Occurrence of Multiple Aberrantly Spliced mRNAs upon a Donor Splice Site Mutation That Causes Familial Lipoprotein Lipase Deficiency*

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A donor splice site mutation was found in the lipoprotein lipase (LPL) gene of a patient with familial LPL deficiency. The mutation, a G → A substitution, occurred at the first nucleotide of intron 2. Northern blot analysis of total RNA from the patient showed strikingly low levels of LPL-specific mRNAs. Using the polymerase chain reaction, the LPL mRNA splicing was analyzed in detail. The results demonstrated that no normal splicing occurred at the authentic splice site; rather a cryptic splice site 18 bases upstream from the mutation site was preferentially utilized. Although the resulting alteration in mRNA was a minute-in-frame 18-base deletion, the amount of the abnormal transcript was only 1/3 that of the normal. In addition to this major cryptic splice site, we also identified multiple minor sites which were utilized at extremely lower efficiencies. Unexpectedly, one of these minor sites was also used as an alternative splice site in the normal subject at a comparably low efficiency. The sequences of these minor cryptic sites possessed many of the characteristics common to those of other normal splice sites, indicating that even such minor sites should have also been selected according to the general rules for splice site selection. These results demonstrate that upon mutation, a broad spectrum of cryptic splice sites is activated in vivo at the sites’ respective efficiencies.

Lipoprotein lipase (LPL) is a principal determinant for the hydrolysis of triglycerides in plasma lipoproteins and is necessary for the supply of free fatty acids to tissues for nutrition and energy source. LPL is a glycoprotein synthesized in most extrahepatic tissues and is anchored to the luminal surface of the capillary endothelium by membrane-bound heparan sulfate. The cDNA coding for LPL was first cloned from human (2), and the corresponding gene has a span of 30 kilobases comprising 10 exons (4). A congenital defect in the enzyme, known as familial LPL deficiency, is a rare autosomal recessive disorder usually diagnosed by a reduced level of postheparin plasma LPL activity and characterized by marked hypertriglyceridemia. Recently, point mutations were identified in the LPL genes of some patients (6–11). In this paper we report a donor splice site mutation identified in the LPL gene of a patient with familial LPL deficiency.

Splice site mutations are common causes of a number of human genetic disorders because of a disturbance in the normal processing of pre-mRNA (12). We found a G → A substitution at the beginning of intron 2 in the LPL gene of an LPL-deficient patient. The change converts the invariant GT motif of the 5′-donor splice site into AT. Similar types of substitutions have been reported previously in many other human genetic diseases (13–17), but the resulting changes in the mRNA transcripts were quite varied. The amounts of the corresponding mRNAs range from markedly decreased (13) to normal levels (17). The pattern of aberrant splicing varies; it includes the activation of cryptic splice sites, exon skipping, and retention of an unspliced intron in the mature transcripts (17). Precise characterization of the abnormally spliced transcripts is often difficult in vivo, especially when the amount of the aberrant transcript is extremely minute (18). Experiments with transfected cells have been used for the analysis of the abnormal transcripts (13). However, there is evidence to suggest that the gene expression of transfected cells is not always the same as that of normal cells in vivo (19).

In the present study we carried out the precise characterization and quantitation of aberrantly spliced mRNA obtained from an LPL-deficient patient. We also demonstrated various cryptic splice sites that were activated upon mutation and discuss factors that affect splice site selection in vivo.

MATERIALS AND METHODS

Subject—The patient, whose parents are first cousins, was first diagnosed as LPL deficient at the age of 34 years, when he had a bout of acute pancreatitis secondary to massive chylomicronemia. His fasting serum triglyceride concentration was 4,928 mg/dl (normal: 40–150), and total cholesterol concentration was 568 mg/dl (normal: 130–220). Postheparin plasma LPL activity was 0.8 μmol of free fatty acids/ml/h (normal: 6.4 ± 2.1), which was measured 10 min after a bolus injection of heparin (10 units/kg) after an overnight fast, as described previously (20). The concentration of plasma apolipoprotein C-II, a co-factor of LPL, was 9.8 mg/dl (normal: 3.6 ± 1.0). LPL enzyme mass was measured by a sandwich enzyme-linked immunoabsorbent assay developed recently in our laboratory. The enzyme-linked immunoabsorbent assay utilizes two separate polyclonal antibodies raised against a synthetic peptide of 16 amino acids corresponding to the N terminus of the mature human LPL and against a recombinant whole human LPL. No immunoreactive mass was detectable

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‡ The abbreviations used are: LPL, lipoprotein lipase; PCR, polymerase chain reaction; bp, base pairs; mRNA, small nuclear RNA.

