Exon 10 skipping caused by intron 10 splice donor site mutation in cholesteryl ester transfer protein gene results in abnormal downstream splice site selection

Naohiko Sakai,[1] Silvia Santamarina-Fojo, Shizuya Yamashita,* Yuji Matsuzawa,* and H. Bryan Brewer, Jr.

Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, 10 Center Drive MSC 1666, Bethesda, MD 20892-1666, and Second Department of Internal Medicine,* Medical School of Osaka University, 2-2 Yamadaoka, Suita, Osaka 565, Japan

Abstract Cholesteryl ester transfer protein (CETP) deficiency is the most common cause of hyperalphalipoproteinemia in Japan. However, the genetic basis of this disorder has not been fully characterized. We have studied a 49-year-old Japanese male presenting with total cholesterol, HDL-cholesterol, and apolipoprotein A-I levels of 300, 236, and 233 mg/dl, respectively, and total absence of CETP activity and mass in plasma. Sequence analysis of the patient's CETP gene revealed that the splice donor consensus GT was substituted by GG in intron 10 (intron 10 splice defect) and by AT in intron 14 (intron 14 splice defect). Restriction digestion of PCR-amplified DNA using NdeI and MaeIII established that the patient was a compound heterozygote for both gene defects. Sequencing of cDNA amplified by RT-PCR from the patient's monocyte-derived macrophage RNA demonstrated abnormal splicing with deletion of exon 10 as well as alternative splicing at a native AG site located 31 nucleotides 5′ of the normal splice acceptor in intron 13. Thus, the intron 10 splice defect results in exon 10 skipping and the insertion of a 31 bp fragment between exon 13 and exon 14, which contains an in frame stop codon. The presence of abnormally spliced mRNA was further confirmed by amplification of patient cDNA using CETP specific primers. Abnormal splicing of exon 14 as a result of the intron 14 splice defect was not detected, indicating potential unstable CETP mRNA derived from that mutation. These findings demonstrate that a novel splice site mutation in intron 10 of the CETP gene results in the skipping of exon 10, as well as disruption of downstream splicing at intron 13 identifying a novel mechanism leading to CETP deficiency.–**Sakai, N., S. Santamarina-Fojo, S. Yamashita, Y. Matsuzawa, and H. B. Brewer, Jr.** Exon 10 skipping caused by intron 10 splice donor site mutation in cholesteryl ester transfer protein gene results in abnormal downstream splice site selection. *J. Lipid Res.* 1996. **37:** 2065–2073.

Supplementary key words cholesteryl ester transfer protein (CETP) • deficiency • hyperalphalipoproteinemia • mutation • splicing • skipping • atherosclerosis • high density lipoprotein (HDL)

High density lipoprotein (HDL) is the major antiatherogenic lipoprotein with increased plasma concentration of HDL associated with low risk of coronary heart disease (1–4). One of several proposed mechanisms by which HDL may protect against the development of atherosclerosis is reverse cholesterol transport (5, 6). In this process, HDL acts as a carrier lipoprotein that mediates the transfer of cholesterol from peripheral cells to the liver (5–8). Various transfer proteins, enzymes, and receptors, such as lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), hepatic triglyceride lipase, low density lipoprotein (LDL) receptors, remnant receptors, and putative HDL receptors may play key roles in this process of reverse cholesterol transport.

CETP is a glycoprotein that mediates mainly the transfer of cholesteryl ester (CE) from HDL to lower density lipoproteins, such as very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and LDL in exchange for triglycerides (TG) in human plasma (9–11). The important role of CETP-mediated lipid transfer in reverse cholesterol transport has been established by the identification and characterization of patients with CETP deficiency (12–17). CETP deficiency is the most common cause of hyperalphalipoprote-

Abbreviations: apo, apolipoprotein; CETP, cholesteryl ester transfer protein; LCAT, lecithin:cholesterol acyltransferase; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PCR, polymerase chain reaction; RT-PCR, reverse transcription coupled with PCR; DTT, dithiothreitol; PBS, phosphate-buffered saline; TG, triglyceride; CE, cholesteryl ester.

[1]To whom correspondence should be addressed.

inemia in Japan (18). To date several mutations involving primarily splicing defects in the CETP gene have been reported in CETP deficiency (19–21). However, the genetic basis of this disorder has not been fully characterized (22). In the present report, we investigate the underlying molecular defect in the CETP gene of a patient presenting with clinical features of this genetic disorder. We describe a novel intron 10 splice defect resulting in exon skipping and CETP deficiency in this patient.

