Alternative pre-mRNA splicing of the sterol 27-hydroxylase gene (*CYP* 27) caused by a G to A mutation at the last nucleotide of exon 6 in a patient with cerebrotendinous xanthomatosis (CTX)

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Abstract A recently identified G to A mutation at the last nucleotide of exon 6 of the sterol 27-hydroxylase gene (*CYP* 27) in a patient with cerebrotendinous xanthomatosis (CTX) was shown here to cause alternative pre-mRNA splicing of the gene. Northern blot analysis of the patient's RNA revealed a broadened band in the human *CYP* 27 mRNA region compared to that of the normal sample, indicating that there may exist differently spliced mRNA species in the patient. RT-PCR produced three fragments in the patient, one was full-length size and the other two were of smaller sizes. Sequence analysis confirmed that the nucleotide of the full-length size was identical to that of the normal full-length cDNA, except for the G to A mutation at codon 362, which corresponds to the last nucleotide of exon 6. One of the smaller size species lacked exon 6 and the other was absent from the 3′ terminal 88 bp of exon 6 due to the use of an activated cryptic 5′ splice site in exon 6. The correctly spliced mRNA harbouring the G to A mutation was responsible for the deficiency of the sterol 27-hydroxylase activity, as confirmed by transfection experiment. Transfection of constructed minigenes, with or without the mutation, showed that correctly spliced mRNA was observed in the normal minigene while the mutant minigene was differently spliced. This is the first report of a G to A substitution at the last nucleotide of an exon resulting in both normal and abnormal pre-mRNA splicings, including exon skipping and activating of a coding region cryptic 5′ splice site. The results reveal a new molecular basis for the CTX and provide information on aberrant splicing of pre-mRNA in multi-exon genes. **—Chen, W., S. Kubota, and Y. Seyama.** Alternative pre-mRNA splicing of the sterol 27-hydroxylase gene (*CYP* 27) caused by a G to A mutation at the last nucleotide of exon 6 in a patient with cerebrotendinous xanthomatosis (CTX). *J. Lipid Res.* 1998. **39:** 509–517.

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

Sterol 27-hydroxylase is a mitochondrial P-450 enzyme that catalyzes the initial step in the hepatic oxidation of the side chain of sterol intermediates in the pathway for metabolism and biliary excretion of cholesterol (1). Mutations in the sterol 27-hydroxylase gene (*CYP* 27) cause cerebrotendinous xanthomatosis (CTX), an autosomal recessively inherited cholesterol metabolic disorder characterized by tendon xanthomatosis, cataracts, neurological manifestations, osteoporosis, and premature atherosclerosis. Since the cloning of human sterol 27-hydroxylase cDNA (2) and determination of its genomic structure (3), several mutations of the sterol 27-hydroxylase gene have been identified in CTX patients from different countries (4–12). Most of the mutations reported to date are point mutations that lead to amino acid substitution or premature termination codon.

Study of de novo mutations that occur at splice site regions in many inherited diseases contributes greatly to understanding of the mechanisms of normal pre-mRNA splicing. A number of mutations at the absolutely conserved intronic 5′ gt or 3′ ag dinucleotide at splice sites have been identified in various genetic diseases. Mutations at these sites usually lead to aberrant pre-mRNA splicing or abolish normal pre-mRNA transcription (13–15). On the other hand, mutations at the moderately conserved −1 position G of a 5′ splice site (corresponds to the last nucleotide of an exon) are relatively rare and the effect of these mutations on the pre-mRNA splicing has been given less attention.

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

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We report here the first example of a G to A substitution at the last nucleotide of exon 6 in the *CYP* 27 gene that leads to alternative splicings of pre-mRNA, including exon 6 skipping, activating of a coding region cryptic 5′ splice site, and also correct splicing of pre-mRNA carrying the G to A mutation. We analyzed the transcription products of this mutant gene in vivo and confirmed that the aberrant pre-mRNA splicings were caused by the mutation in vitro.

MATERIALS AND METHODS

Subject

A 24-year-old female Japanese CTX patient was studied. The patient complained of gradually enlarging bilateral Achilles tendons as well as similar subcutaneous swellings over her triceps, knees, and dextral 2nd finger joint when we examined her at the age of 17. Mild mental retardation with an IQ of 67 was confirmed. No other remarkable neurological defects and visual impairment could be found. Cardiovascular investigations were also normal. Biochemical analysis showed a markedly elevated plasma cholestanol concentration of 40.6 μg/ml. No sterol 27-hydroxylase activity was detected in the fibroblasts from the patient.