2 M. Kawamura, unpublished data.
in the postheparin plasma of the patient.

Genetic Cloning and DNA Sequencing—Gene fragments that cover exons 3-9 were obtained from the genomic library constructed previously for the patient (21). To obtain gene fragments containing the remaining exons, gene amplification was performed by the polymerase chain reaction (PCR) (22) with oligonucleotide primers that were synthesized according to the published sequence of the human LPL gene (4). Directly cloned gene fragments were subcloned into the M13 vector mp18 or mp19 and sequenced by the dideoxy method (23) with Sequenase (U. S. Biochemical Corp.). Direct sequencing of amplified DNA was performed utilizing an unequal ratio of primers for the second PCR (24). After the first PCR, V(DJ) of the reaction was amplified again, together with an unequal ratio (100:1) of the same primers as used in the first reaction. The single-stranded DNA from the second PCR was used for sequencing after chloroform extraction and filtration using a Centricron 100 microconcentrator (Amicon Corp., Danvers, MA).

Preparation of Monocyte-derived Macrophages—Human monocyte-derived macrophages were prepared by culturing human peripheral monocytes. Mononuclear cells were isolated from peripheral blood cells by the Ficoll-Hypaque gradient method as described previously (25). The cells were washed three times with phosphate-buffered saline. The washed cells were suspended in RPMI 1640 and plated in 12-well plastic plates (3 x 10^5 cells/16-mm well). After 2 h of incubation at 37 °C in 5% CO2 and 95% air, nonadherent cells were removed by three washes with phosphate-buffered saline. The cells were then plated in fresh RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The medium was changed twice. The differentiated macrophages were lysed with a 5.5 M guanidine thiocyanate homogenization buffer containing 25 mM sodium citrate (pH 7.0), 0.5% N-laurylsarcosine, and 0.2 M 2-mercaptoethanol. Total cellular RNA was isolated from the cell lysates by the guanidine thiocyanate method (26).

Northern Blot Hybridization Analysis—Total RNAs (30 μg) from both normal subject and the patient were electrophoresed on a 1% agarose gel with 6% formaldehyde, transferred to a nylon membrane, and hybridized in Rapid Hybridization Buffer (Amer sham Corp.) with the human LPL cDNA probe (27) at 65 °C for 1 h. The blot was washed twice with 0.5 x SSC, 0.1% sodium dodecyl sulfate (1 x SSC = 150 mM NaCl and 15 mM sodium citrate) at 65 °C for 20 min. Hybridization with β-actin cDNA probe was carried out similarly.

Reverse Transcription Coupled with PCR Amplification—Total RNA (1 μg) was incubated for 60 min at 37 °C in a reaction mixture (25 μL) containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 5 mM of each deoxyriribonucleoside triphosphate (dNTP), 5 units of ribonuclease inhibitor from human placenta (Takara Shuzo, Kyoto, Japan), 100 pmol of random hexamer primers (Pharmacia LKB Biotechnology Inc.), and 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The total products were subsequently subjected to the first PCR using an equal volume (25 μL) of solution containing 50 mM KCl, 10 mM Tris (pH 8.3), 0.01% gelatin, 5 mM of each dNTP, 5 μL of dimethyl sulfoxide, 15 pmol of 5′ primer, and 25 units of Taq DNA polymerase (Perkin-Elmer Cetus Instruments). PCR amplification (25 cycles) was performed under the following conditions: 1 min denaturation at 94 °C, 2 min primer annealing at 50 °C, and 3 min extension at 72 °C. To amplify target regions, 1/10 of the first PCR products was again subjected to the second PCR with a pair of internal primers.

RESULTS

In the LPL gene of the patient, the G → A substitution was found at the first nucleotide of intron 2 (Fig. 1A). This G → A transition was the only pathological change that was found during sequence analysis of all coding exon and adjacent intron sequences. Homozygosity for the mutation was confirmed by direct sequencing of six products independently amplified by PCR. Northern blot analysis of total RNA from monocyte-derived macrophages revealed a strikingly decreased level of LPL mRNA in the patient although the levels of β-actin mRNA were the same for normal subject and the patient (Fig. 1B). Despite the decrease in the LPL mRNA level, the length of the LPL mRNA of the patient was almost the same as that of the LPL mRNA of the normal subject. Two species of mRNAs with a length of 3.4 and 3.8 kilobases, resulting from an alternative utilization of polyadenylation signals (2), were detected in both cases.

