A Novel Arg[362]Ser Mutation in the Sterol 27-Hydroxylase Gene (*CYP27*): Its Effects on Pre-mRNA Splicing and Enzyme Activity[†]

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ABSTRACT: A novel C to A mutation in the sterol 27-hydroxylase gene (*CYP27*) was identified by sequencing amplified *CYP27* gene products from a patient with cerebrotendinous xanthomatosis (CTX). The mutation changed the adrenodoxin cofactor binding residue [362]Arg to [362]Ser (CGT [362]Arg to AGT [362]Ser), and was responsible for deficiency in the sterol 27-hydroxylase activity, as confirmed by expression of mutant cDNA into COS-1 cells. Quantitative analysis showed that the expression of *CYP27* gene mRNA in the patient represented 52.5% of the normal level. As the mutation occurred at the penultimate nucleotide of exon 6 (−2 position of exon 6−intron 6 splice site) of the gene, we hypothesized that the mutation may partially affect the normal splicing efficiency in exon 6 and cause alternative splicing elsewhere, which resulted in decreased transcript in the patient. Transfection of constructed minigenes, with or without the mutation, into COS-1 cells confirmed that the mutant minigene was responsible for a mRNA species alternatively spliced at an activated cryptic 5′ splice site 88 bp upstream from the 3′ end of exon 6. Our data suggest that the C to A mutation at the penultimate nucleotide of exon 6 of the *CYP27* gene not only causes the deficiency in the sterol 27-hydroxylase activity, but also partially leads to alternative pre-mRNA splicing of the gene. To our knowledge, this is the first report regarding effects on pre-mRNA splicing of a mutation at the −2 position of a 5′ splice site.

Cerebrotendinous xanthomatosis (CTX),[1] an autosomal recessively inherited cholesterol metabolic disorder, is caused by mutation in the sterol 27-hydroxylase gene (*CYP27*). The disease is characterized by tendon xanthomatosis, cataracts, diverse neurologic manifestations (*1*), osteoporosis (*2*), and premature atherosclerosis (*3*), due to the deposition of cholesterol and cholestanol in different tissues (*4*, *5*). Following the cloning of human sterol 27-hydroxylase cDNA (*6*) and determination of its genomic structure (*7*), several types of mutation, including insertion (*8*), deletion (*9*, *10*), and point mutations (*11*−*18*) of the *CYP27* gene, have been identified, in CTX patients from different countries.

Study of de novo mutations occurring at splice site regions in many inherited diseases contributes greatly to understanding of mechanisms of normal pre-mRNA splicing. Although many splice site mutations at the absolutely conserved intronic 5′ gt or 3′ ag dinucleotide have been shown to cause aberrant pre-mRNA splicing or to abolish normal pre-mRNA transcription (*19*−*21*), less attention has been directed to the effects on pre-mRNA splicing of mutations at the moderately conserved exonic positions of a 5′ splice site.

We previously identified a G to A mutation (CGT [362]Arg to CAT [362]His) at the last nucleotide of exon 6 (−1 position of the exon 6−intron 6 splice site) in the *CYP27* gene (*22*), and confirmed that the mutation led to alternative pre-mRNA splicing of the gene, including exon 6 skipping and activating of a cryptic 5′ splice site in exon 6 (*23*). We now report a novel 362 codon mutation (CGT [362]Arg to AGT [362]Ser) of the *CYP27* gene. The C to A mutation occurred at the −2 position of the exon 6−intron 6 splice site, and was responsible for alternative pre-mRNA splicing of the gene, as confirmed by transfection of mutant minigene into COS-1 cells. To our knowledge, this is the first study about the effects of a mutation at the penultimate nucleotide of an exon on pre-mRNA splicing.

MATERIALS AND METHODS

Subject. A 57-year-old Japanese male with CTX was studied. The patient showed typical CTX manifestations including xanthomatosis, cataract, mental retardation (IQ 41), and cerebellar and pyramidal signs. Cardiovascular investigations were normal. Biochemical analysis showed a markedly elevated plasma cholestanol concentration of 26.6 μg/mL with a cholesterol level of 1.12 mg/mL. Informed consent was obtained from the patient.