Northern blot analysis

Thirty μg total RNA extracted from cultured fibroblasts derived from a healthy subject and from the patient was separated by electrophoresis in a 1.6% formaldehyde agarose gel and transferred to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany). After prehybridization in high SDS concentration buffer (7% SDS, 50 mm sodium phosphate, pH 7.0, 50% formamide, 2% blocking reagent, 50 μg/ml transfer yeast RNA, 5× SSC, 0.1% lauroylsarcosine) at 50°C for 24 h, the membrane was hybridized at 50°C for 48 h with the full-length sterol 27-hydroxylase cDNA probe or β-actin probe (243 bp, nucleotide 143–385 in human β-actin cDNA) labeled with digoxigenin-11-dUTP by the random primed method according to the manufacturer's instructions (Boehringer Mannheim). Detection was performed with colorimetric detection reagents from Boehringer Mannheim. Quantitative analysis was carried out by normalizing the signals to that of the β-actin RNA control using the software, NIH Image, 1.61.

Full-length cDNA amplification of the sterol 27-hydroxylase gene

To amplify the full-length cDNA of the sterol 27-hydroxylase gene, 1 μg total RNA was first converted to cDNA in a 20 μl reaction mixture containing 5 mm $MgCl_2$, 1 × PCR buffer II (10 mm Tris-HCl, pH 8.3; 50 mm KCl), 1 mm of each dNTP, 1 U RNase inhibitor, 1 μm oligonucleotide dT and 5 U reverse transcriptase, using a RT-PCR kit (TaKaRa, Otsu City, Japan). The reaction tube was incubated at 42°C for 60 min (annealing and extension), heated at 95°C for 5 min (inactivation of reverse transcriptase and denaturation of RNA-cDNA hybrids), and then soaked at 5°C for 5 min in a thermal cycler (Perkin Elmer, GeneAmp PCR system 9600). PCR amplification was immediately performed after the RT reaction by adding 80 μl of a PCR Master Mix containing 1.25 mm $MgCl_2$, 1 × PCR buffer II, 0.25 μm upstream primer FLup (nucleotide: 20–40, 5′ CCATGGCTGCGCTGGGCTGCG3′) and downstream primer FLd (nucleotide: 1639–1618, 5′CCCAGCAAG GCGGAGACTCAGC3′), 11.4 μl DMSO, and 2.5 U Taq DNA polymerase. The amplification reaction was performed for 30 cycles under the following conditions: 1.5 min at 95°C for denaturation, 30 sec at 68°C for annealing, and 2 min (4 sec increment every successive cycle) at 72°C for extension. Electrophoresis was performed on a 2% agarose gel. Quantitative analysis was carried out by scanning the gel, and the density of the bands was analyzed using the software, NIH Image, 1.61. β-Actin was amplified as a control for quantitative analysis.

Sequence analysis

DNA sequence analysis was performed using the ABI PRISM™ 310 Genetical Analyzer as described previously (16). Briefly, either the PCR product or plasmid was first labeled by Taq terminator sequencing reaction in a thermal cycler, under the following condition: 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min for a total 25 cycles. After removing the excess unincorporated terminators using the Centri-Sep Spin column (ABI, Foster, CA), according to the manufacturer's protocol, the labeled products were dried, resuspended in 25 μl Template Suppression Reaction, and heated to 92°C for 2 min for denaturation. Then, the samples were loaded on an ABI PRISM™ 310 Genetical Analyzer for sequence analysis. All of the fluorescein sequencing reagents were purchased from ABI. All the samples were sequenced in both directions in order to confirm the identified mutations. The primers used for sequencing were: FLup (nucleotide: 20–40, 5′CCA TGGCTGCGCTGGGCTGCG3′), P357 (nucleotide: 382–402, 5′GAGGGAAAGTACCCAGTACGG3′), P359 (nucleotide: 737–758, 5′CCTTCGTCAGATCCATCGGGTT 3′), P372 (nucleotide: 1159–1181, 5′TTTGCCCACATG CCGTTGCTCAA3′), and FLd (nucleotide: 1639–1618, 5′CCCAGCAAGGCGGAGACTCAGC3′.