To investigate further the LPL mRNAs in the patient, PCR technology was adopted for amplification of the regions of interest within the transcripts using a procedure described previously (28). Shortly after reverse transcription of mRNA with random primers (hexamers), the cDNA product was subjected to the first PCR with only a 5′-outer primer (primer A in Fig. 2). Subsequently, 1/1000 of the first reaction product was amplified again by the second PCR; the target regions were amplified by a pair of appropriate internal primers. Fig. 3 shows the results of a second PCR with a pair of oligonucleotide primers (primers C and I in Fig. 2). Staining with ethidium bromide showed a single major band of the expected size for the normal subject, indicating that the PCR accurately amplified the target region of 242-base pair (bp) length. In contrast, the same PCR gave rise to 224 bp, an 18-bp shorter fragment for the patient. Since our previous findings had indicated that there is no deletion in the coding region of the patient’s gene, this abnormal fragment most likely reflects the aberrant splicing event in the mRNA of the patient. Southern blot analysis, performed with a human LPL cDNA probe as described previously (21), confirmed that both the 242- and 224-bp fragments were indeed from LPL mRNAs (Fig. 3). These results suggested that the abnormal 224-bp fragment was probably derived from the misspliced LPL mRNA of patient as depicted in the right panel of Fig. 3.

The amount of the abnormal transcript was measured precisely by the competitive PCR method, which was recently developed for the quantitation of cytokine mRNA by Gilliland et al. (29). Since both the 242- and 224-bp fragments can be co-amplified with the same set of primers and their sizes are only slightly varied, co-amplification of the two fragments should occur in a concentration-dependent manner. As shown in Fig. 4, the same amount of 242- and 224-bp fragments was obtained when cDNAs of the normal subject and the patient were mixed at a ratio of 1:12. The results indicate that the amount of the abnormal transcript is only about 1/25 of that of the normal. Direct sequencing of the 224-bp fragment identified a newly created boundary between exons 2 and 3, which was located 18 bases upstream from the authentic boundary (Fig. 5). The new exon 2-intron 2 junction also contained an alternative GT dinucleotide.

To investigate if the other aberrant splicings could occur at all in vivo, we performed the following experiments. First, we obtained the DNA fragments that migrated faster than

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**Fig. 1. DNA sequence analysis of the patient's LPL gene and Northern blot analysis of macrophage LPL mRNA.** A, DNA sequence of the exon 2-intron 2 boundary in the LPL gene of the patient. The G → A substitution in the donor splice site is indicated by an asterisk. B, Northern blot analysis of monocyte-derived macrophage LPL mRNA. N, normal; P, patient. Longer exposure (right) revealed the markedly decreased levels of LPL mRNA in the patient.
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Primer G  Primer H
Primer F  Primer I
Primer E  Primer J
Primer D  Primer I
Primer C  Primer I
Primer B  Primer I
Primer A  Primer I

EXON 1  EXON 2  EXON 3

Primer A: TCGACGGCTCATGCTGGTGG
Primer F: CACTGTACCTGGGTGGGCC (+208 - +227)
Primer B: AAGCCTCTGCAGTGGTG
Primer G: CTGGAGTGAGTGAGGAAGT (+399 - +418)
Primer C: TTGGCTACAGGCTGAC
Primer H: GGTGGGATTTAAAGAAGCT
Primer D: GTGACCATCGTGGTCAG (+18 - -1)
Primer I: TGACCCACACTCATACACA
Primer E: TAAGGGAGCTCCTTTGGG (+2 - +19)
Primer J: CAGCCAGTCCACACACATAGA

Fig. 2. Location and nucleotide sequence of the oligonucleotide primers used for PCR. To examine the possible occurrence of exon skipping, primer C was used in the second PCR together with primer I. Similarly, to examine the possibilities of the activation of cryptic splice sites and the retention of unspliced whole intron 2 in the LPL mRNA, primers C-G and primer H were used as a 5' primer. The numbers represented in parentheses indicate the exact position of the nucleotide sequence of respective primers. +1 denotes the position of the first nucleotide of intron 2.

Fig. 3. Detection of aberrant splicing in LPL mRNA of the patient. PCR amplification with primers C and I yielded fragments of different length in the normal subject and the patient's cDNA (N, normal; P, patient). Reaction products were analyzed by agarose-gel electrophoresis with DNA molecular size standards (φX174/HaeIII) (left) and by Southern blot hybridization with 32P-labeled human LPL cDNA (middle). Schematic representation of the predicted structure of LPL cDNAs from the normal subject and the patient is given in the right panel.

