroxylase mutations in CTX cases of Moroccan origin

Subjects. Five CTX patients from four unrelated Jewish families (families 201, 203, 204, and 206) of Moroccan extraction were allocated through the main referral center for CTX patients in Israel (Soroka

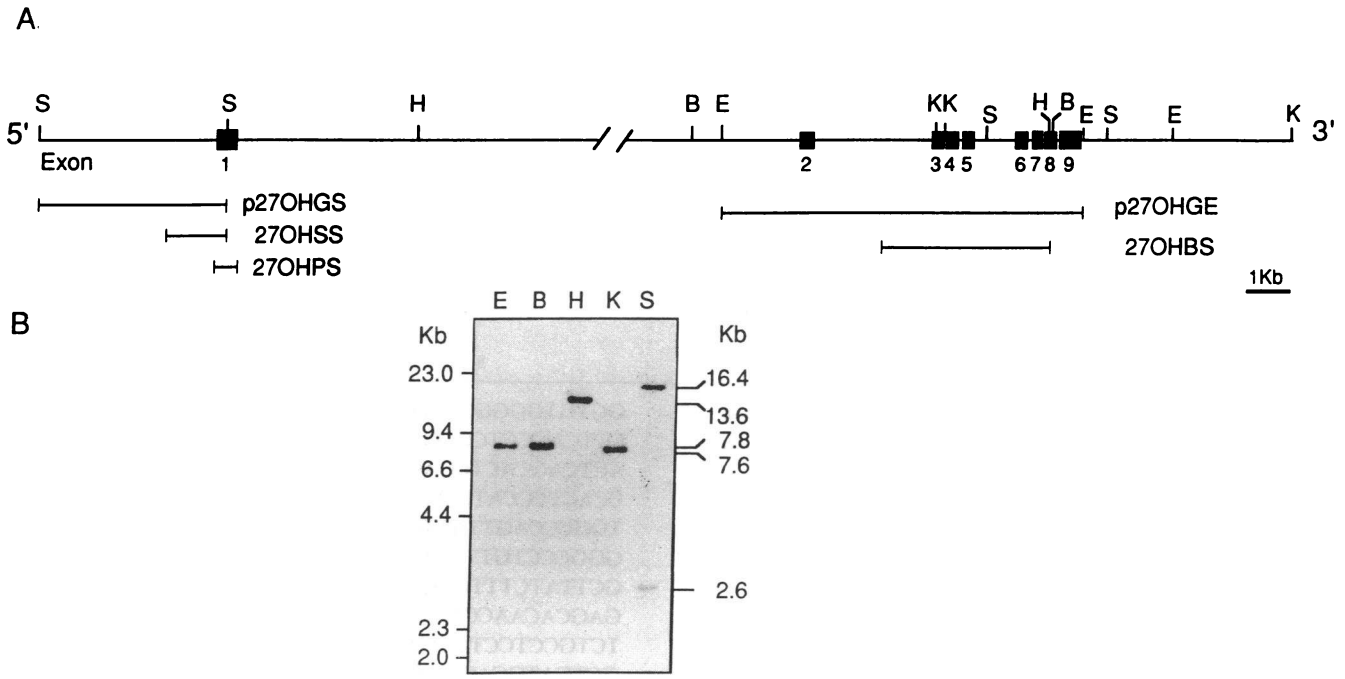

Figure 1. Structure of the human sterol 27-hydroxylase gene. (*A*) The exon-intron organization of the gene is drawn to scale. Exons are indicated by the numbered boxes and introns by the connecting lines. The positions of cleavage sites for the restriction enzymes EcoRI (E), BamHI (B), HindIII (H), KpnI (K), and SacI (S) are indicated along the gene schematic. Cloned fragments described under Methods are marked underneath. (*B*) Southern blot analysis of genomic DNA. 8 μg of high molecular weight DNA from human peripheral blood leukocytes were digested with the indicated restriction enzymes and subjected to Southern blotting analysis with a probe derived from the 3′ region of the human sterol 27-hydroxylase cDNA (exons 3–8). The washed filters were exposed to an X-ray film for 4 d with two intensifying screens. HaeIII-digested bacteriophage λ DNA size standard is shown on the left while the calculated size of each fragment is shown on the right.

Medical Center, Beer-Sheba). The diagnosis was verified according to the previously defined clinical and laboratory criteria (1). The phenotypic characteristics and the pedigree structure of three patients that are included in the current study had been previously reported by Berginer and Abeliovich (19). The following identification numbers are used [current study (previous study)]: 201-7 (B,IV-30), 201-8 (B,IV-31) and 203-3 (C,V-16). The study was approved by the Committee on Research Involving Human Subjects of The Hadassah University Hospital—Hadassah Medical School, Jerusalem.

