Partial deletion of the gene encoding sterol 27-hydroxylase in a subject with cerebrotendinous xanthomatosis

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Abstract An Italian subject with cerebrotendinous xanthomatosis (CTX) was found to have a partial deletion of the gene encoding the enzyme sterol 27-hydroxylase (*CYP27* gene). Southern blot analysis revealed that this deletion (~2 kb) spans from intron 6 to the 3′ flanking (3′FLK) region, eliminating exons 7–9, the last three exons of *CYP27* gene. No sterol 27-hydroxylase mRNA was detected in proband cells, either by Northern blot analysis or by reverse transcription polymerase chain reaction (PCR). This suggests that the mutant mRNA devoid of the exon encoding the whole untranslated sequence (exon 9) might be rapidly degraded in the cytoplasm. We used inverse PCR to obtain a partial sequence of the 3′FLK region of the normal *CYP27* gene; this allowed us to define the mechanism underlying the deletion. The established sequence was used to design suitable primers to perform step-wise sequences of a 1.7 kb segment of the 3′FLK region of the normal gene and of the deletion joint in the CTX patient. The analysis of the sequence data indicate that the deletion might result from a complex mechanism involving two intragenic recombinations between *a)* two 14 nucleotide complementary sequences, one in intron 6 and the other in the 3′FLK region; and *b)* AT-rich complementary sequences of the 3′FLK region, and a slipped mispairing between two 6 nucleotide direct repeats, one in intron 6 and the other in the 3′FLK region. Such repeats are brought close to each other by the formation of the stem-loops induced by the two intragenic recombinations. This is the first example of CTX caused by a rearrangement of *CYP27* gene.—**Garuti, R., N. Lelli, M. Barozzini, M. T. Dotti, A. Federico, S. Bertolini, and S. Calandra.** Partial deletion of the gene encoding sterol 27-hydroxylase in a subject with cerebrotendinous xanthomatosis. *J. Lipid Res.* 1996. **37:** 662–672.

Supplementary key words *CYP27* gene • 3′ flanking region • reverse PCR

Cerebrotendinous xanthomatosis (CTX) is a rare autosomal recessive sterol storage disease leading to the accumulation of cholestanol and cholesterol in many tissues including the central nervous system. The main clinical features of the disease are tuberous and tendon xanthomas, juvenile cataracts, spinal paresis, cerebellar ataxia, and dementia (1–3). In addition, osteoporosis (1, 4) and premature atherosclerosis (5) may be present to a variable extent. If untreated, CTX is a slowly progressive lethal disease (1–3). Early diagnosis allows timely treatment with chenodeoxycholic acid (6), with or without a hydroxymethylglutaryl coenzyme A reductase inhibitor (7), that may prevent or reduce the neurological complications of the disease (1–3, 8, 9).

CTX is due to a deficiency of the sterol 27-hydroxylase (EC 1.14.13.15), a mitochondrial enzyme catalyzing the initial steps in the oxidation of the side chain of sterol intermediates in the pathway for the metabolism of cholesterol (1). Since the cloning of human sterol 27-hydroxylase cDNA in 1991 (10), several point mutations of the sterol 27-hydroxylase gene (*CYP27* gene) causing CTX have been found in various populations (11–18). These point mutations lead to amino acid substitutions (11, 13, 16), frameshift (12, 14, 18), premature termination codon (17), or disruption of the mRNA splicing process (12). Up to now, no major rearrangements of *CYP27* gene have been reported in CTX patients. In this study we report the characterization of the first partial deletion of *CYP27* gene that was discovered in an Italian CTX patient.

Abbreviations: CTX, cerebrotendinous xanthomatosis; CYP27 gene, gene encoding human sterol 27-hydroxylase; 3′FLK, 3′ flanking; PCR, polymerase chain reaction; EEG, electroencephalogram; MRI, magnetic resonance imaging.

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METHODS

Patient

The proband L.M. was a 30-year-old woman born from a consanguineous marriage (her parents were first cousins). Xanthomas of the Achilles tendon were the first clinical signs at the age of 6. Bilateral cataract was diagnosed 3 years later and removed at the age of 20. We examined the patient for the first time at the age of 27. Neurological examination showed mild mental deterioration and brisk deep tendon reflexes in the lower limbs. Serum and cerebrospinal fluid cholestanol levels were elevated (4.2 mg/dl and 0.05 mg/dl vs. < 1 mg/dl and 4 ± 7 μg/dl in the control subjects, respectively). EEG revealed mild generalized aspecific abnormalities and MRI of the brain showed only mild cerebellar atrophy (19). Control subjects were chosen among healthy normolipidemic individuals working in the laboratory. Informed consent was obtained from the proband and the healthy controls.

Southern blot analysis

Genomic DNA was extracted from peripheral blood leukocytes by a standard procedure (20). DNA was digested using 5–10 U/μg DNA of several restriction enzymes (BamHI, EcoRI, SacI, HindIII, and KpnI), separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with a sterol 27-hydroxylase cDNA probe according to a procedure reported previously (21). The full-size sterol 27-hydroxylase cDNA probe (10) was a kind gift from Dr. D. Russell (Dallas, TX).

Cell culture

A skin biopsy was taken from the proband and some healthy control subjects. Explants were cultured in 25-cm^2 flasks in DMEM (Dulbecco's modification of Eagle's medium), 100 IU/ml of penicillin and 50 μg/ml of streptomycin, 2 mM glutamine, 15% fetal calf serum, and 95% air–5% CO_2 (21).

Northern blot analysis

Total cellular RNA was isolated by extraction in guanidine-thiocyanate (22) from cultured skin fibroblasts. RNA (15 μg) was denatured in 50 μl of 50% formamide, 2.2 M formaldehyde, and 1 × MOPS buffer [20 mM 3-(N-morpholino)-propane-sulfonic acid, 5 mM sodium acetate, and 1 mM Na_2EDTA], separated by electrophoresis on a 1.6% agarose gel, and transferred to Hybond-N membranes, which were then hybridized with the full-size cDNA probe. The cDNA clone pHFβ A-I of human β-actin was used to normalize the RNA filters. cDNA probes were labeled with deoxycytidine 5′-[α-^{32}P]triphosphate (3,000 Ci/mmol) by the multiprime method (Amersham). The specific activity of the probes was approximately 2×10^9 to 3×10^9 cpm/μg (21). Northern blotting membranes were prehybridized and hybridized in the following solution: 50% formamide, 3 × standard saline citrate (SSC: 0.45 M NaCl, 0.045 M sodium citrate), 50 mM Tris-HCl (pH 7.5), 5 mM Na_2EDTA, Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 0.1 mg/ml herring sperm DNA. The membranes were subjected to several washings in 2 × SSC and 0.1% SDS; 0.5 × SSC and 0.1% SDS at room temperature; and 0.1 × SSC and 0.1% SDS at 42°C. Washed membranes were then exposed to Hyperfilm-MP (Amersham) X-ray films at -80°C (21).