Full-Length cDNA Amplification of the Sterol 27-Hydroxylase Gene. To amplify full-length cDNA of the sterol 27-hydroxylase gene, 1 μg of total RNA, extracted from the

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[1] Abbreviations: CTX, cerebrotendinous xanthomatosis; *CYP27* gene, sterol 27-hydroxylase gene; pre-mRNA, precursor messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; HPLC, high-performance liquid chromatography; U1 snRNA, U1 small nuclear RNA.

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

primer	location	amplification	5′ to 3′ sequence	position
FRup	5′-flanking	5′-flanking	GGTGTGGGGCTTCCCGATTT	−312 to −293[a]
FRd	exon 1	5′-flanking	CCTCAGCCTCGCGCAGCCCA	51 to 32[a]
E1up	5′-flanking	exon 1	ACTCAGCACTCGACCCAAAGGTGCA	−22 to 3[a]
E1d	intron 1	exon 1	CCATCCCATCCCCAGGACGCGATG	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]
FLup	exon 1	full-cDNA	CCATGGCTGCGCTGGGCTGCG	20−40[a]
FLd	3′-flanking	full-cDNA	CCCAGCAAGGCGGAGACTCAGC	1639−1618[a]
SPup	exon 5		CGAAGATATGGAGGCCCAACT	891−911[a]
SPd	exon 9		TCAGCACTGTCTCTGCAGGAAC	1596−1617[a]

[a] Nucleotide number at the cDNA reported by Cali and Russell (*6*). [b] Minimal distance from exon.

patient's peripheral white blood cells, was first converted to cDNA by oligo-dT, using a RT-PCR kit (TaKaRa, Otsu City, Japan). The reaction tube was incubated at 42 °C for 60 min, heated at 95 °C for 5 min, and then soaked at 5 °C for 5 min in a thermal cycler (Perkin-Elmer, GeneAmp PCR system 9600). PCR amplification was immediately performed after the RT reaction by adding 80 μL of a PCR Master Mix containing upstream primer FLup, downstream primer FLd (Table 1), and 11.4 μL of DMSO. The amplification reaction was run for 30 cycles or 20 cycles (for quantitative analysis) under the following conditions: 1.5 min at 95 °C for denaturation, 30 s at 68 °C for annealing, and 2 min (4 s increment every successive cycle) at 72 °C for extension.

Quantitative Analysis of Transcript. To determine the transcript level in the patient, 8 μL of full-length RT-PCR products, amplified by 20 cycles from the patient and a healthy subject, was separated by electrophoresis on a 5% polyacrylamide gel, and the fragments were confirmed by sensitive silver stain using a kit from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). GAPDH cDNA was used as a control. Quantitative analysis was carried out by scanning the gel, and the density of the bands was analyzed using the software NIH Image, 1.61.

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

Sequence Analysis. DNA sequence analysis was performed using the ABI PRISM 310 Genetical Analyzer, as described (*24*). All of the fluorescein sequencing reagents were purchased from ABI. All the samples were sequenced in both directions to confirm the apparent mutation. The primers used for sequencing are listed in Table 1.

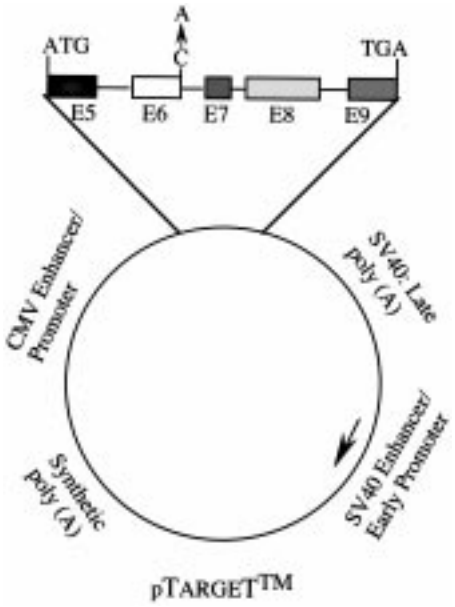

FIGURE 1: Schematic representation of minigene constructs. The insert containing exons 5−9 of the *CYP27* gene was cloned into pTARGET expression vector. The C to A mutation in the penultimate nucleotide of exon 6 is indicated by an arrow.