Biochemical analyses. Plasma total triglyceride, cholesterol, and HDL-cholesterol levels were determined on fasting blood samples using commercially available diagnostic kits (Boehringer Mannheim Gmb H, Mannheim, FRG). Plasma LDL-cholesterol levels were calculated according to the Friedewald-Levy formula (24). Plasma cholestanol levels were determined by the gas chromatographic method (6, 25).

RNA and DNA mutation analysis. Skin biopsies were obtained from the CTX patients, fibroblast cultures were established, and total cellular RNA was extracted in 4 M guanidine thiocyanate (26). The RNA was denatured in 3 M glyoxal, subjected to electrophoresis on 1.6% agarose gel (27) and transferred to a nylon-based membrane (Biotrans) and hybridized with an [α-^{32}P]dCTP-labeled full-length human sterol 27-hydroxylase cDNA probe (22).

Southern blotting analysis was performed as described above using genomic DNA that had been extracted from blood leukocytes obtained from the CTX patients and probed with an identical probe. To identify the mutations that cause CTX in Jewish patients of Moroccan extraction, we have used PCR amplification (20) of genomic DNA and single-strand conformational polymorphism (SSCP) analysis (28). All exons and the 5′ flanking region of the sterol 27-hydroxylase gene were amplified using the oligonucleotides listed in Table I. The PCR reactions included 10 μCi of [α-^{32}P]dCTP and the conditions were as described above. The PCR products of the 5′ region of the gene and exons 6–9 were digested with HaeII and AvaII restriction endonucleases, respectively, before analysis on a 6% nondenaturing polyacrylamide gel containing 10% (vol/vol) glycerol. After the identification of abnormally migrating bands, the appropriate exon was PCR-amplified using similar oligonucleotides that include six-nucleotide 5′ extensions with the consensus sequence for BamHI (upstream oligonucleotides) and SphI (downstream oligonucleotides) restriction endonucleases, respectively, and three additional irrelevant nucleotides (ATA). The PCR products were analyzed on a 1.5% agarose gel, the appropriate band was extracted, purified and subjected to BamHI/SphI digestion followed by subcloning into bacteriophage M13. Sequence analysis of both strands of two independent M13 clones obtained from two separate PCR reactions was performed using the dideoxynucleotide chain termination method (29).

Direct detection of the mutant alleles. Direct detection methods were used to confirm the presence of the two mutations in genomic DNA samples from the four index cases. The mutation in exon 4 creates a new BpmI restriction site and deletes a FokI site. Intron 4 mutation does not change a restriction site and therefore we used the PCR-primer-introduced restriction analysis method (30) to create a new StyI site only in the mutant, PCR-amplified allele. The upstream oligonucleotide primer 27OHMM (5′-CTTTCCTCTTCTCTGTTGCTTTCCC-3′) included a single base substitution (underlined) where adenosine was substituted by cytosine, three nucleotides upstream to the beginning of exon 5. The downstream oligonucleotide primer was identical to the 3′ oligonucleotide primer used for amplification of exon 5 (Table I). We applied the SSCP method (as described above) to detect the mutant gene and determine its frequency in 250 unrelated individuals of Jewish Moroccan extraction.

Results

Structure of the sterol 27-hydroxylase gene. After screening of the human genomic library, four hybridization-positive clones were isolated. The genomic inserts were probed by using ^{32}P-labeled oligonucleotides derived from the human sterol 27-hydroxylase cDNA. Three clones hybridized to oligonucleotides derived from the 3′ coding region and the fourth with an upstream oligonucleotide.

The structure of the gene was derived from plasmid mapping and confirmed by Southern blotting analysis of control human genomic DNA (Fig. 1). The gene spans at least 18.6 kb and includes nine exons and eight introns. The exact locations of the introns were determined by DNA sequencing, and their sizes were verified by the PCR. As no overlapping genomic bacteriophage λ clones were found, the minimal size of intron 1 was estimated from Southern blotting of genomic DNA using SacI restriction analysis and hybridization with an exon 1–specific probe (data not shown). The DNA sequences at the intron–exon junctions are shown in Table II and obey the GT/AG rule of eukaryotic genes (31). As indicated in Fig. 1, the sizes of the DNA fragments generated with five restriction endonucleases agreed with those predicted from the gene map, confirming that the sterol 27-hydroxylase is a single copy gene. The

Table I. Sequence and Locations of Oligonucleotides in Sterol 27-Hydroxylase Gene That Were Used for PCR-SSCP Analysis