MATERIALS AND METHODS

Patient

The patient is a 49-year-old Japanese male. A detailed characterization of the abnormal plasma lipids and lipoproteins has been previously described (16, 23). The patient's total cholesterol and triglyceride levels measured by enzymatic method (Kyowa Medex Co., Tokyo, Japan) were 300 mg/dl and 252 mg/dl, respectively, and the HDL-cholesterol concentration determined by heparin-$CaCl_2$ precipitation method was 236 mg/dl. Apolipoprotein A-I was 233 mg/dl as judged by single radial immunodiffusion assay. CETP mass and activity were not detectable in the patient's plasma (24).

Isolation of genomic DNA, polymerase chain reaction (PCR) and sequencing reaction

Genomic DNA was prepared from whole blood according to the method of Kunkel et al. (25). Each exon of the CETP gene was amplified by PCR using a pair of oligonucleotide primers that had EcoRI restriction site (forward primer) or BamHI restriction site (reverse primer) for the cloning. PCR products were subcloned into BluescriptII KS (-) (Stratagene, La Jolla, CA) after digestion with EcoRI and BamHI (New England Biolabs, Beverly, MA). DNA sequencing was performed according to the dideoxy nucleotide termination method of Sanger, Nicklen, and Coulson (26) using Sequenase (U.S. Biochemical, Cleveland, OH). At least six independent clones for each exon were sequenced in both directions.

Restriction enzyme analysis using MaeIII and NdeI

Intron 10 splice donor site and intron 14 splice donor site were amplified from genomic DNA by PCR using a pair of oligonucleotide primers (5′-CCCTGCGAATTCTTCTTCTGAGGAGTGGAC-3′ and 5′-ATAATTGGATCCATTGGTGGTGTTATTGGC-3′, 5′-CTTCTGTGCTCCAGGGAGGACTCACCATGG-3′ and 5′-GGCACCCAGTTTCCCCTCCAGCCCACACAT-3′, respectively). The reverse primer for intron 14 was designed to generate an NdeI endonuclease cleavage site by inserting a single primer-template mismatch of one base pair when one base at the 5′ splice donor site of intron 14 in the CETP gene is mutated from guanine (G) to adenine (A) (18). PCR products were precipitated and subjected to digestion with the restriction enzymes MaeIII (intron 10) or NdeI (intron 14) (New England Biolabs, Beverly, MA). Digested fragments were separated on a 3% Nu-Sieve GTG-agarose (FMC Bioproducts, Rockland, ME) and 1% standard agarose gel and analyzed after staining gel with ethidium bromide.

Isolation of total RNA and reverse transcription coupled with PCR (RT-PCR)

Total RNA was obtained from the patient's monocyte-derived macrophages. Twenty ml of whole blood was drawn from the patient and mononuclear cells were separated by the Ficoll-sodium metrizoate (LSM, Organon Teknika, PA) gradient method. All mononuclear cells were seeded and cultured in a 10-cm culture dish with 10 ml of RPMI supplemented with 10% heat-inactivated human AB-type serum. After 3 h incubation at 37°C in 5% CO_2 and 95% air, the medium and nonadherent cells were removed and adherent cells were washed 3 times with phosphate-buffered saline (PBS), followed by the incubation at 37°C in 10 ml of the same medium as described above. The medium was replaced every 3 days. After 14 day culture the differentiated macrophages were washed with PBS and dissolved in 5 ml of 4 M guanidine thiocyanate/25 mM sodium citrate (pH 7.0)/0.5% (w/v) Sarcosyl/0.1% (v/v) 2-mercaptoethanol. Total cellular RNA was isolated using the guanidine thiocyanate method (27).