Construction of Expression Vector. To observe the effects of the identified mutation on pre-mRNA splicing, two minigene constructs, with or without the mutation (Figure 1), were generated for transfection into COS-1 cells. Genomic sequences from exon 5 to exon 9 of the *CYP27* gene were amplified from the patient and from a healthy subject, using primers SPup (5′CGAAGATATGGAGGCCCAACT3′, nucleotides 891−911) and SPd (5′TCAGCACTGTCTCTGCAGGAAC3′, nucleotides 1596−1617). The PCR amplification reaction was run for 30 cycles, under the following conditions: 1 min at 95 °C for denaturation and 4 min at 68 °C for annealing and extension. After confirmation of successful amplification of the 2111 bp band by agarose gel

electrophoresis, the product was directly ligated into the pTARGET expression vector (Promega, Madison, WI). Plasmids of the minigene constructs were prepared using JETSTAR Plasmid Kit (Genomed, Research Triangle Park, NC). The two minigenes have the same structure, except for the C to A mutation at the penultimate nucleotide of exon 6 (−2 position of exon 6−intron 6 splice site), as confirmed by sequence analysis.

For expression analysis of mutant and normal full-length cDNAs, the above full-length RT-PCR products of the patient and the healthy subject were subcloned into the pTARGET expression vector. Plasmids of the mutant and normal full-length cDNA construct were prepared. The two expression constructs had the same structure except for the C to A mutation at codon 362 in the mutant one, as confirmed by sequence analysis.

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

To determine the effect of the C to A (CGT ^{362}Arg to AGT ^{362}Ser) mutation on sterol 27-hydroxylase activity, 20 μg of plasmids carrying normal cDNA or mutant cDNA and a mock vector were transfected into 1×10^6 cells, as described above. After 48 h, cells were harvested for immunoblot analysis and sterol 27-hydroxylase assay.

Immunoblot Analysis. Mitochondrial fractions were isolated from COS-1 cells transfected with the normal and mutant full-length cDNA. A total of 20 μg of protein was loaded on a 10% SDS−polyacrylamide gel and was transferred into a Hybond ECL nitrocellulose membrane (Amersham LIFE SCIENCE, Little Chalfont, U.K.) after electrophoresis. An anti-peptide antibody directed against residues 15−28 of the mature sterol 27-hydroxylase, extracted from rabbit serum (a kind gift from Dr. David W. Russell), was diluted 1/1000 and incubated with the membrane for 1 h at room temperature. Incubation with 1/2000 secondary antibody, anti-rabbit IgG horseradish peroxidase-conjugated whole antibody from goat, was carried out for 1 h at room temperature. Development was performed using the Amersham ECL detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Assay of Sterol 27-Hydroxylase Activity. Assay of sterol 27-hydroxylase activity was performed using a procedure described by Skrede et al. (*25*). Labeled substrate, 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol with specific activity 150 cpm/pmol, was synthesized as described and purified by HPLC (*26*). The substrate (480 000 cpm, 3.2 nmol) was dissolved in 10 μL of acetone (which was then evaporated under a nitrogen stream), and solubilized in 250 μL of 5% bovine serum albumin followed by addition of the following incubation mixture: 33 mM Hepes (pH 7.4); 5 mM ATP; 5 mM potassium malate; 1 mM glucose 6-phosphate; 0.5 IU of glucose-6-phosphate dehydrogenase; 1.2 mM NADPH and 15 mM $MgCl_2$. Transfected COS-1 cells were harvested and washed 3 times with PBS. The cell pellet was then suspended in 250 μL of 0.25 M sucrose, and added to the incubation mixture to start the reaction. After incubation at 37 °C for 1 h, the reaction was terminated by adding 0.1 mL of 1 M HCl. Extraction with 5 mL of ethyl acetate was performed twice, the converted 27-hydroxylated product was separated by HPLC (LC-10A Shimadzu, Kyoto, Japan) using a LC-18 column (250 × 4.6 mm, Supelco, USA), and the radioactivity was measured using a radiodetector (RLC-700, Aloka, Tokyo, Japan). The activity was expressed as a percentage of the conversion of the substrate.