Oligonucleotide target	Location	Amplification	Sequence 5′ to 3′	Position*
RL14	5′-flanking	5′-flanking	GGTGTGGGGCTTCCCGATTT	−335 to −315
RL15	Exon 1	5′-flanking	CCTCAGCCTCGCGCAGCCCA	+30 to +10
1a	5′-flanking	Exon 1	ACTCAGCACTCGACCCAAAGGTGCA	−42 to −17
1b	Intron 1	Exon 1	CCACTCCCATCCCCAGGACGCGATG	14‡
2a	Intron 1	Exon 2	TGGCCCAGTTATTCAGTTTTGATTG	10‡
2b	Intron 2	Exon 2	GGGCCCTGTTCCAGTCCCTTCAGGC	10‡
3a	Intron 2	Exon 3	GCTTATCTTTGTGCTGTTCCTCTGC	9‡
3b	Intron 3	Exon 3	GAGCACAACCTCTCCCTGACCCATT	33‡
4a	Intron 3	Exon 4	TCTGCCTCCTGTGATGGCCTCTGTG	10‡
4b	Intron 4	Exon 4	GCTGATGCACAGACCTGGAGTCACC	39‡
5a	Intron 4	Exon 5	GCTCTTGGTCCTTGGAGATCATGAC	40‡
5b	Intron 5	Exon 5	ACTGGTTACGGTTGGGAGCTGGGGG	30‡
6a	Intron 5	Exon 6-9	TTCCTAGAATCGCCTCACCTGATCT	17‡
9b	3′-untranslated, exon 9	Exon 6-9	CCCAGCAAGGCGGAGACTCA	3§

* The A of the ATG initiation codon is number +1. ‡ Minimal distance from exon. § Downstream from the TGA (termination codon).

Table II. Exon-Intron Organization of the Human Gene Encoding Sterol 27-Hydroxylase

Exon number	Exon size	Sequence at the exon-intron junction			Amino acid interrupted
		5′ splice donor		3′ splice acceptor	
	bp				
1	>255	TTACAGgtaacccgcg 255	. . . 12.20 kb . . .	aactccacagGTGCTT 261	Gln (52)
2	191	CACCACgtgagctggg 446	. . . 2.70 kb . . .	cgtcctgcagGGAAGG 452	Thr (116)
3	200	TGGAAGgtacccttgc 646	. . . 0.11 kb . . .	cactactcagCTATTT 652	Ala (183)
4	198	CCTTTGgtgaggactc 844	. . . 0.15 kb . . .	gctttcacagGGAAGA 850	Gly (249)
5	174	GACACGgtgcgtgaag 1018	. . . 0.96 kb . . .	tatcttctagACATCC 1023	Thr (306)
6	167	TCTGCGgtaggacaga 1184	. . . 0.24 kb . . .	ttccctgcagTCTCTA 1190	Arg (362)
7	79	AAGAACgtgagtgggc 1263	. . . 0.06 kb . . .	tcctttatagACCCAG 1269	Asn (388)
8	213	GCAAGGgtgagctggg 1476	. . . 0.15 kb . . .	tacccccagCTGATC 1482	Arg (459)
9	344	CCTTGGgtcagaatat 1820			

Capital letters and numbers refer to exon sequences, with the A of the ATG initiation codon being assigned number 1; small letters refer to intron sequences; amino acids are numbered according to Cali and Russell (17).

sequence encoding the "extrapeptide" mitochondrial signal is located in exon 1, the putative ferredoxin binding site in the 3′ end of exon 6, and the predicted heme binding cysteine residue in exon 8. These sites were determined based on the structure of the cDNA and protein (18).

DNA sequencing of the 5′ flanking region. The DNA sequence of the 5′ untranslated region is shown in Fig. 2. This sequence was derived from two partially overlapping bacteriophage M13 clones (27OHSS and 27OHPS). The sequence includes 500 bp, displays a high G+C content and possible binding sites for the transcription factor Sp1 and the liver transcription factor, LF-B1. A canonical TATA sequence and CAAT box did not appear to be present in the immediate 5′-flanking region.

Clinical manifestations and biochemical analyses. The four Jewish families were traced back to their origin from Morocco. Family 201 emigrated from Taroudant, family 203 from Mogador, family 204 from Marrakech, and family 206 from Sefrou. Consanguinity, at the first cousin level, was demonstrated in all families except for family 204. All five Moroccan patients were females aged 23–47 yr (Table III). The clinical manifestations appeared during childhood. Although the physical findings were similar, it is striking that patient 201-8 did not have xanthomatosis and patient 206-3 had only mild thickening of the Achilles tendons. Patient 206-3 also differed in the distribution of her neurological manifestations. She had only mild dementia but significant cerebellar dysfunction. The degree of brain atrophy as determined by computerized tomography and magnetic resonance imaging was most striking in the older patients (203-3 and 204-8). Plasma cholestanol levels were elevated in all patients, the highest in patient 203-3 and the lowest in 204-8. Other plasma lipid and lipoprotein levels were normal except for patient 201-7 that had abnormally elevated total cholesterol and LDL cholesterol levels.