Construction of expression vector

To observe the different splicing patterns caused by the G to A mutation at the last nucleotide of exon 6, two minigene constructs, with or without the mutation, were generated and transfected into COS cells. Genomic sequences from exon 5 to exon 9 of the *CYP* 27 gene was amplified from the patient and a healthy subject using primers SPup 5′CGAAGATATGGAGGCCCAACT3′ (nucleotide: 891–911) and SPd 5′TCAGCACTGTCTCT GCAGGAAC3′ (nucleotide: 1596–1617). The PCR amplification reaction was performed for 30 cycles under the following conditions: 1 min at 95°C for denaturation and 4 min at 68°C for annealing and extension. The upstream primer SPup contains an initial codon ATG and the −3 position relative to the ATG is a G which is considered to ensure efficient translation (17). The downstream primer SPd contains the stop codon TGA. After confirmation of successful amplification of the 2111 bp band by agarose gel electrophoresis, the insert was directly ligated into the pTARGET™ expression vector (Promega, Madison, WI) which contains CMV immediate-early enhancer/promoter region, a chimeric intron for high level expression of insert, and the SV40 late polyadenylation signal. Plasmids of the minigene constructs were prepared using JETSTAR Plasmid Kit (Genomed, Research Triangle Park, NC). The two minigenes have the same structure except for the G to A mutation at the last nucleotide of exon 6, as confirmed by sequence analysis.

Creation of mutant full-length cDNA by in vitro site-directed mutagenesis

For expression analysis of the mutant full-length cDNA, the G to A mutation was recreated in an expressible cDNA encoding human sterol 27-hydroxylase. Site-directed mutagenesis of the cDNA was accomplished using the pKF18k vector and a kit from TaKaRa. Then, the normal and mutant cDNAs of the sterol 27-hydroxylase were ligated into the pTARGET™ expression vector by *Eco*R I sites. The nucleotide sequences of the normal and mutant cDNAs were confirmed by sequence analysis. Plasmids for transfection were prepared using a JETSTAR Plasmid Kit.

Transfection analysis

COS-1 cells obtained from JCRB cell Bank (Tokyo, Japan) were maintained in DMEM containing 10% fetal calf serum and used for transfection. Twenty μg plasmids of the normal and mutant minigene constructs and a mock vector were transfected in triplicate into 1×10^6 COS cells by the method of calcium phosphate co-precipitation. 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. Electrophoresis was performed on a 5% acrylamide gel.

To determine the effect of the G to A mutation on the sterol 27-hydroxylase activity, 20 μg plasmids carrying normal cDNA or mutant cDNA were transfected in triplicate into 1×10^6 cells, as described above. After 48 h, cells were harvested and the mitochondrial fraction was prepared, as described (5). The isolated mitochondrial fraction was used for sterol 27-hydroxylase assay.

Assay of sterol 27-hydroxylase activity

Assay of sterol 27-hydroxylase activity was performed using a procedure described by Skrede et al. (18). 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 (19). The substrate (480,000 cpm, 3.2 nmol) was dissolved in 10 μl acetone (which was then evaporated under a nitrogen stream), and solubilized in 250 μl of 5% bovine serum albumin followed by the addition of the following incubation mixture: 33 mm HEPES (pH 7.4); 5 mm ATP; 5 mm potassium malate; 1 mm glucose 6-phosphate; 0.5 IU glucose-6-phosphate dehydrogenase; 1.2 mm NADPH, and 15 mm $MgCl_2$. The mitochondrial pellet isolated from the transfected COS cells was suspended in 250 μl of 0.25 m sucrose and added to the incubation mixture to start the reaction, giving a final volume of 608 μl. After incubation at 37°C for 1 h, the reaction was terminated by adding 0.1 ml 1 m HCl. Extraction with 5 ml ethylacetate was performed twice and the converted 27-hydroxylated product was detected by HPLC (LC-10A Shimadzu, Kyoto, Japan) using an LC-18 column (250 × 4.6 mm, Supelco, Bellefonte, PA). The radioactivity of the product was measured using a radiodetector (RLC-700, Aloka, Tokyo, Japan).