Reverse transcription and PCR amplification

RNA (1 μg) from cultured fibroblasts of the proband L.M. and a normal subject were reverse-transcribed in a 20-μl reaction mixture containing 8 mM $MgCl_2$, 1 mM of each dNTP, 1 unit of RNAsin, 100 pmol random hexamers, and 12 units of AMV reverse transcriptase in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9). After heating the sample at 95°C for 5 min, 80 μl of 1 × PCR buffer containing 20 pmol of each primer was added as well as 2.5 units of Taq polymerase. The $MgCl_2$ concentration in the reaction mixture ranged from 1 to 2 mM. The following primers were used: 5′ TCC GGC GGC GGC AAC GGA GCT TAG A 3′ (forward primer corresponding to nucleotides 170–194 of the cDNA) (exon 1) (*1s*); 5′ CGC AGA GTC TCC TTA AGC ACA GC 3′ (reverse primer complementary to nucleotides 1205–1182 of the cDNA) (exon 6) (*6as*). The conditions were 95°C for 5 min, 70°C for 1.30 min for the first time, and subsequently 95°C for 1.30 min, and 70°C for 1.30 min for 28 cycles. PCR products were analyzed by 1.5% agarose gel electrophoresis.

Inverse PCR

In order to sequence the unknown 3′ flanking region of the normal *CYP27* gene, the procedure known as "inverse PCR" (23) was used. Genomic DNA from a normal control subject was digested with BamHI and subjected to 0.8% agarose gel electrophoresis in 1 × TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). The DNA digestion fragments migrating in the 2–3 kb region of the gel were purified with JetSorb (Genomed GmbH, Bad Oeynhausen, Germany) and incubated overnight at 15°C with 2000 U of T4 DNA ligase (New England Biolabs, Beverly, MA) in 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 50 μg/ml BSA buffer, pH 7.8. The circular DNA fragments were then digested with 40 U of XhoI at 37°C for 1 h and then amplified by PCR using the following primers: 5′ GGA GTA GCT GCA TCT CCA GCT CT 3′ (complementary to nucleotides 1489–1467 of cDNA in exon 8) (*8as*); and 5′ GCA

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CAC ACC CTG AGC TTT TG 3′ (corresponding to nucleotides 1751–1770 of the cDNA in the 3′ untranslated region) (*9s1*) (**Fig. 1**). The conditions were: 95°C for 5 min, 80°C for 1 min with the addition of 2.5 U of Taq polymerase, and subsequently 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min for 30 cycles. The amplified fragment (approximately 2 kb) was separated by 1% agarose gel electrophoresis, purified by JetSorb, and sequenced directly using a commercial kit (fentmol Sequencing System) and the oligonucleotide *8as* as a primer (see above). The nucleotide sequence obtained by this procedure included part of exon 8 (up to the BamHI site) and 153 nucleotides (nts) of the 3′ flanking (3′FLK) region adjacent to the same BamHI site (Fig. 1). On the basis of the sequence data of the 3′FLK region close to the BamHI site, an oligonucleotide was designed: 5′ AGG GGA TTC ACA GTT GGT TGG GG 3′) (*3′FLK as1*) which allowed us to perform a direct PCR from genomic DNA of a control subject in order to obtain a DNA fragment spanning from the 3′ of exon 9 to the BamHI site in the 3′FLK region (see Fig. 5). The other primer used for the amplification was *9s1* (see above). The conditions were 95°C for 5 min, 80°C for 1 min with the addition of Taq polymerase, and subsequently 95°C for 1 min, 55°C for 1 min, and 72°C for 3 min for 30 cycles. The amplified fragment obtained by this direct PCR was digested with StuI, EcoRI, and SacI to construct a partial restriction map (see Fig. 5).

Nucleotide sequence of the 3′ flanking region of the normal *CYP27* gene

The PCR fragment spanning from exon 9 to the BamHI site in the 3′FLK region (see above) was sequenced step-wise using the following primers:

(*3′FLK as1*) (see above);
5′ TCC AAG AGT GGG GAC AGG AGG TAC 3′ (*3′FLK as2*);
5′ GTT CAA ACC CTA TCA TTC CCA CAG G 3′ (*3′FLK as3*);
5′ GCT TGA GCC TGG GTT AAG GAG GCA 3′ (*3′FLK as4*);
5′ CAA GGA GGC TGG ACA GTT AAA AGC 3′ (*3′FLK as5*);
(*9s1*) (see above);
5′ TAT CGC ATT GCT GTC CTT GGG TAG 3′ (*9s2*);
5′ ACC AAG CAG GAA CTG CAG TGA CTC 3′ (*9s3*).

Sequence of the deletion joint in proband L.M.

To amplify the region encompassing the deletion joint in proband L.M., 1 µg of genomic DNA was amplified in a 100-µl mixture containing 0.2 mM of each dNTP, 50 pmol of each primer, 2.5 units of Taq DNA polymerase in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$). The following primers were used: 5′ GAG ATC CAG GAG GCC TTG CAC GA 3′ (forward primer corresponding to nucleotides 1087–1109 of the cDNA in exon 6) (*6s*) and the *3′FLK as1* (see above). The conditions were 95°C for 1 min 68°C for 1 min × 30 cycles. The PCR-amplified product (approximately 1 kb) was purified using JetPure (Genomed GmbH) and sequenced directly, as specified above. The primer used for the sequence of the deletion joint was the exon 6 forward primer (*6s*) (see above). The 3′FLK region downstream of the deletion joint was sequenced step-wise using the following primers: *3′FLK as1; 3′FLK as2; 3′FLK as3; 3′FLK as4* (see above). The sequence obtained with the primer *3′FLK as4*, encompassing the deletion joint, confirmed the sequence obtained with the exon 6 forward primer (*6s*) (see above).