RNA and DNA mutation analyses. RNA blotting analysis revealed the absence of sterol 27-hydroxylase mRNA in the CTX samples and Southern blotting analysis showed that the

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-500 CCAGGGATCAGATGACTGGCCCCCCTCGCTCCGAACTGACTCCGGGATCA

-450 ATCCGGAAGGCCATTGGGAGAAGCCGAGGGCAGCTTAGCCACGGCCGGTT

-400 CCCGTTCCCTCCAGGACGCGAGGGTCGCCTTGGGTGGGGAACCCGCGACC

-350 GGGCGAGGACCTATCCCGGTGTGGGGCTTCCCGATTTCGGAAAGAATCTC

-300 GCTGCACCCCCGCCCAGAGTTCAGACCAAGCGAAAAGTTATTTGAGAGGC

-250 CTCGGGGGCGCGGGGTGAGGAGTCGTGGCGGAGGCTTGGTCGGGGCGCCG

-200 TGGATATCCCCGAGTCACCGCGTCCCTCTCCTGCAGCTCCCGCGTCGCTG

-150 GGAGGAGCGAGGGAGCGAGCGGGAAGGGGTCTAGCTGGCCTTTGCTCGGC

-100 CCTCCCCAGCGCCCGGCTTTGAACCCGCCCTGCACTGCTGTCTGGGCGGG

-050 TCCGGGGACTCAGCACTCGACCCAAAGGTGCAGGCGCGCGAGCACAACCC

     MetAlaAlaLeuGlyCysAlaArgLeuArgTrpAlaLeuArgGly
+001 ATGGCTGCGCTGGGCTGCGCGAGGCTGAGGTGGGCGCTGCGAGGG..
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Figure 2. DNA sequence analysis of the 5′-flanking region of the human sterol 27-hydroxylase gene. The nucleotide sequence of the 5′-flanking region of the gene is shown. The sequence is numbered on the left with negative numbers assigned to the 5′-flanking nucleotides. A consensus CCGCCC or GGGCGG present in the recognition sequence for the transcription factor Sp1 are overlined as are GTTATT, the nucleotides present in the recognition sequence for liver transcription factor LF-B1.

Table III. Molecular Identification and Clinical and Laboratory Manifestations of the CTX Patients before Treatment

Patient number	201-7	201-8	203-3	204-8	206-3
Sterol 27-hydroxylase mutation	Splice junction (homozygote)	Splice junction (homozygote)	Frameshift (homozygote)	Splice junction and frameshift (compound heterozygote)	Frameshift (homozygote)
Background data					
Sex	F	F	F	F	F
Age (*yr*)	27	25	47	34	23
Age of onset (*yr*)	4 or earlier	3 or earlier	early childhood	late childhood	8–9
Consanguinity	+	+	+	−	+
Physical findings					
Myopathic facial expression	+++	+++	+++	+	+
Pes cavus	++	++	++	++	++
Tendon xanthomata	+++	−	++++	++	+
Cataracts	++++	++++	++++	++++	++++
Dementia	+++	+++	++++	++	+
Pyramidal signs	++	++	++	+	++
Cerebellar signs	+	+	++	+	+++
Sensory loss	−	+	+	−	−
Convulsions	febrile up to 11 yr	febrile up to 5 yr	+	−	grand mal attack at age 20
Neurological studies					
EEG abnormality*	+++	+++	+++	+++	+++
Diffuse brain atrophy‡	+	+	+++	++	+
Plasma lipids and lipoproteins	7.1 (276)	3.4 (131)	4.9 (189)	4.9 (190)	4.4 (173)
Cholesterol, *mmol/liter* (*mg/dl*)					
Cholestanol, *mmol/liter* (*mg/dl*)§	0.19 (7.3)	0.14 (5.4)	0.23 (9.0)	0.06 (2.2)	0.07 (2.6)
Triglyceride, *mmol/liter* (*mg/dl*)	2.0 (180)	0.5 (43)	1.8 (159)	1.0 (86)	1.1 (98)
HDL-cholesterol, *mmol/liter* (*mg/dl*)	1.8 (70)	1.8 (70)	1.3 (50)	1.3 (52)	1.8 (70)
LDL-cholesterol, *mmol/liter* (*mg/dl*)‖	4.4 (170)	1.3 (52)	2.7 (107)	3.1 (121)	2.1 (83)