RESULTS

Mutation of the *CYP* 27 gene in the CTX patient

We recently identified a G to A substitution at the last nucleotide of exon 6 in the patient (20). The same mutation was confirmed this time by Taq terminator sequencing reaction on the ABI PRISM™ 310 Genetical Analyzer. Except for this nucleotide change, the sequences of all 9 exons, splice site regions, and promoter region in the patient were identical to that in the healthy subject (data not shown).

Transcription analysis of the mutant gene in vivo and in vitro

As the mutation occurred at the last nucleotide of exon 6, its effect on pre-mRNA transcription was first analyzed in vivo. Northern blot analysis of the patient's

RNA showed a broadened band compared to that in the normal sample (2.2 kb), indicating that there may exist differently spliced mRNA species (**Fig. 1**). No obvious decrease of transcription efficiency was observed in the patient (0.90) compared to that in the normal sample (0.97) as determined by quantitative analysis of the signals.

We next performed RT-PCR for the *CYP* 27 gene using RNAs derived from the patient and a healthy subject. Similar results were obtained in three separate experiments. One typical example is shown in **Fig. 2**. In the patient's full-length RT-PCR product, two smaller fragments were observed in addition to the normal size fragment compared to that in the normal sample (Fig. 2). The ratio of the three species was 1:0.84:0.16 (normal size:middle size:the smallest size) by quantitative analysis as described in Materials and Methods. The amount of normal size in the patient accounted for 54.1% of that in the healthy subject as determined by quantitative analysis. The β-actin RT-PCR product was used for correction of the quantitation. Each of the RT-PCR products was subcloned into pTARGET™ vector. Sequence analysis of the subcloned RT-PCR product revealed three species of different insert sizes in the patient: full-length cDNA carrying the G to A substitution at codon 362 (CGT ^{362}Arg to CAT ^{362}His) (**Fig. 3B**); 3′ terminal 88 bp of exon 6 deleted-cDNA due to use of an activated 5′ splice site in exon 6 (Fig. 3C); and exon 6 skipped cDNA (Fig. 3D). The three types of cDNA corresponded to the three fragments observed in electrophoresis of the RT-PCR products (Fig. 2). No abnormal cDNA inserted colony was found in the sample from the healthy subject (data not shown).

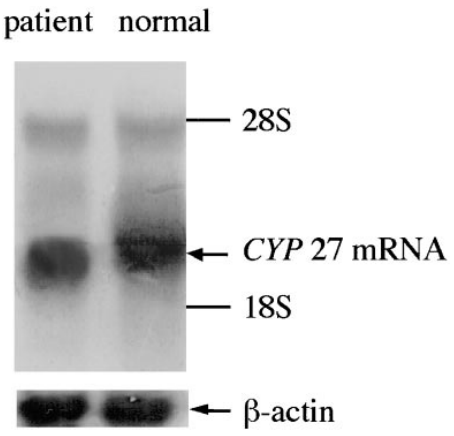

Fig. 1. Northern blot analysis of the sterol 27-hydroxylase mRNA. Thirty μg total RNA extracted from cultured fibroblasts from a healthy subject and from the patient were separated by electrophoresis in a 1.6% formaldehyde agarose gel and transferred to a positively charged nylon membrane. Hybridization was carried out at 50°C for 48 h with the full-length sterol 27-hydroxylase cDNA probe labeled with digoxigenin-11-dUTP. Detection was performed with colorimetric reaction. β-Actin was used as a control.

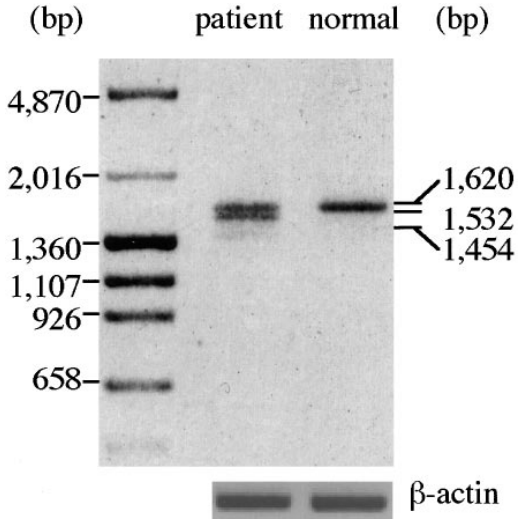

Fig. 2. RT-PCR analysis of the sterol 27-hydroxylase mRNA. Full-length cDNA of the *CYP* 27 gene was amplified as described in the Materials and Methods. β-Actin mRNA was amplified at the same time and used for correction of quantitative analysis. pHY DNA marker was used.