To perform RT-PCR, 1 μg of total RNA was incubated at 70°C for 15 min in a reaction mixture (100 μl) of 10 mM Tris-HCl (pH 8.3)/50 mM KCl/1.5 mM $MgCl_2$/2 mM DTT/200 μM dNTP/80 units RNAsin (Promega, Madison, WI)/0.01% (w/v) gelatin containing 100 nM reverse primer (R1; 5′-ACACCAGGGTTCCAGCTGTGA-3′). After cooling down, 32 units of Molony murine leukemia virus reverse transcriptase (Pharmacia Biotech, Piscataway, NJ) was added in the reaction, followed by the incubation at 37°C for 1 h. PCR reaction was performed in the same reaction mixture by adding 100 nM forward primer (F1: 5′-GGGCCACTTACACACCAC-3′) and Taq DNA polymerase (Amplitaq, Perkin Elmer Cetus, Foster City, CA) under the following conditions: 1-min denaturation at 94°C, 2-min primer annealing at 50°C, and 3-min extension at 72°C. The second PCR was performed using 1 μl of total PCR product as a template and a pair of nested primers (F2: 5′-GGCCACTTCTAGACCACTGCCTGATAACCATG-3′, R2: 5′-TTCCAGCTGTTAACCTGGTGCTTGCCTTCTGC-3′) or other pairs of primers for the specific target region. The

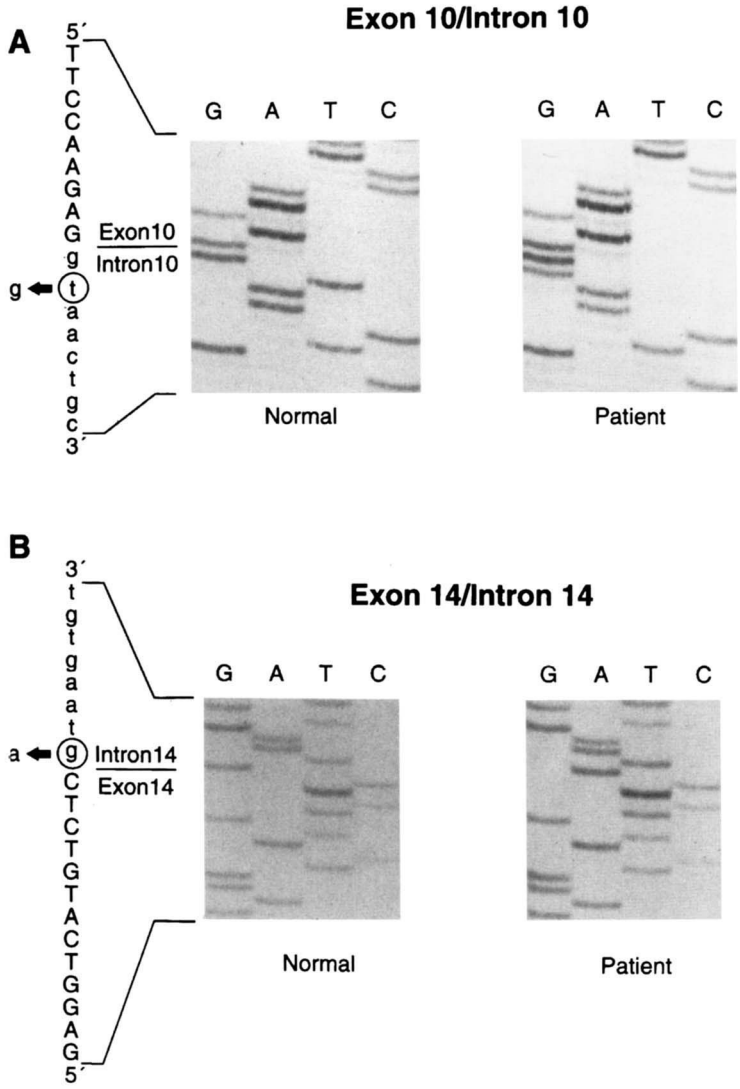


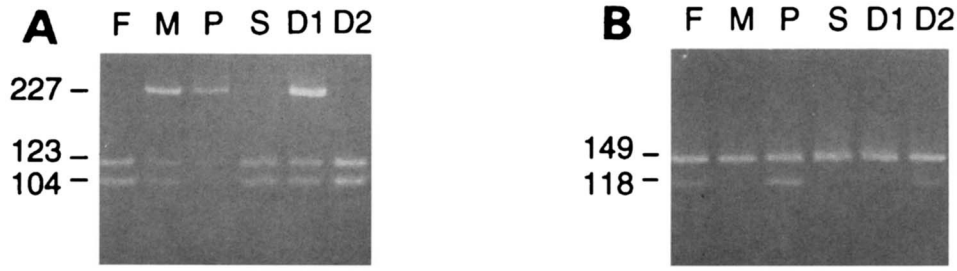
Fig. 1. Genomic DNA sequences of splice donor sites of intron 10 (A) and intron 14 (B). Each exon and exon–intron junction of CETP gene was amplified by PCR, cloned into Bluescript, and sequenced. The patient's mutant CETP allele (right) and control normal CETP allele (left) are shown. Capital letters represent exonic sequences and lower case letters represent intron sequences. The mutant nucleotides at the second nucleotide of intron 10 and the first nucleotide of intron 14 are circled.

second PCR product was cloned into pUC 18-derived vector (pCMV) and sequenced as described above.