Scoring system: (++++) to (−) = very severe to absent. * Irregular diffuse slow activity with periodical sharp waves discharges. ‡ Confirmed by magnetic resonance imaging and computerized tomography. § normal level < 0.03 mmol/liter (1 mg/dl). ‖ Calculated according to Friedewald et al. (24).

size of the BamHI restriction fragments was identical for all genomic DNA samples that were analyzed (Fig. 3). It was thus concluded that the absence of mRNA in the Moroccan CTX patients does not result from a major gene rearrangement. SSCP analysis of the 5′ region of the gene and all exons and flanking sequences was performed. Abnormally migrating bands were found only in the analysis of exons 4 and 5 (Fig. 4). SSCP analysis of exon 4 showed that patients 203-3 and 206-3 had a different band pattern while patient 201-7 had identical band pattern as compared to the control. Patient 204-8 showed both band patterns. Analysis of exon 5 revealed that patient 201-7 had an abnormal band pattern, patients 203-3 and 206-3 were identical to the control and again patient 204-8 had both band patterns. The SSCP analysis suggested therefore that patients 203-3 and 206-3 are homozygous for a mutation in exon 4, patient 201-7 is homozygous for a mutation in exon 5, and patient 204-8 is a compound heterozygote harboring both mutations.

Sequence analysis revealed that the mutation in exon 4 is a deletion of thymidine that results in a frameshift and in premature termination codon 35 nucleotides downstream, the second mutation, a null mutation, is a guanosine to adenosine substitution at the 3′ splice acceptor site of intron 4 (Fig. 5). PCR and restriction analysis were then used for the analysis of the five CTX cases that participated in the study. BpmI restriction analysis confirmed that CTX cases 203-3 and 206-3 were homozygote and 204-8 heterozygote for the frameshift mutation. By using PCR-primer-induced restriction analysis and StyI restriction analysis it was confirmed that CTX cases 201-7 and 201-8 were homozygote and 204-8 heterozygote for the splice junction mutation (data not shown).

DNA samples from 250 unrelated individuals of Jewish Moroccan origin were screened to determine the heterozygote frequency of the two mutations. The sample includes 457 parental Moroccan alleles (43 individuals had one parent that originated from another country), revealed that one carries exon 4 and two carry intron 4 mutations. Both parents of each one of the three heterozygotes originated from Morocco. The point estimate of the Moroccan mutations allele frequency is therefore 0.00658 (95% confidence interval 0.000–0.013). The

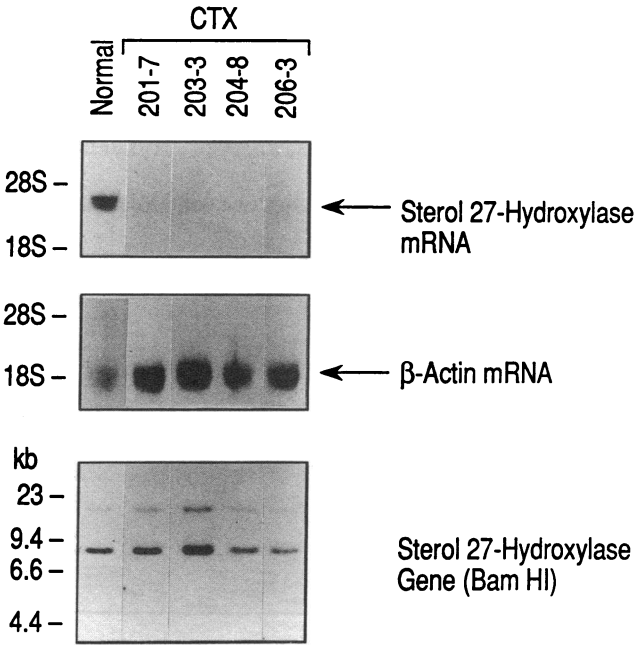

Figure 3. Blot hybridization of CTX fibroblast RNA and DNA. Total cellular RNA was prepared from fibroblast cultures and genomic DNA from blood leukocytes. RNA and Southern blotting analysis were performed and the nylon membranes were probed with a full-length ^{32}P-labeled human sterol 27-hydroxylase cDNA probe. For comparative analysis, the RNA blot was also probed with a β-actin cDNA probe.

estimate for homozygosity is therefore 0.0000433. Inasmuch as the current Jewish population that originated from Morocco in Israel is 498,000 (32) and the degree of interethnic mixture (calculated from our sample) is only 8.6%, it suggests that 20 CTX cases ($0.0000433 \times 498{,}000 \times 0.914$) that result from the two mutant alleles in nonconsanguineous marriages may exist. So far, only two nonconsanguineous Jewish Moroccan CTX families are known: family 204 and another family which is not included in this study (Berginer, unpublished observation).