To confirm that the abnormal cDNA fragments were generated by the alternative pre-mRNA splicing due to the mutation, minigene constructs consisting of genome sequences form exon 5 to exon 9 of the *CYP* 27 gene, with or without the mutation, were transiently transfected into COS cells. RT-PCR analysis of the total RNA extracted from the transfected cells using primers SPup and SPd showed a single 726 bp band in normal minigene, while in the mutant minigene, the most abundant band was 412 bp one, with 2 faint 560 bp and 638 bp bands (**Fig. 4B**). 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 major band observed in the mutant minigene lacked exons 5 and 6, and one of the faint bands was absent from exon 6 and the other was absent from the 3′ terminal 88 bp of exon 6 due to use of an activated 5′ cryptic splice site (Fig. 4A). Thus, the in vitro data confirmed that the normal minigene could be spliced correctly, while the G to A mutation at the last nucleotide of exon 6 was responsible for the abnormal pre-mRNA splicing.

Transfection analysis of the mutant full-length cDNA

Of the three species of spliced mRNA observed in vivo in the patient (Fig. 2), the two smaller ones led to frame-shift, and translations of these mRNAs synthe-

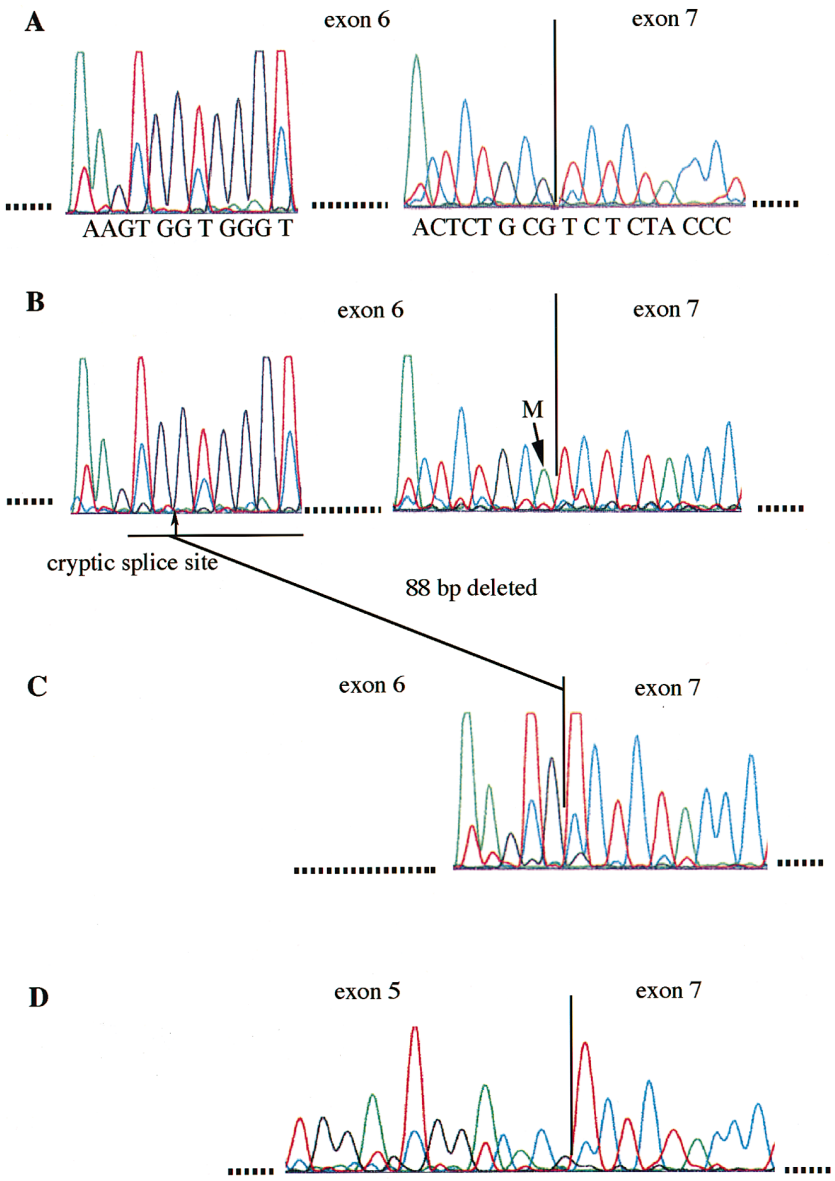

Fig. 3. Sequence analysis of the subcloned full-length RT-PCR product. Full-length RT-PCR products amplified from RNAs derived from the patient and a healthy subject were subcloned into pTARGET™ vector. Plasmids were prepared from colonies and sequenced. A: normal sample; B, C, D: patient sample.