![Detection of aberrant splicing in LPL mRNA of the patient.](image)

Fig. 4. Quantitation of the aberrantly spliced LPL mRNA of the patient by the competitive PCR method. Normal cDNA (N) and the patient's cDNA (P) were mixed in a series of ratios as indicated, and the mixtures were PCR amplified with primers C and I. The relative amount of the two products reflects the original contents of normal and the patient's LPL mRNAs.

| cDNA (N) | 1 | 1 | 1 | 1 | 1 | 1 |
| cDNA (P) | 2 | 4 | 10 | 12 | 15 | 20 | 100 |

242bp 224bp

![Quantitation of the aberrantly spliced LPL mRNA of the patient.](image)

Fig. 5. Comparison of the cDNA sequences coding for normal and the patient's LPL. Each autoradiogram represents the cDNA sequence encompassing the exon 2-exon 3 boundary of LPL gene. The nucleotide sequence of cDNA from the patient revealed an in-frame 18-bp deletion at the 3' terminus of exon 2. Horizontal arrows indicate the exon 2-exon 3 boundary in the sequences of cDNA from the normal subject and the patient.

amplification, four species of fragments were visible in the gel, as shown in Fig. 6. These DNA fragments must have been generated by the activation of the minor cryptic splice sites present in intron 2. The four fragments were eluted separately from the agarose gel and were sequenced directly. These experiments not only identified the exact location of four minor cryptic splice sites (+43, +146, +250, and +387 in intron 2) but also confirmed the presence of "alternative GT" within the sequence of the respective sites. Longer exposure of the autoradiograph in Fig. 3 also demonstrated the presence of minor bands with an intensity, as measured by a densitometer, of approximately 1/1000 of that of the major band corresponding to the 224-bp fragment. In addition to these four minor sites, more infrequently utilized sites were identified by sequence analysis of the total PCR products which were subcloned into M13 vectors. Of 100 clones examined, two contained the inserts derived from the activation of sites other than the four minor sites. These results indicated that the patient's LPL mRNA consisted of multiple species of transcripts which resulted from aberrant splicing with various degrees of frequency. To examine the possibility of the occurrence of further downstream splicing, we performed second PCRs with 5' primers (primers E, F, G and H represented in Fig. 2). However, as shown in Fig. 7 (lanes 1, 3, 4, and 5), the
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RESULTS



FIG. 6. Detection of minor transcripts present in vivo. Four species of fragments derived from patient's cDNA were amplified to a visible state by a second PCR with primers D and I. To avoid amplification of the transcripts made with the major cryptic site, the 5' primer (primer D) was located immediately downstream of the major site. The number indicated on the right vertical axis represents the position of the cryptic splice sites responsible for the generation of respective fragments. DNA size marker = φX174/HaeIII. Schematic representation given on the right illustrates the four minor cryptic splice sites.

results clearly denied the possibility of further downstream splicing as well as the possibility of retention of intron 2 in the mRNA.

For reference, we also examined the LPL mRNA prepared from normal subjects. The results unexpectedly demonstrated low levels of aberrant splicing in the normal genes. As shown in Fig. 7 (lane 2), the PCR-amplified cDNA from the normal gene contained an aberrant transcript derived from an alternative splice site. The site (+250) was identical to one of the minor sites identified in the mutant gene, and the levels of utilization of the +250 site appeared comparable in both cases. In addition, the occurrence of downstream splicing (farther than +387), which was not detected in the patient, was observed in the normal subjects (data not shown). This finding indicates that low levels of aberrant splicing could occur in vivo even in the presence of the normal splice site. Fig. 8 illustrates the splicing events that occur in the normal and mutant genes as well as the estimated relative amount of each transcript.

Splicing is mediated by the initial interaction of primary transcripts with U1 small nuclear RNA (snRNA) (12). To gain insight into the mechanism of splice site selection in vivo, we compared the seven cryptic sites described above with other GT-containing sequences in intron 2, with respect to the degree of nucleotide homology, location, and their free-energy changes (30) upon binding to U1 snRNA. The degree of homology to the other known 5' splice sites was rated according to the report of Shapiro and Senapathy (31). The results are shown in Fig. 9 along with other possible cryptic site sequences that contain GT dinucleotide. The cryptic sites generally showed more favorable values for splice site selection than the other sequences in the two parameters (homology score and free-energy value). It should be noted that the major cryptic site, although showing unexpectedly unfavorable values for these two parameters, contained the GT dinucleotide nearest to the authentic splice site.