Discussion

Here we show that in Moroccan Jews, CTX is caused by two distinct sterol 27-hydroxylase gene mutations. These two mutations, a deletion of thymidine in exon 4 and a guanosine to adenosine substitution at the 3′ splice acceptor site of intron 4, in addition to an estimate of carrier frequency, provide an indication of the expected prevalence of CTX in the Israeli Jewish Moroccan community.

Moroccan Jews have been socially isolated from non-Jews in Morocco and it has been established that they resemble other Jewish populations with regard to several polymorphic genetic systems (33). The possibility of the existence of a founder mutation in the Jewish Moroccan population is related to the unique demographic characteristics of this ethnic group. Some of its ancestors immigrated to Morocco before the destruction of the Second Temple (34) where they intermarried with the Berber tribes (35, 36). After the Arab conquest of Morocco in the 8th century the Jews lived in ghettos where inbreeding was common (37). This population was diluted with a massive Jewish refugee immigration from the Iberian peninsula at the end of the 15th century. This Moroccan Jewish community then migrated almost exclusively to Israel.

The fact that two mutations are prevalent in this community is also compatible with a founder mechanism as has recently been shown for familial hypercholesterolemia in Afrikaners (38) and in the Finnish population (39). Based on the screening results of 250 unrelated individuals for carrier fre-

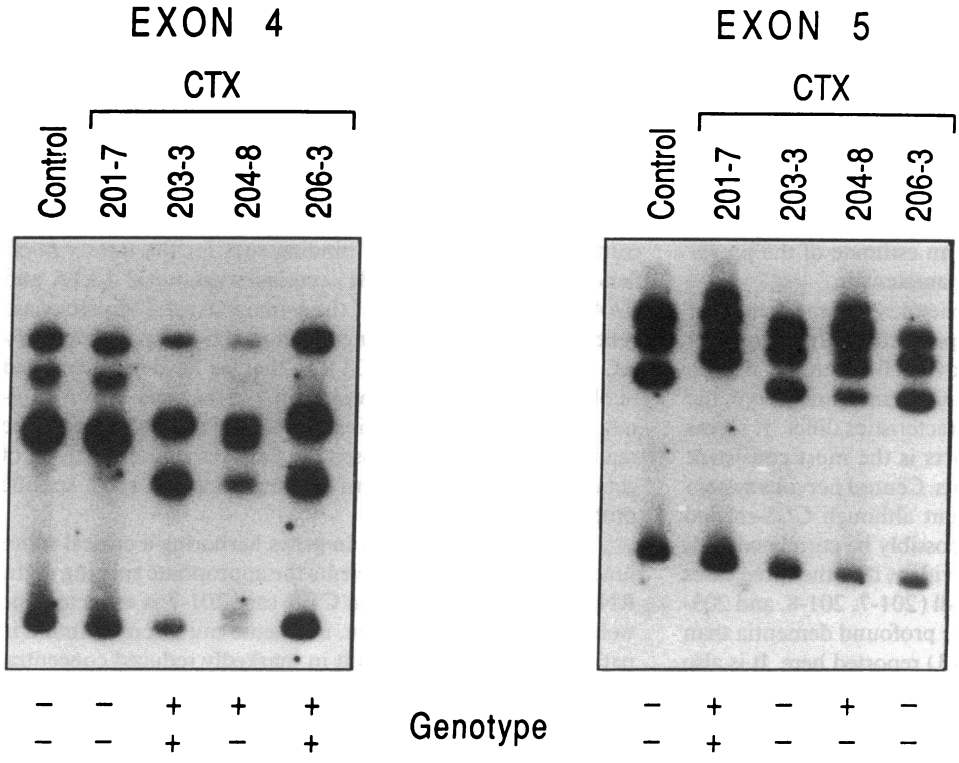

Figure 4. SSCP analysis of genomic DNA. Exon 4 of the sterol 27-hydroxylase gene was amplified using oligonucleotide EXINT45 (5′-CTATTTGCTACATCCTGTTC-GAGAA-3′) that is homologous to the first 25 bases of exon 4 and oligonucleotide 4b (Table I). Exon 5 was amplified using oligonucleotides 5a and 5b (Table I). After amplification, the PCR products were heat denaturated and subjected to electrophoresis on a 6% nondenaturing polyacrylamide gel containing 10% glycerol followed by autoradiography for 24 h. For the determination of the possible genotypes, the band pattern obtained for the control DNA was designated: – – (two normal alleles), for the cases that showed a completely different band pattern and therefore may be homozygote for a mutation; + + (two mutant alleles); and + – (heterozygote), for a case with both band patterns.