RESULTS

Figure 1 illustrates the autoradiogram of sequencing gels of DNA isolated from the patient and a normal control. About 100 bp of the 5′ and 3′ flanking regions as well as all exons and exon–intron junctions were sequenced. The DNA sequences of splice donor sites of introns 10 (Fig. 1A) and 14 (Fig. 1B) for the CETP gene are shown. In the patient, the second nucleotide T of intron 10 is substituted by G (intron 10 splice defect) in 4 clones out of 6 clones sequenced and the first nucleotide G of intron 14 is substituted by A (intron 14 splice defect) in 3 clones out of 6 clones sequenced. Sequence analysis of other exons as well as intron–exon junctions of the patient's CETP gene did not reveal any other mutations.

To establish that these two mutations were not on the same allele, restriction analysis of PCR-amplified DNA was performed in the patient as well as in his immediate family members. The identified T to G mutation in intron 10 eliminates a MaeIII restriction enzyme site. Thus, a 227 bp DNA fragment containing the mutant region was amplified by PCR from genomic DNA of a normal control and the patient and digested with MaeIII (**Fig. 2A**). Digestion of PCR-amplified DNA for a control resulted in the generation of two fragments, 123 bp and 104 bp in length, while digestion of the patient's amplified DNA resulted, in addition, in the formation of an uncut 227 bp fragment. Figure 2B illustrates the digestion products of a 149 bp DNA after incubation with NdeI. This fragment was amplified by PCR using a pair of primers designed to have an NdeI restriction site when the intron 14 splice donor site G is mutated to A.



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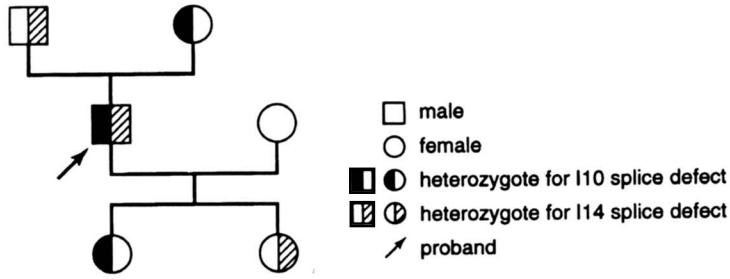

	Age	HDL-C*	CETP activity**
Father	77	114	9.4
Mother	71	160	4.8
Proband	43	236	0.0
Spouse	40	58	27.0
Daughter 1	12	94	12.8
Daughter 2	10	113	15.2
Control (n=20)	42±15	52±11	21.1±3.8

*mg/dl, **%/10μl/18hr

Fig. 2. Restriction analysis of PCR-amplified DNA for the intron 10 and intron 14 splice defects. The family members were screened for intron 10 splice defect (A) and intron 14 splice defect (B) as described in Materials and Methods. (A) Normal allele results in 123 bp and 104 bp fragments, whereas mutant allele shows an 227 bp uncut band after digestion with MaeIII because of the destruction of the MaeIII restriction site (GTNAC) at the mutation site. (B) Normal allele results in a 149 bp uncut band, while mutant allele shows 118 bp and 31 bp bands after digestion with NdeI because of the introduction of the cleavage site (CATATG). (C) Summary of restriction analyses, HDL-cholesterol concentration, and CETP activity in family members.

Digestion of the 149 bp fragment with NdeI results in an uncut 149 bp band for the normal allele, and in addition, in the formation of abnormal fragments 118 bp and 31 bp in length for the patient's amplified DNA (Fig. 2B). Thus, analysis of the restriction fragments present after digestion for both patient and family members demonstrated that the patient was a compound heterozygote for both mutations, with the intron 10 splice defect derived from the maternal gene and the intron 14 splice defect from the paternal gene. Direct sequencing of the CETP gene also confirmed that the patient was heterozygous for both mutations (data not shown). Consistent with the genetic analysis, plasma HDL-cholesterol concentrations of the heterozygotes were approximately half of control values (Fig. 2C).