sized truncated sterol 27-hydroxylases lacking enzyme activity. As correctly spliced mRNA harboring the G to A mutation was also observed in the patient, its effect on the sterol 27-hydroxylase activity was checked in a transfection experiment. As shown in **Fig. 5**, transfection with a vector carrying the normal full-length cDNA led to 10.6 ± 1.7% (mean ± SD) conversion of substrate 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol into 27-hydroxylated product 5β-[7β-^{3}H]cholestane-3α,7α,12α,27-tetrol. In contrast, no converted substrate was detected in COS cells transfected with the full-length cDNA plasmid carrying the G to A mutation and a mock plasmid. The results suggest that the mutation is responsible for the deficiency of enzyme activity.

DISCUSSION

Although a G to C transversion at the last nucleotide of exon 3 (−1 position of the 5′ splice site of intron 3) in the *CYP* 27 gene has been reported (9), this muta-

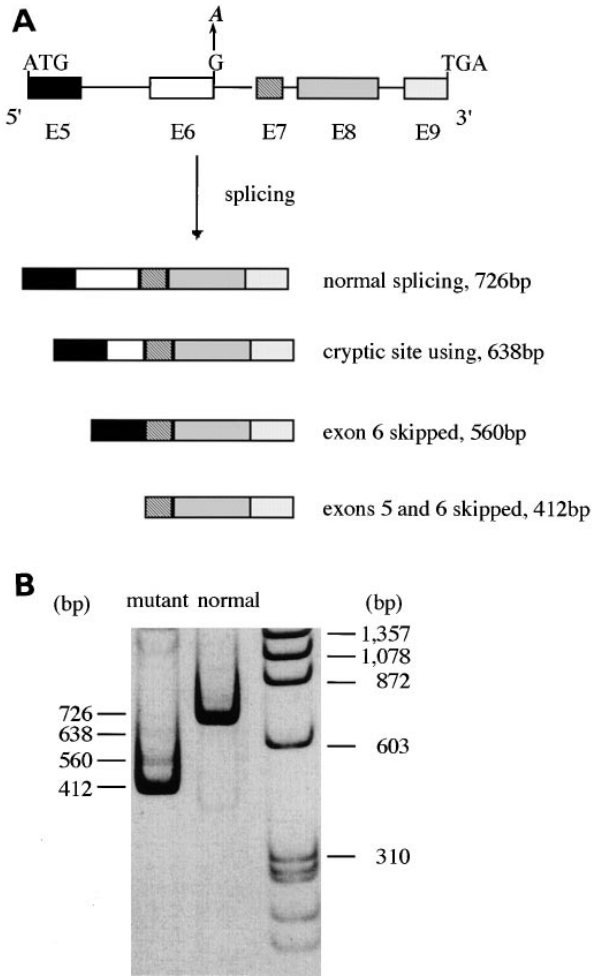

Fig. 4. Transcription analysis of the mutant gene in vitro. Minigenes with or without the mutation were transfected into COS cells. RNA was extracted from the transfected COS cells and RT-PCR was performed to detect the alternative splicing. A: Scheme of the minigene construct and the different splicing patterns. B: RT-PCR analysis of RNA extracted from transfected COS cells. 0x174 *Hae* III DNA marker was used.