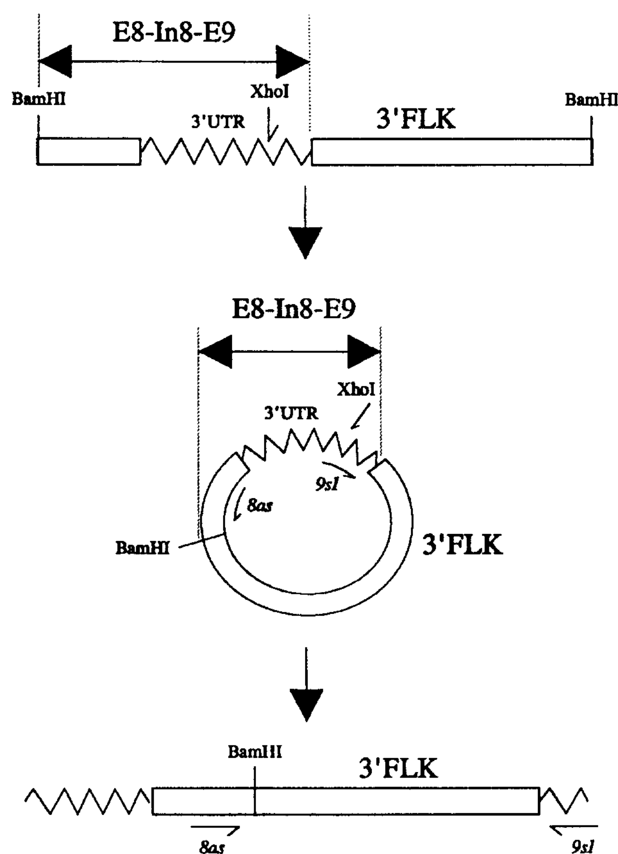

Fig. 1. Method of "inverse PCR" used as a starting point for sequencing the 3′ flanking region of sterol 27-hydroxylase gene (*CYP27* gene). Genomic DNA of a normal subject was digested with BamHI and subjected to agarose gel electrophoresis. Fragments of 2–3 kb in size were eluted from the gel and ligated with T4 DNA ligase. The circular fragments were digested with XhoI and then amplified by PCR using primers *8as* and *9s1* as indicated by the arrows. The region encompassing exon 8, intron 8, and exon 9 is indicated. 3′ UTR is an untranslated region encoded by exon 9; 3′FLK is the 3′ flanking region. The fragment of *CYP27* gene is not drawn to scale.

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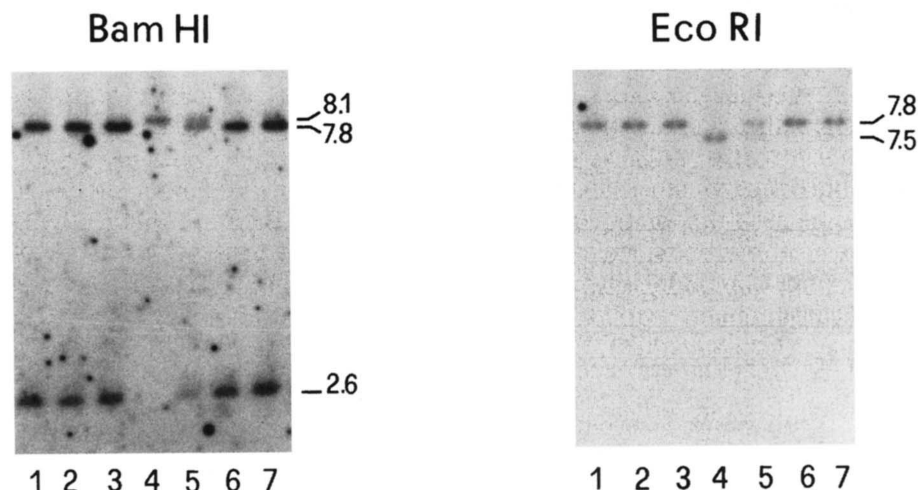

Fig. 2. Southern blot analysis of *CYP27* gene. DNA was digested with BamHI and EcoRI and hybridized with a full size cDNA probe. Lanes 1–3 and 6–7: control subjects; lane 4: proband L.M.; lane 5: proband's father. Abnormal fragments were also observed after the digestion of proband DNA with SacI, HindIII, and KpnI (see restriction map in Fig. 3).

Single primer PCR of the deletion joint in proband L.M.

Yet another strategy was adopted to define the sequence of the deletion joint, i.e., a PCR amplification using a single primer (24). The primer used was the following: 5′ TTC CTA GAA TCG CCT CAC CTG ATC T 3′ (intron 5) (*6A*) (ref. 12). Genomic DNA from the proband was amplified by PCR under three conditions to maximize the amount of the misprimed PCR products: *a)* 95°C for 30 sec, 65°C for 30 sec, and 72°C for 2 min (60 cycles); *b)* 95°C for 30 sec, 55°C for 30 sec, and 72°C for 2 min (60 cycles); *c)* 95°C for 30 min, 45°C for 30 sec, and 72°C for 2 min (60 cycles). PCR products were pooled, purified with JetPure, and sequenced directly using the *6s* primer (see above).

Screening of the deletion in healthy carriers

Genomic DNA (1 μg) from the proband's father (CTX heterozygote) and some healthy controls was amplified in a 100-μl mixture containing 0.2 mM of each dNTP, 50 pmol of each primer, 2.5 units of Taq DNA polymerase in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$). The following primers were used: forward primer complementary to exon 6 (*6s*) and the reverse primer complementary to the 3′FLK (*3′FLK as4*). In other experiments, a third primer (forward primer complementary to exon 9) (*9s3*) was used in addition to primers *6s* and *3′FLK as4*. The conditions were 95°C for 1 min, 70°C for 1 min for 30 cycles.

Computer search and gene bank

The sequence of the 3′FLK region of *CYP27* gene was compared with the National Institute of Health Genbank by DNAsis software (Hitachi, Brisbane, CA) to search for homology to Alu sequences and for the presence of secondary structures.