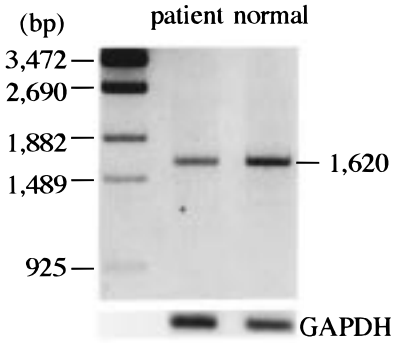

FIGURE 2: RT-PCR analysis of the full-length sterol 27-hydroxylase mRNA. Full-length cDNA of the *CYP27* gene was amplified, using primers FLup and FLd, as described under Materials and Methods. Electrophoresis was performed on a 2% agarose gel. *λ-Eco* T14 I DNA marker was used. GAPDH cDNA was amplified simultaneously for quantitative analysis.

RESULTS

Full-Length RT-PCR Analysis. Although full-length RT-PCR analysis showed a normal 1620 bp band in the patient (Figure 2), the mRNA level, standardized with respect to GAPDH, represented 52.5 ± 8.0% of that in the healthy subject, as determined by quantitative analysis of the RT-PCR products amplified by 20 cycles. cDNA band amplified from alternatively spliced mRNA species was not evident, probably due to instability of the species.

Mutation of the CYP27 Gene. Direct sequence analysis of the patient's full-length RT-PCR product showed a C to A mutation at codon 362 (CGT ^{362}Arg to AGT ^{362}Ser) (Figure 3), which corresponds to the penultimate nucleotide of exon 6 in genomic DNA (−2 position of the exon 6−intron 6 splice site) (Figure 4). The mutation was confirmed on both strands of duplicately amplified PCR products, both in cDNA and in genomic DNA. Except for this mutation, sequences of the 5′-flanking region and all nine exons including the splicing−junction regions of the *CYP27* gene were identical to that found in the healthy subject (data not shown).

Transcription Analysis of the Mutant Gene in COS-1 Cells. We hypothesized that as the mutation occurred at the penultimate nucleotide of exon 6 (−2 position of the exon 6−intron 6 splice site), it may partially affect the normal splicing efficiency in exon 6 and cause alternative splicing elsewhere, which may account for the decreased mRNA observed by quantitative analysis in vivo. This was examined by transfection of the mutant minigene into COS-1 cells. Minigene constructs consisting of genome sequences from exon 5 to exon 9 of the *CYP27* gene, with or without the mutation, were transiently transfected into COS-1 cells. RT-PCR analysis of RNA extracted from the transfected cells using primers SPup and Spd showed a single 726 bp band

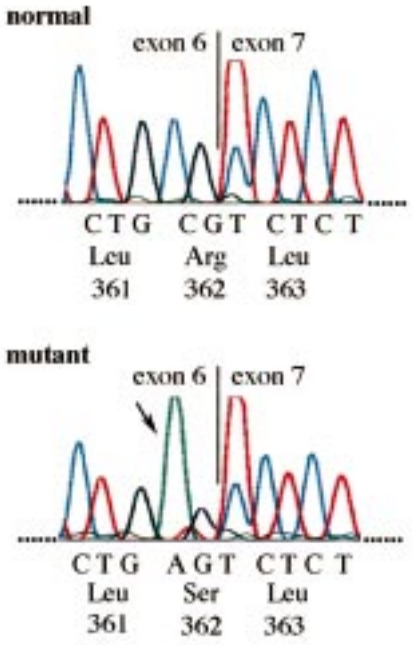

FIGURE 3: Direct sequence analysis of the full-length RT-PCR product. Full-length cDNA products amplified by RT-PCR were directly sequenced as described under Materials and Methods. The C to A mutation is indicated by an arrow.