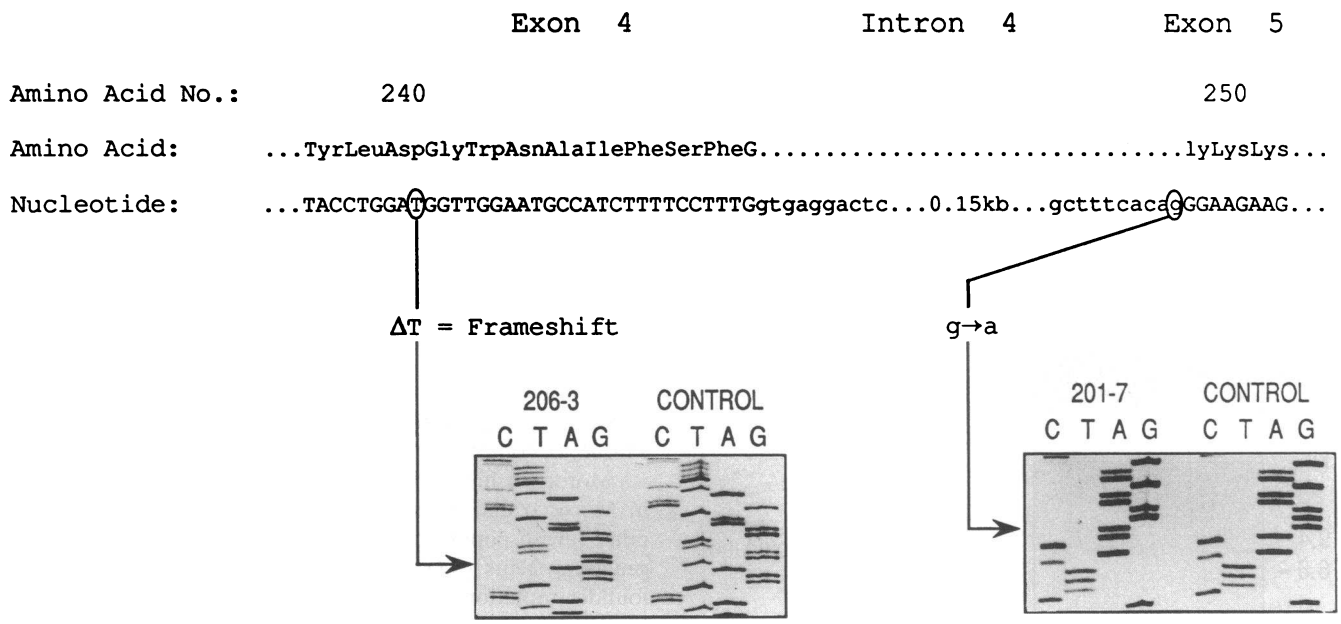

Figure 5. DNA sequence analysis of the mutant gene in CTX cases 206-3 and 201-7. Exons 4 and 5 and their flanking sequences were PCR-amplified using genomic DNA from CTX cases 206-3 and 201-7, respectively. Control DNA samples were included for comparison. Sequence analysis was performed on cloned DNA (see Methods). The normal sequence is shown and the exact location and nature of both mutations indicated. Upper-case letters indicate exon sequences and lower-case letters intron sequences. The sequences shown are of the coding strands of both mutant alleles.

quency of the two mutations, we predict the existence of 20 CTX cases that result from random matings in Jews of Moroccan origin in Israel. Inasmuch as only two nonconsanguineous families with CTX from this community are known, it is expected that additional families will be identified in the near future.

CTX is a rare autosomal recessive disease that does not interfere with fertility (40). Its increased prevalence in Moroccan Jews may result primarily from an exceedingly high rate of consanguinity. In Moroccan Jews, cousin marriage is traditionally acceptable, a fact which appears to be shown in Jews and Moslems (41). The high consanguinity rate of 10.7% reported three decades ago (42) may also explain the increased frequency of several other distinct recessive genetic diseases including steroid 11β-hydroxylase deficiency (43), complement deficiency (44), and Tay-Sachs disease (45). The present consanguinity rate in the Moroccan Jewish community is unknown and thus does not allow for an estimate of the prevalence of CTX based on this social characteristic.

Analysis of the clinical and biochemical characteristics of five CTX cases of Moroccan origin presented here (Table III) reveals that, although all five cases have null mutations that do not produce any detectable mRNA and therefore no enzymatic activity is expected, the clinical characteristics differ. It seems as though the appearance of cataracts is the most consistent characteristic of CTX in these patients. Central nervous system (CNS) involvement is always present although CNS-related clinical signs may differ and could possibly be correlated with the plasma cholestanol levels. It is evident that the three cases with plasma cholestanol of > 5 mg/dl (201-7, 201-8, and 203-3) at the time of diagnosis have more profound dementia than the other two cases (204-8 and 206-3) reported here. It is also evident that the degree of dementia does not necessarily correlate with the degree of brain atrophy as demonstrated by magnetic resonance imaging and computerized tomographic scanning. These observations suggest that the mechanism for the development of dementia in CTX is complex and may be related at least in part to the toxic effect of bile alcohols.