RESULTS

Southern and Northern blot analyses

Abnormal restriction fragments were seen after the digestion of proband's DNA with several restriction enzymes (**Fig. 2**). The presence of abnormal bands in the absence of the normal counterparts suggested that the proband was homozygote for a major rearrangement of the *CYP27* gene. In the proband's father we observed the normal fragments as well as the abnormal ones, as one would expect in a heterozygote. By comparing our results with the published restriction map of *CYP27* gene (12) we concluded that proband L.M. was homozygote for a deletion of approximately 2 kb spanning from intron 6 to the 3′ flanking region of the gene and eliminating exons 7–9 (**Fig. 3**). To ascertain whether the *CYP27* gene carrying the deletion was transcribed into a shorter mRNA, Northern blot analysis was carried out using RNA isolated from proband's skin fibroblasts. **Figure 4** shows that no mRNA was detectable in proband cells by Northern blot. To establish whether minute amounts of sterol 27-hydroxylase mRNA were present, RT-PCR was performed using primers complementary to exons 1 and 6. Even adopting this procedure we were unable to detect the presence of sterol 27-hydroxylase mRNA in proband cells, whereas the expected 940 bp fragment was observed in control cells (Fig. 4).

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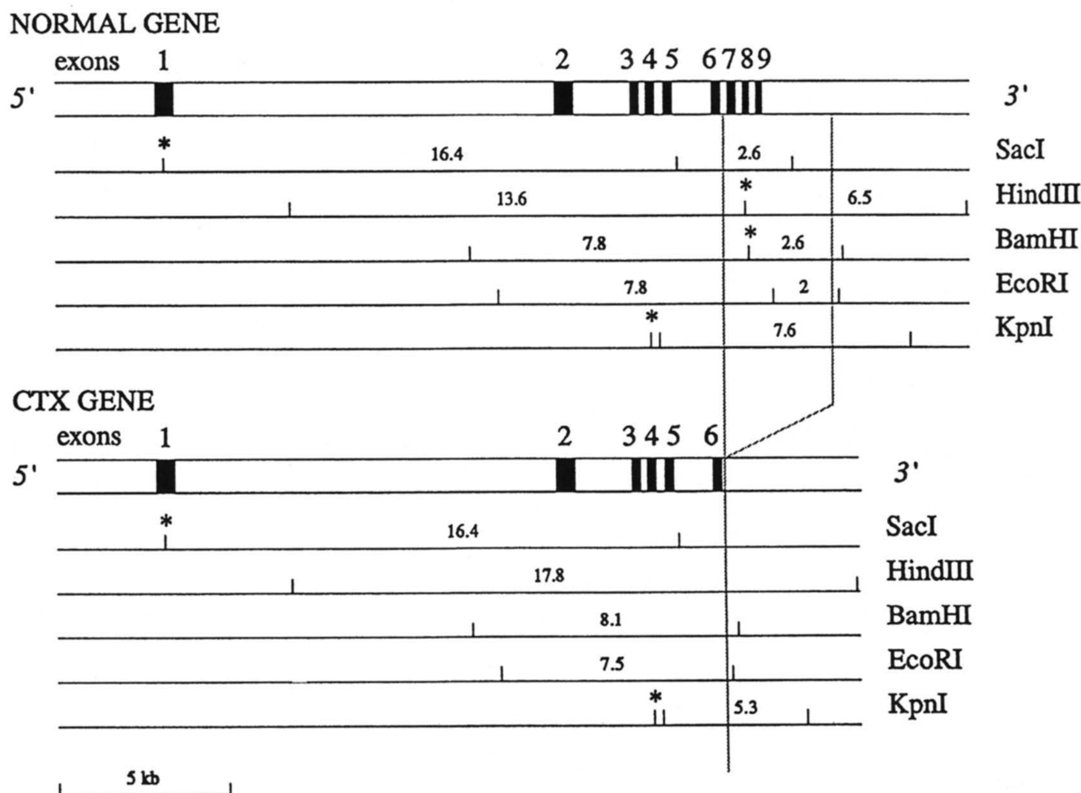

Fig. 3. Partial restriction map of *CYP27* gene in a control subject and in proband L.M. (CTX). Stars indicate the restriction sites in the cDNA (see ref. 12).

Sequence of the deletion joint

As we had no knowledge of the 3′ flanking sequence of *CYP27* gene, we could not design a suitable oligonucleotide to perform a simple and direct PCR amplification of the deletion joint from proband genomic DNA. To overcome this problem, we adopted two parallel strategies that had been previously used for short chromosomal walkings. The first (inverse PCR) (Fig. 1) (see

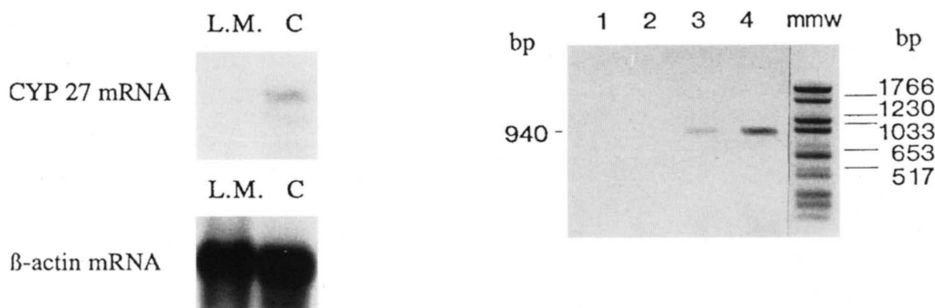

Fig. 4. Content of sterol 27-hydroxylase mRNA in cultured skin fibroblasts. The left panel shows the Northern blot of RNA isolated from cultured skin fibroblasts and hybridized with sterol 27-hydroxylase and β-actin cDNAs. Lane LM: proband L.M.; lane C: control subject. The sterol 27-hydroxylase mRNA consists of a major band (2.2 kb) and a minor band (1.8 kb) as previously reported by Cali and Russell (10). The right panel shows the reverse transcription and PCR amplification (RT-PCR) of sterol 27-hydroxylase mRNA. Lanes 1–2: proband L.M.; lanes 3–4: control subject. The concentration of $MgCl_2$ in the reaction mixture was 1 mM (lanes 1 and 3) and 2 mM (lanes 2 and 4). The primers used for RT-PCR were complementary to exon 1 (*1s*) and exon 6 (*6as*), respectively (see Methods for details).