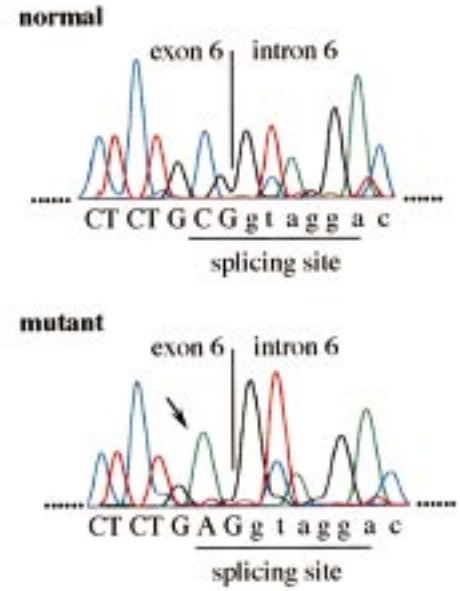

FIGURE 4: Direct sequence analysis of PCR products amplified from genomic DNA. The 5′-flanking region and all nine exons, including the splicing−junction regions of the *CYP27* gene, were amplified by PCR, respectively, using the primers listed in Table 1, and then subjected to direct sequencing analysis as described under Materials and Methods. The C to A mutation is indicated by an arrow.

in the normal minigene, while in the mutant minigene an alternatively spliced 638 bp band was observed (Figure 5). Direct sequence analysis confirmed that the single 726 bp band observed in the normal minigene corresponded to the correctly spliced RNA species. On the other hand, the mutant band was missing the 3′-terminal 88 bp of exon 6 due to use of an activated cryptic 5′ splice site (Figure 6). This alternatively spliced species resulted in a frameshift and formed a premature terminal codon 47 bp downstream from the 5′ end of exon 7. Thus, the data confirmed that the normal minigene could be spliced correctly, while the C to A mutation at the penultimate nucleotide of exon 6 (−2 position of the exon 6−intron 6 splice site) was responsible for the alternative pre-mRNA splicing in COS-1 cells.

Transfection Analysis of the Mutant Full-Length cDNA. The mutation changed the codon ^{362}Arg to ^{362}Ser. Its effect on the sterol 27-hydroxylase activity was checked by transfection of the mutant cDNA into COS-1 cells. As shown in Figure 7A, both of the cells transfected with the normal and the mutant cDNAs expressed two immunoreactive proteins corresponding to the 59 kDa precursor (upper band) and the 56 kDa mature (lower band) forms of the mitochondrial sterol 27-hydroxylase. Mock transfected cells did not produce any immunoreactive protein. Assay of the enzyme activity showed that transfection with the normal full-length cDNA led to a 21.4 ± 1.2% (mean ± SD) conversion of the substrate 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol into the 27-hydroxylated product 5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol. In contrast, no converted substrate was detected in mutant cDNA and mock transfected COS-1 cells (Figure 7B). The results suggest that the mutation is responsible for deficiency in the enzyme activity.

DISCUSSION

By direct sequencing analysis, a C to A mutation at codon 362 of the sterol 27-hydroxylase gene (CGT ^{362}Arg to AGT ^{362}Ser) was identified in a Japanese CTX patient. The normal ^{362}Arg is highly conserved among mitochondrial cytochrome P-450 and serves as a binding region for the adrenodoxin cofactor of the sterol 27-hydroxylase (*27*). The mutation could then be expected to affect normal sterol 27-hydroxylase activity by interfering with the binding of the adrenodoxin cofactor. This expectation was confirmed by transfection analysis of mutant cDNA into COS-1 cells, as shown in Figure 7.