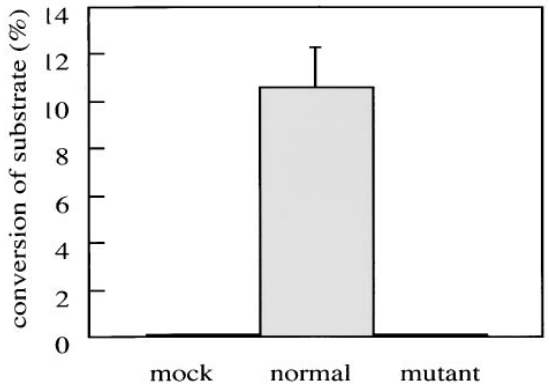

Fig. 5. Expression of normal and mutant full-length sterol 27-hydroxylase cDNAs. COS cells were transfected with 20 μg of normal full-length cDNA plasmid, mutant full-length cDNA plasmid, and mock plasmid by the calcium phosphate co-precipitation method. After 48 h, the cells were harvested and the isolated mitochondrial fraction was used for assay of sterol 27-hydroxylase activity. The results shown were representative of two separate transfection experiments.

tion was only associated with a barely detectable level of mRNA and no alternatively spliced mRNA species was found. To our knowledge, no alternative splicing due to the use of an activated 5′ cryptic splice site in the coding region has been reported in the eight cases with G to A mutation at the −1 position of a 5′ splice site in other genetic diseases (see below). The present mutation is the first example that a G to A mutation at the last nucleotide of an exon resulted in both normal splicing with the mutation and aberrant splicing, including exon skipping and activating of a coding region cryptic 5′ splice site. Transfection of the minigenes into COS cells confirmed that the mutation led to aberrant splicing in vitro. The different mRNA species observed in vivo (Figs. 2 and 3) and in vitro (Fig. 4) may result from different splicing conditions in vivo and in vitro. It has been suggested that alternative splicing in the cell could be modulated by subtle cell-specific variations. Reed and Maniatis (21) observed that splice site selection can sometimes be affected by diluting the splicing extract. Similar findings have also been obtained by varying ionic conditions in the in vitro splicing reaction (22). Weil et al. (23) reported that temperature could effectively and specifically suppress the expression of a splicing defect in the cell. These observations suggest that many factors are involved in the splicing process that may affect the splicing pattern.

Early studies indicated that exon sequences may not play a major role in RNA splicing (24). However, such a notion has been challenged by more accurate investigations of sequence requirements using artificially generated RNA substrates that contain various combinations and manipulations of *cis*-competing splice sites (21, 25, 26), and also by the accumulation of aberrant splicing cases caused by de novo mutations in the exon region of a splice site in some genetic diseases, as in the present case. It is now widely accepted that pre-mRNA splicing is carried out in a two-step process. First, the pre-mRNA is cleaved at the 5′ splice site with concomitant joining of the 5′ end of the intron by a 5′–2′ phosphodiester bond to a conserved A residue some 30–40 nucleotides upstream of the 3′ splice site. Second, the 3′ splice site is cleaved, releasing the intron as a branched lariat, and the upstream exon is then joined to the downstream exon (27–29). Although little is un-