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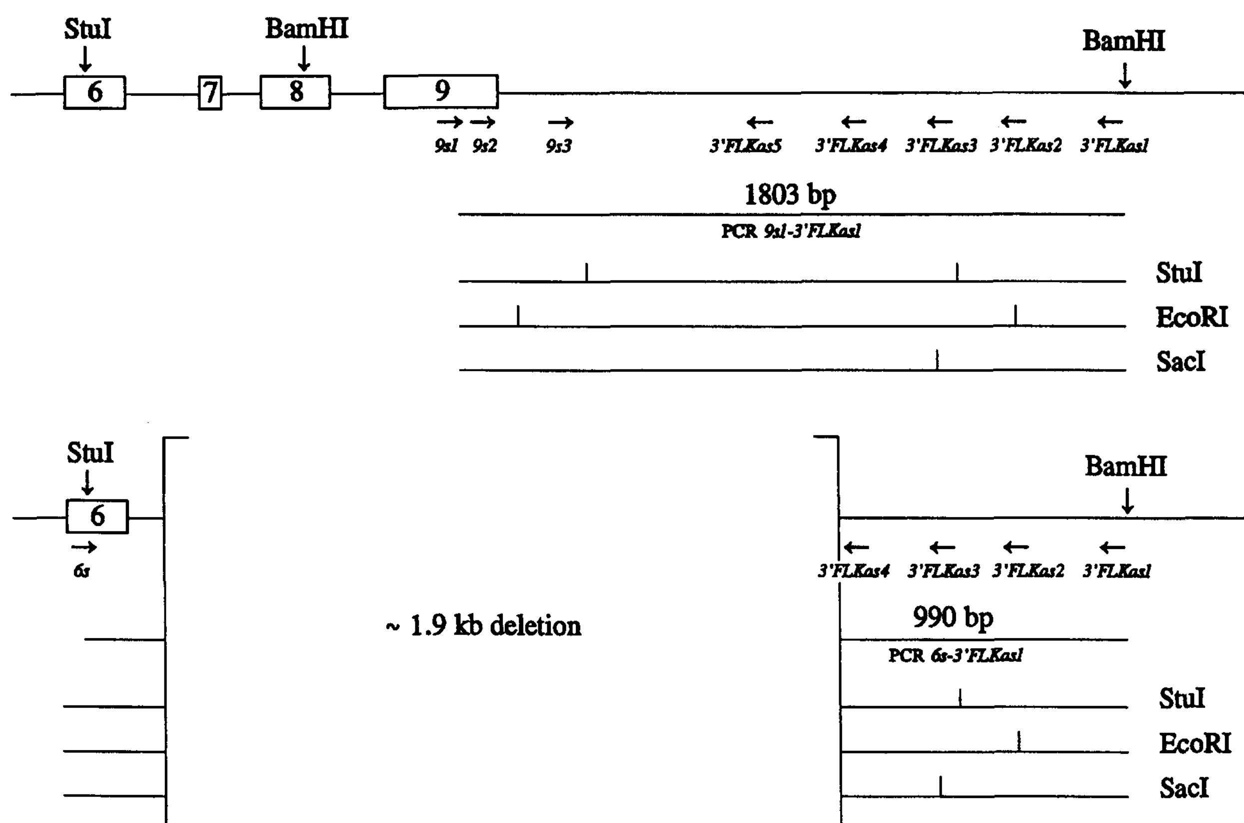

Fig. 5. Strategies used for sequencing the 3′FLK region of *CYP27* gene in a normal subject (upper panel) and in proband L.M. (lower panel). The oligonucleotides used for sequencing indicated by the arrows are given in the Methods. The size of the PCR fragments obtained in a normal subject (PCR *9s1–3′FLK as1*) and in proband L.M. (PCR *6s–3′FLK as1*) are also indicated.

Methods for details) allowed us to obtain a partial sequence of the distal 3′ flanking region of the normal *CYP27* gene, close to the BamHI site (Fig. 3). On the basis of this sequence we designed an oligonucleotide (*3′FLK as1*) which, in combination with a primer complementary to exon 6 (*6S*), was used for the direct PCR amplification of the putative deletion joint from proband genomic DNA (**Fig. 5**). This PCR amplification generated a fragment of 1 kb that was sequenced directly.

The second strategy ("single primer PCR") is a procedure that allows some unknown sequences adjacent to regions of known sequence to be amplified. By using a single primer and taking advantage of the mispairings of this primer to imperfect complementary sequences of the adjacent unknown region, it is possible to generate several PCR products of different length. It is likely that some of these products result from the amplification between the true complementary binding site at one end and a false priming site at the other end.

Both inverse PCR and single primer PCR were successfully applied to sequence the deletion joint that is shown in **Fig. 6.** The sequence of the deletion joint allowed us to define more precisely the size of the deletion with respect to the map derived from Southern data (Fig. 3 and Fig. 5).

Sequence of the 3′ flanking region of the normal *CYP27* gene

As previously pointed out, before the present study the sequence of the 3′ flanking region of the normal *CYP27* gene was unknown. As mentioned above, the strategy of inverse PCR allowed us to obtain the basic information on a partial sequence of the distal 3′FLK region close to the BamHI site (Fig. 5). By using the oligonucleotide *3′FLK as1* we were able to amplify a region of genomic DNA of a normal subject spanning from exon 9 to the BamHI site of the 3′ flanking region (1.8 kb). The sequence of this region (Fig. 7) revealed several interesting features such as *a)* a 14-nucleotide sequence (from nt 813 to nt 800) that is in an opposite orientation with respect to a 15-nt sequence in intron 6 (see below); *b)* a 280-nucleotide inverted Alu sequence (from nt 1163 to nt 883) that has an 82% homology with