Codon 362 (CGT) of the sterol 27-hydroxylase is composed of nucleotides from exons 6 and 7. The CG is from the 3′ last two nucleotides of exon 6 and T from the 5′ first nucleotide of exon 7. Mutations at this codon provide a good model to investigate the effecct of splice site mutation on pre-mRNA splicing. Besides the current mutation, two other point mutations at codon 362 have been noted in CTX patients. One occurred at the last nucleotide of exon 6 (−1 position of the exon 6−intron 6 splice site) and changed the G to A (CGT ^{362}Arg to CAT ^{362}His) previously identified by our group (*22*). The mutation caused alternative pre-mRNA splicing, including exon 6 skipping and activating of a cryptic 5′ splice site 88 bp upstream from the 5′ end of exon 6, both in vivo and in COS-1 cells transfected with the mutant minigene construct (*23*). The other occurred at the same position as the current case (−2 position of the exon

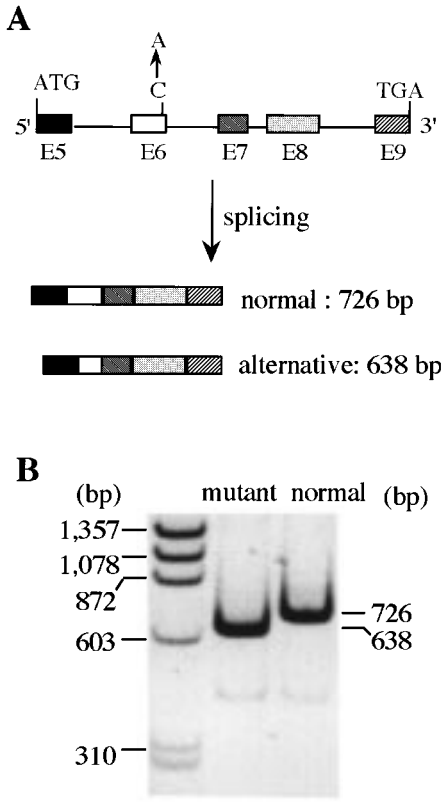

FIGURE 5: Transcription analysis of the mutant minigene constructs transfected in COS-1 cells. Minigenes, with or without the mutation, were transfected into COS-1 cells. After 48 h, RNA was extracted from the transfected cells, and RT-PCR was done to detect alternative pre-mRNA splicing. (A) Scheme of the minigene construct and the different splicing patterns. (B) RT-PCR analysis of RNA extracted from transfected COS-1 cells. øx174-*Hae*III DNA marker was used.

6−intron 6 splice site), but changed the C to T (CGT ^{362}Arg to TGT ^{362}Cys) as reported by Cali et al. (*11*). Information about the effect on pre-mRNA splicing of the mutation and about the mutant mRNA level was not presented. However, the current mutation, which changed the same C to A, was responsible for a mRNA species alternatively spliced at an activated cryptic 5′ splice site 88 bp upstream from the exon 6−intron 6 splice site in COS-1 cells transfected with the mutant minigene (Figures 5 and 6). This alternatively spliced species resulted in a frameshift and a premature terminal codon 47 bp downstream from the 5′ end of exon 7. We assume that this species may also occur in vivo, but it is difficult to detect due to instability. It is evident that mRNA species with a premature termination codon are unstable and are degraded rapidly (*28*). This was supported by the data that the mutant *CYP27* mRNA in the patient was 52.5% of the normal level by quantitative analysis. It is surprising that only the alternatively spliced mRNA species was observed in COS-1 cells transfected with the mutant minigene without any detected normally spliced species. Also, no difference in the mRNA amount was found between the mutant and normal minigene transfectants (Figure 5), although the alternative species harbors a premature termination codon. Same data were obtained when we transfected the COS-1 cells with 5 and 10 μg of the minigene constructs (data not shown). This inconsistency between in vivo and in COS-1 cell studies may be partially related to the overexpression of the minigenes in COS-1 cells, and to the different splicing conditions between in vivo and in COS-1 cells. It has been suggested that alternative splicing could be modulated by subtle cell-specific variations. Reed and Maniatis observed that splice site selection can sometimes be affected by diluting the extracted splicing solution (*29*). Similar findings were obtained by varying the ionic conditions in the in vitro splicing reaction (*30*). Weil et al. found that temperature had a significant effect on different expres-