To further investigate the consequences of the molecular defects caused by these two mutations, RT-PCR was performed using total RNA isolated from control and patient's macrophages as a template (**Fig. 3**). Control cDNA amplified by RT-PCR consisted of a 1575 bp major band and a 1395 bp minor band, representing the presence of alternatively spliced cDNA of CETP gene. Compared to control cDNA, patient cDNA exhibited a slightly reduced apparent size of 1555 bp on agarose gel electrophoresis. In addition, the minor cDNA product (1395 bp) could not be detected.

To further characterize the spliced products, the cDNAs were cloned into a plasmid vector. Analysis of the DNA sequence of these spliced products demonstrated that in controls the major band (1575 bp) represented the full length CETP cDNA (data not shown), while the minor band (1395 bp) was an alternatively spliced cDNA which lacked exon 9 (**Fig. 4,** left panel). The exon 10 splice defect mutation present in the patient's gene resulted in exon 10 skipping (Fig. 4, center panel). In addition, an insertion of the 31 bp of intron 13 splice acceptor site sequence between exons 13 and 14 in all 16 sequenced clones (Fig. 4, right panel)

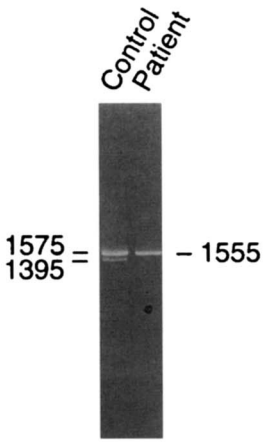

Fig. 3. Agarose gel electrophoresis of the CETP cDNA from a normal control and the patient. After RT-PCR amplification as described in Materials and Methods, 10 μl of total product was loaded on 0.8% agarose gel and run.

was demonstrated, indicating that cryptic splice acceptor sequence present in intron 13 was utilized. Interestingly, sequence analysis of either control or patient cDNA failed to reveal exon 14 skipping.

Figure 5A illustrates the mutant allele in the patient's CETP gene and CETP cDNA as well as the sequence and location of the oligonucleotide primers used for RT-PCR amplification of mRNA. In order to determine whether the intron 13 cryptic splice acceptor site is also used in controls, we amplified exons 9 through 16 using primers A and B (Fig. 5A), followed by digestion with NcoI which cuts uniquely within the 31 bp inserted sequence. As shown in Fig. 5B, the patient's cDNA fragment (541 bp) was completely digested with NcoI. However, the normal cDNA fragment (561 bp) was not digested by NcoI indicating the absence of the 31 bp insertion for control cDNA. To further establish these findings, we performed RT-PCR using a primer pair based on the 31 bp inserted sequence (oligos C and D, Fig. 5). As shown in the left panel of Fig. 5C, the expected 532 bp fragment could be amplified from patient RNA but no amplified fragments were detected after the control RT-PCR reaction. Thus, the cryptic splice site at intron 13 splice acceptor site is not used in splicing of normal CETP mRNA.

In order to evaluate the consequences of the intron 14 splice defect, the PCR-amplified fragment containing exons 9 through 16 utilized previously for digestion with NcoI was digested with PstI, which contains a unique restriction site in exon 14. As illustrated in Fig. 5B, both control and patient's amplified cDNA fragments were completely digested by PstI indicating the presence of exon 14 in both groups of amplified cDNA.