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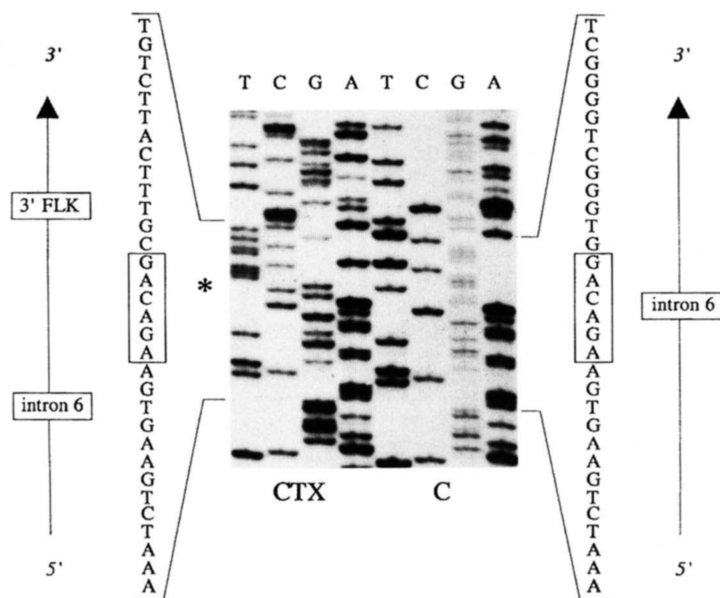

Fig. 6. Nucleotide sequence of the deletion joint. In proband L.M. (CTX) intron 6 joins to the 3′FLK region of *CYP27* gene, whereas in the control subject (C) the sequence of intron 6 is not interrupted (see also Fig. 8 for the complete sequence of intron 6). In the control subject, the boxed six-nucleotide sequence is present in intron 6 and also in the 3′FLK region (see Fig. 7). In proband L. M., the deletion eliminates one of these six-nucleotide repeats so that the remaining one (boxed) might belong either to intron 6 or to the 3′FLK region (see also Fig. 9).

the consensus Alu sequence (23); *c)* a 71-nucleotide sequence (from nt 814 to nt 884) that is rich in AT (77%); and *d)* a 6-nucleotide sequence (AGACAG) that is identical to the one found in intron 6 (see below).

Sequence of introns 6, 7, and 8 of normal *CYP27* gene

The complete sequences of introns 6, 7, and 8 that were unknown before the present study are shown in **Fig. 8.** Intron 6, which is involved in the deletion found in proband L.M., contains: *a)* a 15-nucleotide sequence (from nt 86 to nt 100) that is complementary to a 14-nucleotide sequence in the 3′ flanking region of the gene; *b)* a 6-nucleotide sequence (AGACAG) that is repeated in the 3′ flanking region of the normal *CYP27* gene (see above) and is located at the deletion joint in proband L.M. (Fig. 6 and **Fig. 9**).

Screening of the deletion of *CYP27* gene in healthy carriers

The sequences of the deletion joint and the 3′ flanking region of *CYP27* gene in proband L.M. (Figs. 5 and 6) suggested the rationale for a nonradioactive method for the screening of the deletion in healthy carriers. By using a forward primer complementary to exon 6 (*6s*) and a reverse primer complementary to a sequence in the 3′FLK region (*3′FLK as4*) (Fig. 5), we expected to get two PCR fragments of 2186 bp and 275 bp in the proband's father, corresponding to the normal and the deleted allele, respectively. While we could clearly detect the 2186 bp fragment in the control subjects, in the proband's father we obtained the expected 275 bp fragment but we failed to detect the 2186 bp fragment (data not shown). PCR amplification carried out in the presence of an additional forward primer complementary to exon 9 (*9s3*) (Fig. 5), a condition that could facilitate the amplification of the normal allele, failed to produce the fragment corresponding to the normal allele.

The amplification of the normal and the deleted allele in the heterozygote was possible, however, by performing two independent PCR amplifications, one using primer *6s* and primer *3′FLK as4* (for the detection of the deleted allele) and the other using primer *9s3* and primer *3′FLK as4* (for the detection of the normal allele).

DISCUSSION

Since the first report by Cali et al. (11), who documented the presence of missense mutations of *CYP27* gene in two patients with CTX, several point mutations have been characterized in various countries (11–18). Some of these mutations have been found at a relatively high frequency in some ethnic groups such as the Jews of North African origin (13) or the Israeli Druze (14). The frequency and type of mutations of *CYP27* gene in