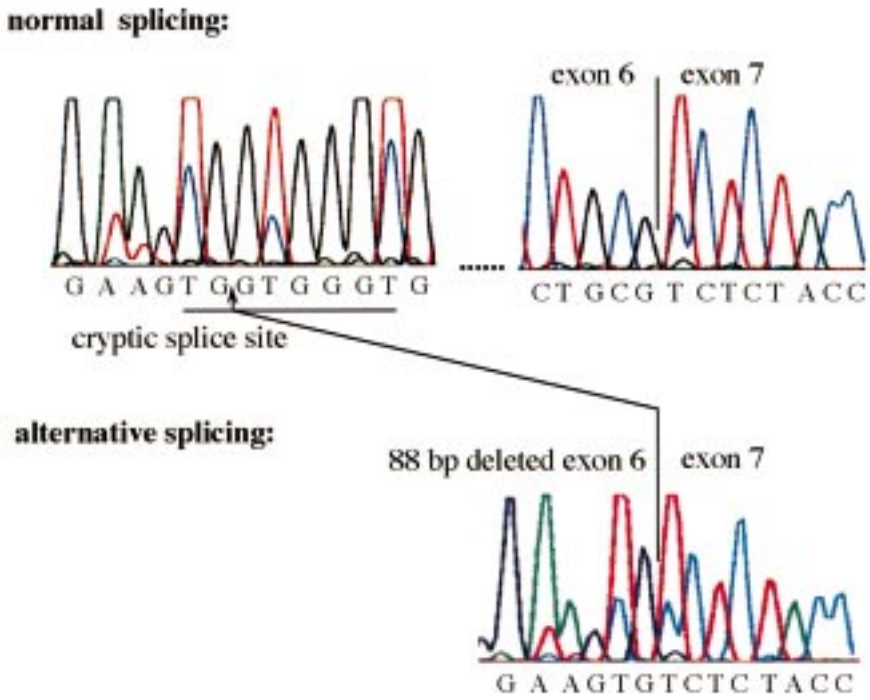

FIGURE 6: Direct sequence analysis of RT-PCR products from transfected COS-1 cells. Minigenes, with or without the mutation, were transfected into COS-1 cells. After 48 h, RNA was extracted, and RT-PCR was done. The amplified RT-PCR products were directely sequenced.