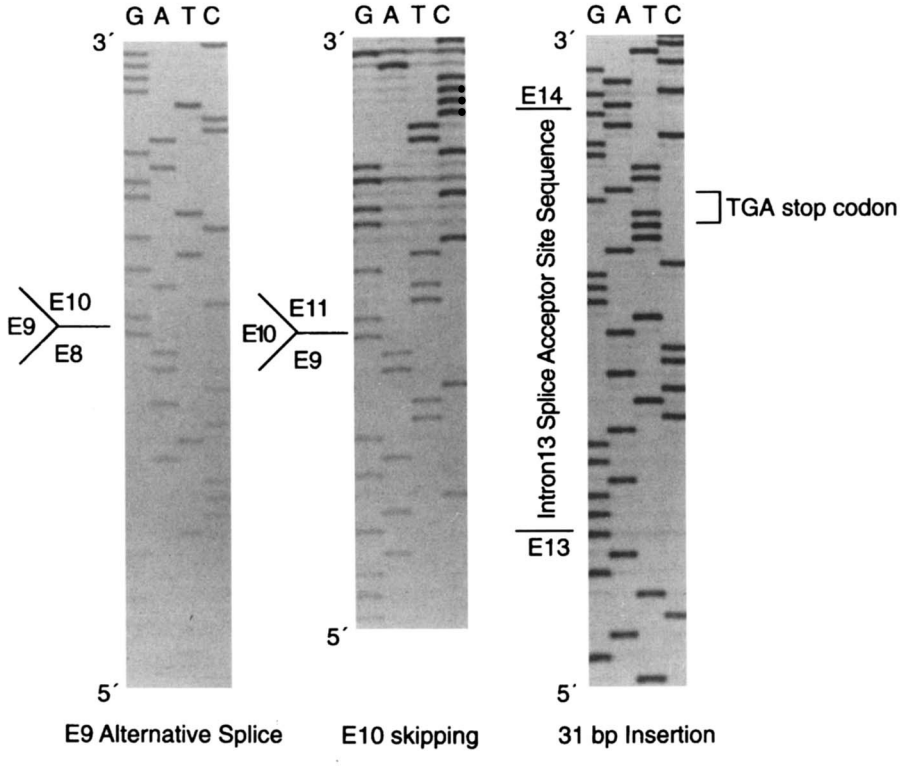

Fig. 4. Parts of CETP cDNA sequence from a normal control or the patient. CETP cDNA were cloned into pCMV and sequenced using the specific primers. An alternative splicing of exon 9 in the minor band of normal control cDNA (left), a skipping of exon 10 (middle), and an insertion of 31 bp sequence of intron 13 (right) in the patient's cDNA are shown.