DISCUSSION

In the present study we showed that the LPL mRNAs in this patient were misspliced by activation of nearby cryptic splice sites, and their amounts were markedly decreased to 1/12 of the normal level. These abnormalities explain the molecular basis of the LPL-deficient state in the patient.

In some splicing mutations, two or more cryptic sites are activated simultaneously (17, 19). Furthermore, competition among cryptic sites has been demonstrated recently for the transfected cells by a superb mutagenic technique (32). These findings led us to hypothesize that a series of candidate introns is used in vivo although this competition would be undetectable by conventional methods. To test this hypothesis we attempted to detect minor transcripts present in vivo in the affected cells of this patient with the PCR method. The experiments successfully amplified four minor transcripts to a visible state in gels (Fig. 6). The selection of the minor cryptic sites would not be accidental but would rather follow the same rule that applies to major sites. Indeed, the sequences of all the minor sites showed a degree of favorable values similar to those of other established splice sites in two parameters (Fig. 9). In addition, the sequence analysis of the subcloned PCR product revealed the presence of two additional rarer transcripts, indicating the possibility that a number of transcripts could
exist in vivo at barely detectable levels. These results enabled us to establish a model for 5'-cryptic site selection in vivo whereby a spectrum of sequences can compete with each other for activation and can be utilized at their respective efficiencies. The resultant transcripts are quantitatively so diverse that only a few can normally be found and some can be detected by PCR, but the majority are undetectable.

Unexpectedly, similar low levels of aberrant splicing event were observed in normal gene expression. In normal subjects, it was found that the cryptic site (250) was used for alternative splicing as well. Although we have not yet analyzed for other genes, such low levels of alternative splicing may be associated with normal gene expressions in vivo. Another unexpected finding was that the utilization of the alternative site (+250) was not augmented by the disruption of the authentic splice site in the patient, suggesting that such low levels of alternative splicing should not be directly linked to the activation of cryptic splice sites caused by splicing mutations.

Recently, a number of nonsense mutations have been reported to cause a considerable reduction in the corresponding mRNA (33, 34). A reduction in mRNA associated with splicing mutations (13, 35) might be explained partly by a similar mechanism (i.e. mRNA instability) because roughly two-thirds of the splicing mutations should alter the reading frame of mRNA, which would introduce a stop codon downstream. The concept was supported by the fact that the splicing mutations with normal levels of mRNA usually cause no frameshift in the coding sequence (17, 30). In contrast, the major change in the LPL mRNA of the patient was shown to be a simple in-frame deletion of 18 nucleotides (Fig. 5); however, the amount of the abnormal transcript was greatly reduced to about 1/2 of the normal level (Fig. 4). This reduction can hardly be explained by the extent of the structural change of the transcripts since the deletional alteration in the present patient is small and thus is unlikely to affect mRNA stability seriously. One possible explanation is that the binding between the sequences of the major cryptic site in this patient and U1 snRNA may be weak. In fact, the predicted free-energy value of the major cryptic site (−2.8 kcal/mol) is not a particularly favorable value for stable binding to U1 snRNA (Fig. 9). Since such binding is one of the rate-limiting steps in RNA processing, instability of the binding would be disadvantageous for sufficient production of mature transcripts.

These results also provide some insight into the factors that affect splice site selection. As has been shown in other studies (17), we found a significant difference in the average values of the two parameters between the seven cryptic site sequences and other candidate sequences (average homology score: 73.6 versus 58.2%; average free-energy value: −5.1 versus −1.7 kcal/mol) (Fig. 9). In the individual case, however, there are some interesting contradictions. For example, the major cryptic site (68.6% and −2.8 kcal/mol) is less favorable than the minor other sites (74.4% and −5.5 kcal/mol on average). In addition, utilization of the sequence GTGGTGAGG at position +38 could not be detected despite the high values in the two parameters. Therefore, in addition to these two parameters, there must be other undetermined factors crucial for splice site selection in vivo. In the selection of the cryptic splice sites, the distance from the authentic site must be another important determinant. In fact, in the present study we could never detect the activation of the cryptic site farther than +387 (Fig. 7). The overall RNA structure of the candidate sequences may also be another important factor which should be considered.

In this study, we demonstrated that one major and multiple minor cryptic sites can be activated at their respective efficiencies in vivo. Since even such minor cryptic splice sites apparently follow the common rules for splice site selection, extensive analysis of these sites will provide new insight into the mechanism governing splice site selection.

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