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5'GCATTGCTGT CCTTGGGTAG AATATAAAAT AAAGGGACTT TTATTTCTTA
          10         20         30         40         50
 TTGGAGACCT TTGTCATTCT TTTTGACTAC CCAGCATCTT AGGGAATTCC
         60         70         80         90        100
 TCTCCTCCAG TGAGTCACGG TGAGAGGCAC AGCCGCCCTG TACCTTGGAA
        110        120        130        140        150
 ACTGAGAACA CCTTTTAGCT TTCCCAGACC TCACAGAGAG GCCTTGGTTT
        160        170        180        190        200
 CACGAGGCAC TGGTTGAGGC CACACCAAGC AGGAACTGCA GTGACTCCCA
        210        220        230        240        250
 AGCACTGTGT CAGTCAAAGC TGCGGTTGTA GAGAATATAA ACCACCCTCT
        260        270        280        290        300
 TTGAACAGAA AAAGCAGATA CAGGCAATGG GGTGCTACAA AAATTATTGG
        310        320        330        340        350
 AGGTGCTCGA GACCAGGCTA GAGTTGACCT CCAGCAGGCA CTGAGCAGCT
        360        370        380        390        400
 TGGGGAGCAC CATGTTGGCC ACAATCAGGA GGATGAGAGT CTAGAAGCTA
        410        420        430        440        450
 TTGCTACAAC TATTGGCTCT AGAGCTACTC CATGTCAATG AAGTTCAAGT
        460        470        480        490        500
 AGGTCTGTGC CGGCAAAACA AAACAAAACA GGTCACGCAG GATGACCCCC
        510        520        530        540        550
 ATCCTGTCTC TCAATACCCA TGAAGCCAGC AGTAACTGGA CTTGGACCTC
        560        570        580        590        600
 TGCAAATGCC TCGCGACTGC TCCTGACAGC AGAAAGGGCA GGAGCTTCTA
        610        620        630        640        650
 CCTCTCTTCC ACCTTTCACA TCTCTCACAT ACAGCTGAGA GGAGGAACCT
        660        670        680        690        700
 AAGTCCTGCC TGGAGCACTA GCTGTAAGGG AGCCTAGGGA TTATAGCTTT
        710        720        730        740        750
 TAACTGTCCA GCCTCCTTGC AACAAGAAGC CCACTTGAAG GAAAGTGGGT
        760        770        780        790        800
 TGCCATTGAG AGCCGCCAGT GCATCACAGC ACTAGCCTGG AAATTCAGAG
        810        820        830        840        850
 TCCAAGCCCC AGCAGTAAAT TTTGGTGGAG TCATAGAAAA CAATGAAGTT
        860        870        880        890        900
 CACTTTCTTT TTTAAAAATT AATTTGATTT TTTG[AGACAG]CGTTTCATTC
        910        920        930        940        950
 TGTCACCCAG ACTGGAGTGC AGTGGCACCA TCTTGGCTCA CTGCCTCCTT
        960        970        980        990       1000
 AACCCAGGCT CAAGCGATTC TCCCACTTTA GCCCCCCGAG TAGCTGGAAC
       1010       1020       1030       1040       1050
 TACAGGCACG CACCACCTCG CCTGGCTAAT TTTTGTATTT TTTTTGTAGA
       1060       1070       1080       1090       1100
 GATGGGGTTT TGCCATGTTG GCCAGGCTGG TCTCGAACTC CGGAGCTCAA
       1110       1120       1130       1140       1150
 GGGATCTGCC TGCCTTGGTC TCCTGAAGTG CTGGGATTAC AGGCGTGAGC
       1160       1170       1180       1190       1200
 CACTGCGACA ACCTAAAGTT CACTTTCTCA GGCCTGTGGG AATGATAGGG
       1210       1220       1230       1240       1250
 TTTGAACTCA TTTAAAAAAT TGTGTCATTG CCCGGATTAA AATACTCCAC
       1260       1270       1280       1290       1300
 AACTGCCCTC AGCCCTCAGG TCAAGTTCAA AGTTGTTACT GTGGCTCATA
       1310       1320       1330       1340       1350
 AGACCTACCG TGCCCTGGCA CCCTGCACCC TGACCGTGGT CACCCTTTCA
       1360       1370       1380       1390       1400
 CAGCTGAGCC TGAATTCTTT GCATGTCTCT GAACACATTA TGTTCTTTCC
       1410       1420       1430       1440       1450
 AGCCTCAATA CTTTGTACCT CCTGTCCCCA CTCTTGGAAC ACCCTCTTCC
       1460       1470       1480       1490       1500
 CTAACTGTCA TGTCATCACA GAGAGCTTGC TCTTTCTTAG GATTCAGCTC
       1510       1520       1530       1540       1550
 AAATGTCACT GCCTCTGCAG GGCCCTCCCT GACCTCTCTG AGCAATCCTG
       1560       1570       1580       1590       1600
 TGCCCCACCT TCCCCTCCTC TGCTTACAGA GCTGAGCTGT CAGCATGCTC
       1610       1620       1630       1640       1650
 CTTGTTAGAG ATCCAGCTCT CTCCCCAACC AACTGTGAAT CCCCTGAAGG
       1660       1670
 CCACAGTCAG GCTTCTTTAA TGTTG (BamHI) 3'
```

Fig. 7. Nucleotide sequence of the 3′ flanking region of *CYP27* gene. The last nucleotides of the 3′ untranslated region encoded by exon 9 (ref. 10) are underlined by a broken line. The polyadenylation signal is indicated by an overline. The 14-nucleotide sequence (from nt 813 to nt 800) and the AT-rich region (from nt 814 to nt 884) involved in the formation of the two stem-loop structures (see also Fig. 9) are underlined. The six-nucleotide sequence also present in intron 6 (see Fig. 8) is boxed. An inverted Alu sequence is indicated by a dotted line.

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INTRON 6

```
           10         20         30         40         50
5' GAGACTCTGC Ggtaggacag aatgctgttc tggggggcac aggatctctt
           60         70         80         90        100
   tgtggggagg gaatcagagg aggaaatctg aagtgaagac aggtgggctg
          110        120        130        140        150
   gggctagtga caaggatgag atgggagagg taggggagaa ggagtggggc
          160        170        180        190        200
   actttgtacc cccatgaatc cagagcaaga ctccagacat tctttccctg
          210
   cagTCTCTAC CCT 3'
```

INTRON 7

```
           10         20         30         40         50
5' CCCAAGAACg tgagtggggc tagagagccc gattgcccag gagtgcccta
           60         70         80         90        100
   tgcccccgaa gagaggcatt catgctgccc aatcttcctt tatagACCCA
          110
   GTTTGTGTTC 3'
```

INTRON 8