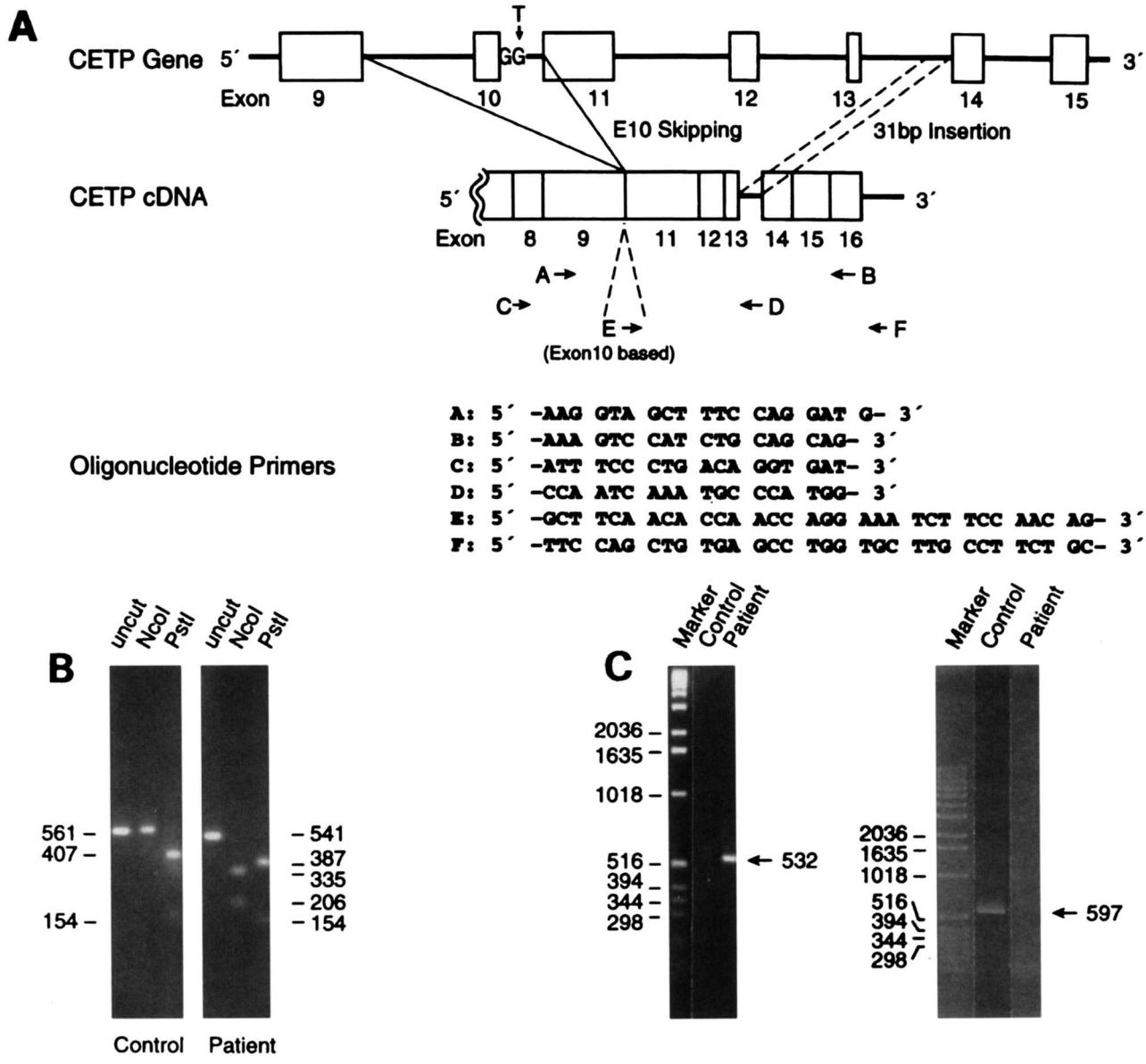

Fig. 5. (A) Schematic representation of splicing event detected for the patient's gene and the location of primers used in (B) and (C). (B) Restriction analysis of CETP cDNA with NcoI and PstI in a normal control and the patient. After amplification using a pair of primers (A and B), the product was subjected to digestion with NcoI or PstI. (C) Detection of minor transcripts with the 31 bp insertion in control RNA (left panel) and of transcripts from the intron 14 mutant allele in patient's RNA (right panel). Primer pairs C and D (left panel), or E and F (right panel) were used for the PCR amplification.

To further confirm the absence of transcripts from the intron 14 mutant allele in the patient, another RT-PCR was carried out with oligo primers E and F in Fig. 5. The sense primer E was designed in exon 10 to amplify the transcripts from the intron 14 mutant allele which do not lack exon 10. As shown in the right panel of Fig. 5C, the expected 597 bp fragment could be amplified from control RNA but no fragment was amplified from the patient RNA. Thus, utilizing this approach, exon 14 skipping as a result of the intron 14 mutation was undetectable in the patient, indicating that transcripts containing exon 14 skipping are unstable.

DISCUSSION

As the major transfer protein involved in the exchange of CE and TG between HDL and apoB-containing lipoproteins, CETP plays a central role in HDL metabolism. Thus, patients with CETP deficiency present with high plasma concentrations in TC, HDL-cho-

Sequence of Intron 13/Exon 14 Junction

5′ -agcatctgcc ttgtgggtca cttctgtgct ccagggagga ctcaccatgg
gcatttgatt ggcagAGCAG CTCCGAGTCC -3′

3′ Splice Acceptor Consensus Sequence

5′ $-(c/t)_n$ n (c/t) ag G -3′

Fig. 6. Sequence around the authentic and cryptic splice acceptor sites in intron 13 of CETP gene. Capital letters represent exon 14 sequence and lower case letters represent intron 13 sequence. The authentic and cryptic splicing acceptor consensus ag is bolded. Mismatched nucleotides from the consensus sequence were underlined for both splice acceptor sites. Splice acceptor consensus sequence was quoted from ref. 31.

lesterol, and apoA-I as well as low LDL and apoB concentrations (16, 23). To date, several mutations of CETP gene have been reported in CETP deficiency (19–21, 28), including an intron 14 splice defect, exon 15 missense mutation (442D:G), and exon 10 nonsense mutation. The incidence of the first two mutations has been demonstrated to be quite frequent in the Japanese population (18, 28, 29). In the present study, we describe the underlying molecular defect in a 49-year-old Japanese patient presenting with total cholesterol, HDL-cholesterol, and apoA-I levels of 300, 236, and 233 mg/dl, respectively.