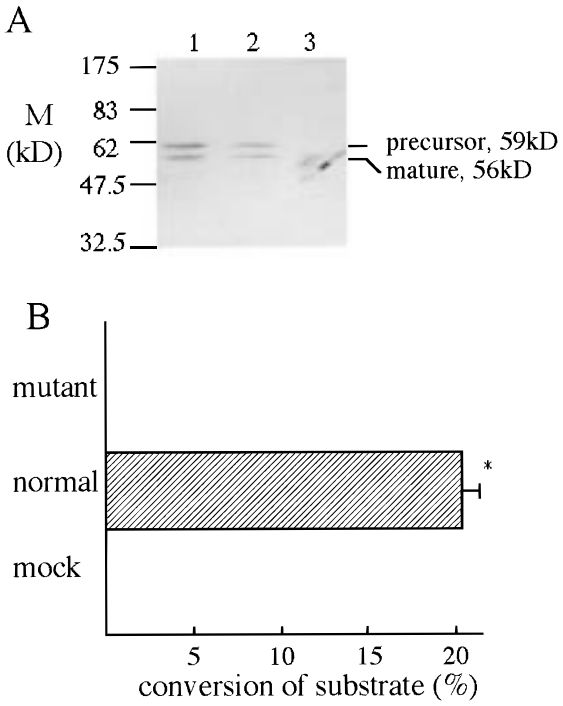

FIGURE 7: Expression of normal and mutant full-length sterol 27-hydroxylase cDNAs. COS-1 cells were transfected with 20 μg of normal full-length cDNA plasmid, mutant full-length cDNA plasmid, and mock plasmid, by the calcium phosphate coprecipitation method. After 48 h, the cells were harvested, and mitochondrial fractions were isolated for immunoblot analysis (A; lane 1, normal; lane 2, mutant; lane 3, mock) and for enzyme activity assay (B), as described under Materials and Methods. The enzyme activity is shown as a conversion (%) of substrate. The results shown are means of two separate transfection experiments.

sion of a mutant splice site in the pro-α2(I) collagen gene (*31*).

The ^{362}Arg codon appears to be a mutational hotspot as three different mutations attacked this residue. The three mutations occurred at the CG dinucleotides of the 362 codon (CGT), which are considered to be hypermutable in the human genome (*32*). The C to T mutation reported by Cali et al. was identified in a Canadian. The current C to A mutation and the G to A one as we reported previously were each evident in one Japanese patient. The most common mutation identified in Japanese is a C to T (CGG ^{441}Arg to TGG ^{441}Trp) mutation (*33*). It is interesting to survey whether the three cases harboring the ^{362}Arg codon mutations share similar phenotypes. However, the manifestations among the three cases were quite different, which indicates that in addition to genetics, other factors such as individual and environmental differences may contribute to the phenotype of CTX.

Sequence complementarity between U1 small nuclear RNA (U1 snRNA) and the consensus sequences (AGgtaagt) around a 5′ splice site is considered to be crucial for splicing (*34*). Although mutation at any position of a 5′ splice site could be expected to influence normal pre-mRNA splicing, by interfering with the correct base pairing between U1 snRNA and the splice site, the effect of a mutation on pre-mRNA splicing differed from the positions mutated. Mutations at the absolutely conserved intronic gt region (+1 and +2 positions of a 5′ splice site) usually blocked the normal 5′ splice site and led to abolition of normal pre-mRNA splicing or aberrant splicing, for example, exon skipping, as noted in many inherited diseases (*19*−*21*). On the other hand, mutations at the exonic region of a 5′ splice site did not completely block the normal 5′ splice site. To date, only 12 mutations at the last nucleotide of an exon (−1 position of a 5′ splice site) have been reported to be associated with alternative pre-mRNA splicing, in some genetic diseases. The G to A substitution was noted in nine cases (*23*, *31*, *35*−*41*), and G to C (*15*, *42*) and G to T (*43*, *44*) were both found twice. Normal splicing was also observed in 8 of the 12 cases although aberrant splicing with exon skipping was found in all studies. It shows that sequences in the intronic gt region play a more important role in splicing processes, by stabilizing the complementarity between the U1 snRNA and splice site, as compared to events in the exonic region. To our knowledge, the current case is the first report regarding effects of a mutation at the −2 position of a 5′ splice site on pre-mRNA splicing. Our data showed that the mutation at the −2 position of the 5′ splice site of exon 6 in the *CYP27* gene was responsible for the alternative pre-mRNA splicing in transfected COS-1 cells, although this type of mRNA species was not detected directly in vivo probably due to instability or other unknown reasons.

A novel 362 codon mutation (CGT ^{362}Arg to AGT ^{362}Ser) in the sterol 27-hydroxylase gene was identified in a Japanese CTX patient and was confirmed to be responsible for deficiency in the enzyme activity by expression of the mutant cDNA into COS-1 cells. The mutation occurred at the −2 position of the exon 6−intron 6 splice site, and, although the alternatively spliced mRNA species was not detected in vivo due to instability, transfection of the mutant minigene into COS-1 cells confirmed that the mutation was responsible for an alternative splicing at an activated cryptic 5′ splice site in exon 6.

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