```
           10         20         30         40         50
5' CTCGCAAGGg tgagctggga gaggctagta gggtgtgtgg gcagggaggg
           60         70         80         90        100
   gtggaggagt cctgggagga gaggaaggga ggcacagggt aggagtgtgc
          110        120        130        140        150
   agagcgggga gtggatggca aacacacaat ccacccaacc acatgtgctc
          160        170
   tttacccccc agCTGATCCA 3'
```

Fig. 8. Nucleotide sequence of introns 6, 7, and 8 of *CYP27* gene. The upper-case letters indicate the last and the first nucleotides of the exons. In intron 6 the six-nucleotide sequence, also present in the 3'FLK region, is boxed. The 15-nucleotide sequence involved in the formation of the stem-loop structure with the complementary 14-nucleotide in the 3'FLK region (Fig. 7) is underlined (see also Fig. 9).

genetically heterogenous populations are still unknown. We have started a survey of *CYP27* gene mutations in Italian CTX patients in an attempt to define the most frequent mutations and to investigate the genotype–phenotype relationship in both the homozygous and the heterozygous subjects. Out of 7 apparently unrelated probands we have investigated so far, only one was found to carry a major deletion of *CYP27* gene. The case we report in this study is, to the best of our knowledge, the first major rearrangement of *CYP27* gene observed in a CTX patient. The results of Southern blot analysis unequivocally demonstrated that the proband L.M. was homozygous for a 2-kb deletion spanning from intron 6 to the 3' flanking region of the gene and eliminating its last three exons (exons 7–9). We assume that this deletion produces a null allele as no sterol 27-hydroxylase mRNA was detected in proband's skin fibroblasts. Even if present in minute amounts, the mutant mRNA would presumably encode a truncated protein of 362 amino acids. The joining of exon 6 to the unspliced 5' half of intron 6 would introduce a premature termination codon following arginine at position 362. This truncated enzyme, if present, would be unfunctional, as it is devoid of the heme binding domain (10). The absence of the mRNA in the presence of a major deletion is not surprising, as several reports have demonstrated that the content of structurally abnormal mRNAs containing premature termination codons is frequently reduced in proband cells probably as the result of an increased degradation rate (25). This mechanism may have occurred in proband L.M. in whom the deletion eliminates exon 9 that encodes the 3' untranslated region of the mRNA containing the polyadenylation signal (10). As the shortening of poly-A tail (deadenylation) appears to be the first step in mRNA decay (26), it is most likely that a truncated mRNA devoid of the polyadenylation signal (i.e., completely deadenylated) is degraded shortly after transcription.

By using several strategies we were able to sequence the deletion joint in proband L.M. as well a fragment of 1.7 kb of the 3'FLK region of the *CYP27* gene in a normal subject. The analysis of these sequences has provided the basis for a reasonable hypothesis concerning the mechanism underlying the deletion in proband L.M. As illustrated in Fig. 9, the presence of a stretch of 14 oppositely oriented nucleotides located in intron 6 and in the 3' flanking region may induce the formation of a first stem-loop structure. A second stem-loop structure may occur further downstream in the gene where an AT-rich sequence is present. As observed in other major deletions of the LDL-receptor gene or of the β-globin cluster (27–31), these secondary structures generated by intragenic recombinations might be the underlying mechanism of the deletion observed in proband L.M.. Figure 9 also shows that the formation of the two stem-loops joins two six-nucleotide direct repeats (AGACAG) that under normal circumstances are located far apart, one in intron 6 and the other at the beginning of an inverted Alu sequence in the 3'FLK region. It is conceivable that these direct repeats play an important role in the deletion of proband L.M. because they are thought to cause slipped-mispairing during DNA duplication (32). Slipped-mispairing involves complementary base pairing between the temporarily melted template and the copied strands. During reannealing the two strands slip out of correct register and anneal again. If the copied strand slips 5' and reanneals, then a duplication results, whereas if it slips 3' on the template strand, then a deletion occurs (32).

The deletion described in this report was found in a CTX patient who belongs to a large family that has been living for several generations in a small district in southern Italy. When a rapid method of screening is made available, the accurate identification of the heterozy-

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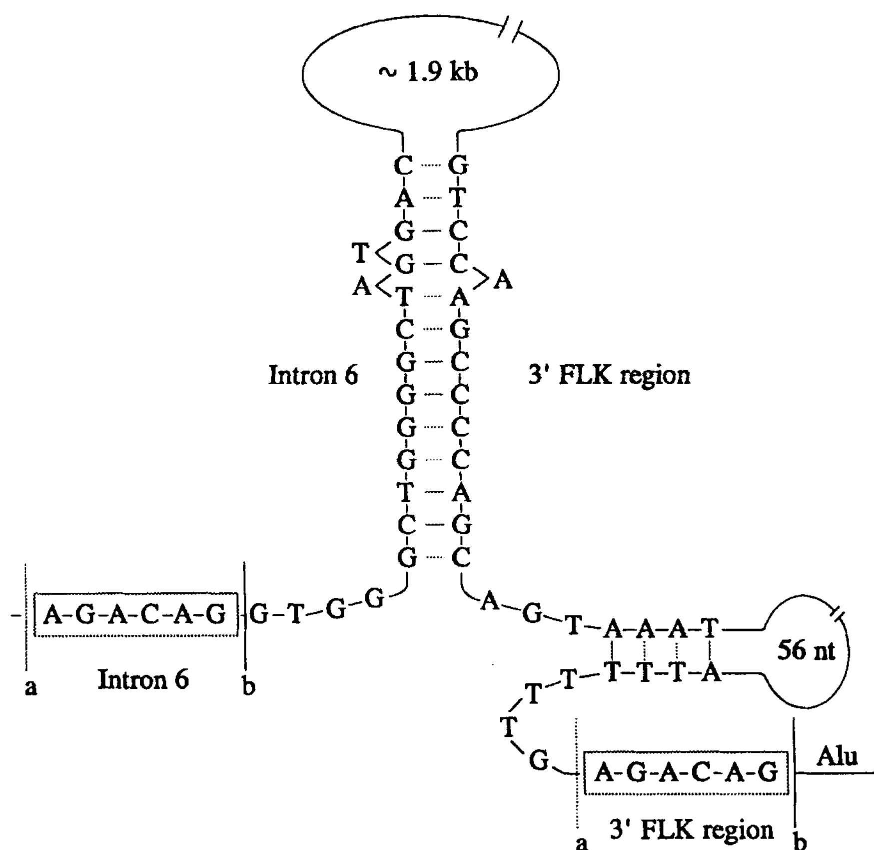

Fig. 9. Possible secondary structure in the region of the breakpoints of *CYP27* gene in proband L.M. The figure represents two hypothetical stem-loops with their potential hairpin structures that may cause the deletion. The formation of hairpin structures brings two six-nucleotide direct repeats (boxed) close to each other. The vicinity of these direct repeats may cause slipped-mispairing during DNA replication (32) with the elimination of one of these repeats at the deletion joint. The discontinuous (a) and continuous (b) vertical lines indicate the possible breakpoints. If the breakpoints are at position a, the AGACAG sequence present at the deletion joint (Fig. 6) belongs to the 3'FLK region; if the breakpoints are at position b, the AGACAG sequence present at the deletion joint belongs to intron 6.

gotes in that area will be feasible. We have attempted to devise a simple nonradioactive method based on PCR amplification of a region of *CYP27* gene spanning from exon 6 to the 3'FLK region, a method that could allow the simultaneous identification of the normal and the deleted allele. However, in view of the large size difference between the fragment corresponding to the normal allele (2186 bp) and the one corresponding to the deleted allele (275 bp), there was a selective amplification of the deleted allele and no amplification of the normal one, regardless of the experimental conditions used. The only way to detect the heterozygote for the deletion was to perform two independent PCR amplifications. In the first amplification the normal allele is amplified (792 bp fragment) using *9s3* as forward primer and *3'FLK as4* as reverse primer (Fig. 5). The deleted allele is amplified in the second amplification using *6s* as forward primer and *3'FLK as4* as reverse primer, as specified above (Fig. 5). A genetic study of the family of proband L.M., besides its obvious practical implications, will allow us to identify the heterozyotes and to study their phenotype on a more sound genetic basis than was possible in the past.

The financial support of Telethon-Italy (grant E.156 to S.C.) is gratefully acknowledged.

Manuscript received 17 October 1995 and in revised form 14 December 1995.

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