Analysis of control and patient's genomic DNA as well as cDNA revealed that the patient is a compound heterozygote for both a novel intron 10 splice defect and the intron 14 splice defect (19). In addition, the presence of alternative splicing of exon 9 was demonstrated in control cDNA, as previously reported (30), but not in the patient's cDNA. The intron 10 splice defect results in the skipping of exon 10 in the patient's cDNA as well as the insertion of a 31 bp sequence from intron 13 splice acceptor site, indicating an alteration of splice site selection and the use of an alternative splice site acceptor sequence at intron 13. Exon 14 skipping could not be found in either patient's or control cDNA.

Thus, for the first time skipping of exon 10 in CETP mRNA was demonstrated. It is well known that conservation of the 5′ and 3′ splice sites nucleotide sequence as well as the branch site are critical for accurate splicing (31–34). Exon skipping is predominantly attributed to the mutations identified in the consensus sequence of the 5′ donor and 3′ acceptor splice sites (35). In this patient the substitution of the splice donor consensus sequence GT in intron 10 by GG resulted in skipping of exon 10. However, a more striking finding is that the intron 10 splice defect has a remote effect on the downstream splicing of intron 13 and exon 14. Thus, in addition to exon 10 skipping, an insertion of 31 bp of intron 13 sequence was detected in the patient's cDNA. The sequence of the patient's intron 13 splice acceptor site and the cryptic splice acceptor site used are shown in **Fig. 6.** The homology of the cryptic splice site to the consensus sequence described by Mount (31) is higher than that of the natural splice site. Recently, Will et al. (36) reported that a G to T transversion of the first base of exon 4 in the CFTR gene activates a cryptic CFTR exon in intron 3, showing the importance of the first nucleotide of exon 4 in the 3′ splice site selection. Thus, the cryptic splice site used in the patient may be preferentially utilized over the authentic one because the first nucleotide of the 31 bp insertion is a G, while that of the native exon 14 is an A. The in-frame stop codon included in this 31 bp inserted sequence can result not only in the abnormal translation of mRNA but also in a decrease in the steady state levels of mRNA, leading to CETP deficiency. The exact mechanism by which premature termination mutations lead to decreased amounts of mRNA is unclear but may involve altered intranuclear stability, abnormal nuclear to cytoplasmic transport of the mRNA, or decreased stability of intracellular mRNA (37, 38).

We also investigated whether the cryptic splice site was used to any extent in the splicing of control mRNA. Restriction analysis and PCR studies demonstrated that only the native intron 13 acceptor splice site was utilized in the splicing of control cDNA. Thus, we speculate that this cryptic splice acceptor site is inactivated during the normal splicing process, but in the patient, skipping of exon 10 may have a remote effect on the splice site selection at the intron 13 splice acceptor site, activating the cryptic splice acceptor site in this intron.

This hypothesis is supported by recent studies which suggest that exonic sequences as well as the secondary structure of the RNA precursor have essential information required for accurate splice site selection (39, 40). Thus, not only are the conservation of the sequences

surrounding the splice donor, splice acceptor, and the branch sites essential, but in addition, the secondary structure of pre-mRNA may be necessary for maintaining accurate splicing. The importance of secondary structure of pre-mRNA in accurate splicing is also demonstrated by the finding of an exonic mutation that results in exon skipping (41–43). It is also noted that the normal alternative splicing of exon 9 could not be observed in the patient's cDNA. Thus, the alteration of the secondary structure caused by exon 10 skipping appeared to affect the splice site selection at both intron 9 and 13 splice acceptor sites.

Another interesting finding in the analysis of this patient's CETP mRNA was that despite the presence of a mutation in the intron 14 splice site, abnormally spliced exon 14 was not identified. Thus, as supported by the fact that no CETP mass is detectable in CETP-deficient patients homozygous for the intron 14 splice defect (24, 44), the transcripts from the intron 14 mutation may be easily degraded by cells. This is also consistent with previous studies that indicate that the structure of mRNA may also determine its susceptibility to degradation (45, 46).

In the current study we report a novel mutation in the CETP gene of a patient presenting with CETP deficiency and hyperalphalipoproteinemia. The mutation in the donor splice site sequence of intron 10 leads to exon 10 skipping. In addition, this mutation results in the disruption of downstream splicing at intron 13, indicating for the first time a possible remote effect of exon skipping on the downstream splice site selection and providing a new mechanism leading to CETP deficiency.

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