The absence of tendon xanthomas in patient 201-8 is striking especially when compared to her sister (201-7) and may be related to her very low LDL-cholesterol levels. Low LDL-cholesterol levels are commonly found in CTX and could possibly be related to overexpression of LDL receptors (46). In some cases LDL-cholesterol levels are elevated as in patient 201-7 where the markedly increased plasma LDL-cholesterol concentration could contribute to the development of severe tendon xanthomas in this relatively young patient.

The current investigation reveals the structure of the human sterol 27-hydroxylase gene that spans at least 18.6 kb of genomic DNA. The gene includes nine exons and eight introns. The 500-bp sequence immediately upstream of the translation initiation codon has a high guanosine and cytosine content, contains potential binding sites for the transcription factors, Sp1 (47) and LF-B1, and lacks canonical TATA and CAAT boxes. The exons of the human sterol 27-hydroxylase gene are bounded by sequences that match the splice sites GT/AG consensus sequences (31). In that we have not succeeded in obtaining clones that span intron 1 of the gene, only a minimal estimate for the size of intron 1 and of the gene itself can be made. This estimate is based on SacI restriction analysis of genomic DNA and Southern blotting using an exon 1 specific probe.

The absence of mRNA in genes harboring a critical splice junction mutation that prevents the appropriate splicing of the RNA precursor as found in CTX case 201-7 is expected and well understood. In addition, non-sense mutations as found in patient 206-3 may also result in markedly reduced concentrations of mRNA. Although the molecular mechanisms leading to this phenomenon are not well understood, several possibilities have been suggested (48–50).

The gene for the sterol 27-hydroxylase (*CYP27*) belongs to a group of now over 150 cytochrome P450 genes (51). The sterol 27-hydroxylase gene that includes nine exons, is similar in structure to *CYP11B1* and *CYP11B2* genes encoding the human steroid 11β-hydroxylase (52). To date, cytochrome P450 genes have been classified into 27 families, each defined as unique when having < 40% resemblance to members of other families. All genes within a given family are predicted to have the same number of exons and similar exon/intron boundaries (53), suggesting that the rabbit 27-hydroxylase (54) and the rat 27-hydroxylase (55, 56) genes will be similar in structure to the human gene.

The enzyme sterol 27-hydroxylase belongs to the mitochondrial P450s which require ferredoxin as a co-factor and ferredoxin reductase for electron transfer. Several conserved domains have been recognized in the P450 mitochondrial proteins, suggesting that the organization of the exons might correlate with functional domains of the protein. We found that the sequence encoding the hydrophobic "extrapeptide" mitochondrial signal is located in exon 1 and that the predicted heme-binding cysteine residue resides in exon 8 of the gene. The putative ferredoxin binding site (57) is located at the 3′ end of exon 6. Three potential binding sites for the transcription factor SP1 and one for the liver transcription factor LF-B1 were also identified. This is significant as the expression of the sterol 27-hydroxylase gene in the liver appears to be independent of regulation by cholesterol (17, 54). SP1 has been widely described as playing a role in the expression of "house-keeping" genes (58), while the liver transcription factor LF-B1 is required for the expression of liver specific genes. The importance of these sites for the transcriptional control of the human sterol 27-hydroxylase remains to be elucidated.

Elucidation of the sterol 27-hydroxylase gene structure and analysis of mutant alleles that underlie CTX may provide the basis for future research in several important directions. These directions include the analysis of the regulatory mechanisms of sterol 27-hydroxylase gene expression, the molecular diagnosis of CTX at the pre-symptomatic stage, and the study of pathogenetic mechanisms that lead to the major manifestations of the disease in molecularly defined CTX cases. These studies are currently underway in our laboratory.

Acknowledgments

We thank Dr. D. W. Russell (Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, TX) for most helpful discussions and for providing the human sterol 27-hydroxylase cDNA clones and other DNA probes used in these experiments, Dr. G. Salen (University of Medicine and Dentistry, New Jersey Medical School, Newark, NJ) for the analysis of plasma cholestanol levels in the study patients, and Dr. H. Giladi and Ms. M. Ben-Naim for their valuable technical assistance.

This research was supported by grant no. 88-0186 from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel, and by the Sarah Mayer Research Foundation.

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