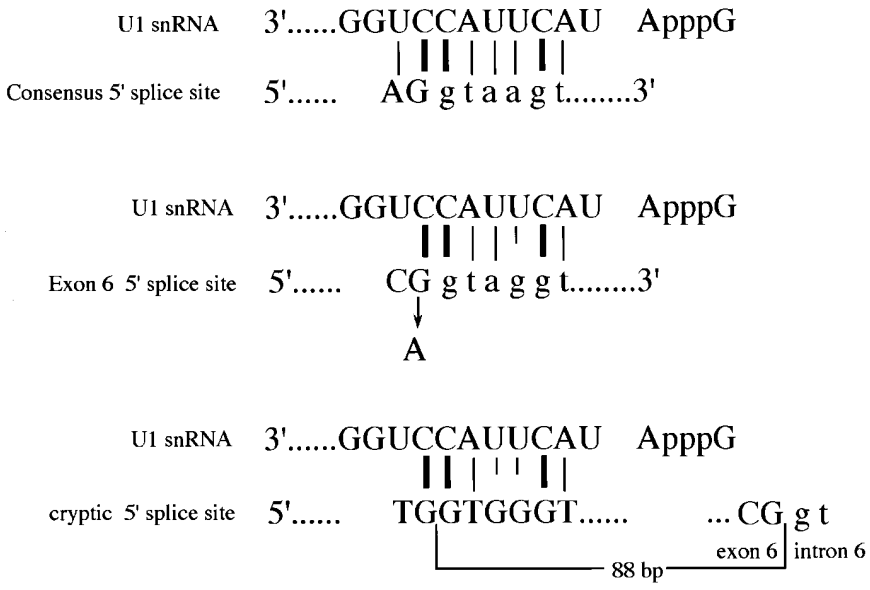

Fig. 6. Scheme of the complementarity of the 5′ end of U1 snRNA to the 5′ splice site region. A: consensus 5′ splice site. B: intron 6 5′ splice site of the sterol 27-hydroxylase gene. The G to A mutation is indicated by an arrow. G–C pairing is indicated by thick bars, A–U pairing by thin bars, and G–U pairing by short bars.

derstood about how the different RNA regions involved in this process are recognized and how precise cleavage sites are determined, the sequence complementarity between the consensus sequences around the splicing sites and the small nuclear RNAs (snRNAs) is considered to be crucial for splicing (30). For example, U1 snRNA is believed to recognize consensus sequences at the 5′ splice site (31). As shown in **Fig. 6**, the 5′ terminal single-strand nucleotides of U1 snRNA completely base pair with the consensus 5′ splice region of pre-mRNA. The G to A mutation in our case disrupts one of these base pairings and could be expected to reduce the efficiency of normal splicing. In order to obtain more precise information on the use of normal, mutant, and cryptic 5′ splice sites, we calculated the scores of these splice sites according to Shapiro and Senapathy (32). Scores of the mutant 5′ splice site (CAgtaggt) and the cryptic 5′ splice site (TGGTGGGT) were 71.3 and 73.5, respectively, a little lower than the 75.4 of normal 5′ splice site of intron 6 (CGgtaggt). This may explain the observation that three spliced mRNA species exist simultaneously in our case. To date, only 11 mutations at the last nucleotide of exon have been identified in some genetic diseases. The G to A substitution was reported in eight cases (23, 33–39) and G to C (9, 40) and G to T (41, 42) were both reported twice. Exon skipping was found in all of the eight G to A mutant cases, and normal splicing carrying the mutation was also observed in five of the eight cases. However, no aberrant splicing due to use of an activated cryptic 5′ splice site in a coding region was reported in any of the G to A mutation cases. The present study is the first example that a G to A mutation occurring at the last nucleotide of an exon can lead to aberrant splicing by use of an activated 5′ splice site in coding region. Different from the mutation at the intronic 5′ gt regions, which usually blocks the normal 5′ splice site (13–15), mutation at the last nucleotide of exon at a 5′ splice site does not seem to block the normal 5′ splice site completely. G is present at the last nucleotide of exon in 78% of cases and A is present in 11% (32). It is unclear why the G to A mutation at the last nucleotide of exon results in different splicings, while exons normally containing A at the last nucleotide can be cleaved correctly. These observations suggest that although pairing to U1 small nuclear RNA seems to be a requirement for splicing, other factors may also exist which interfere with stabilization of the U1 small nuclear RNA–5′ splice site interactions or to modulate accessibility of these sites.

According to the two-step splicing model, the G to A substitution at the last nucleotide of exon 6 in our case could be expected to result in accumulation of an abnormal transcription species with intron 6 unremoved but correctly spliced elsewhere. Such a species was not observed in our case and in all of the eight cases reported so far. Akli et al. (35) explained that due to the large length of intron 5 (1.5 kb) of the β-hexosaminidase α subunit gene, the putative intron 5 retaining

species mRNA does not cross the nucleus membrane and is degraded in the nucleus. In our case, intron 6 was only 195 bp and the abnormal species retaining intron 6 was also not observed. On the other hand, intron unremoved mRNA species could be observed in case of mutations occurred at the intronic 5′ gt positions (14, 15).

We have described here the first example that a G to A mutation at the last nucleotide of exon 6 of the *CYP* 27 gene leads to differently spliced mRNA species including correctly spliced full-length mRNA carrying the mutation, exon 6 skipped mRNA and 3′ terminal 88 bp of exon 6 deleted-mRNA, due to the use of an activated 5′ cryptic splice site in exon 6. The results provide a new molecular basis for CTX and also novel information on aberrant splicing in multi-exon genes.

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