A novel A → G mutation in intron I of the hepatic lipase gene leads to alternative splicing resulting in enzyme deficiency

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Abstract We have identified the underlying molecular defect in a patient with hepatic lipase (HL) deficiency presenting with hypertriglyceridemia and premature cardiovascular disease. DNA sequencing of polymerase chain reaction (PCR) amplified DNA and digestion with BsrI established homozygosity for an A → G mutation in intron I of the patient's hepatic lipase gene. This mutation introduces an additional AG motif within a potential branch lariat signal located 13 bp upstream of the native 3′ splice site. Two minigene constructs (normal and mutant) consisting of exons 1 and 2 as well as 192 bp of intron I of HL were generated by the overlap PCR extension method and transfected in human 293 cells. Sequence analysis of reverse transcribed, amplified cDNA generated from total RNA isolated from transfected cells demonstrated the presence of abnormally spliced products containing 13 and 78 additional bases as well as the accumulation of unspliced mRNA. No normally spliced mRNA was identified. Thus, the A → G mutation disrupts normal splicing of intron I and generates a new AG site that is utilized as an alternative 3′ splice signal leading to the most prominent RT-PCR product in vitro. Translation of these alternatively spliced products leads to premature termination resulting in the synthesis of a truncated, non-functional enzyme. The absence of normal HL protein in post heparin plasma of this patient was confirmed by Western blotting. DNA restriction analysis demonstrated that all four of the proband's children, who exhibit HL activity levels between those of the HL-deficient father and the mother with normal HL activity, are heterozygotes for the splice site mutation. Thus, our studies establish the functional significance of a novel mutation in the HL gene of a patient presenting with HL deficiency.—**Brand, K., K. A. Dugi, J. D. Brunzell, D. N. Nevin, and S. Santamarina-Fojo.** A novel A → G mutation in intron 1 of the hepatic lipase gene leads to alternative splicing resulting in enzyme deficiency. *J. Lipid Res.* 1996. **37:** 1213–1223.

Supplementary key words expression studies • hepatic lipase deficiency • premature atherosclerosis • splicing defect

Hepatic lipase (HL) is an important lipolytic enzyme synthesized primarily by the liver that mediates the hydrolysis of triglycerides and phospholipids present in plasma lipoproteins (1–4). Its action as both an acylglycerol hydrolase and phospholipase results in the conversion of intermediate density lipoprotein (IDL) to low density lipoprotein (LDL) and high density lipoprotein $(HDL)_2$ to HDL_3 (5–7). Thus, HL plays an important role in the metabolism of remnant lipoproteins and HDL. It has recently been shown that the expression of human HL in transgenic mice reduces HDL particle size and lowers HDL, total cholesterol as well as aortic cholesterol levels (8).

The DNA sequence of the human HL gene has been described. It consists of 9 exons interrupted by 8 introns (9–11) that encode an mRNA of 1.6 kb (12–14) and has a genomic organization similar to that of other members of the lipase family (15). The mature human HL protein has a molecular weight of 56 kD and consists of 476 amino acids which share a high degree of primary amino acid sequence homology to lipoprotein lipase and pancreatic lipase (12–15).

The important role of HL in lipid metabolism has been established by the identification of patients with HL deficiency. This rare genetic disorder, which appears to be inherited as an autosomal recessive trait, has been identified in only four kindreds to date (16–21). However, due to difficulty in identifying patients with HL deficiency, the frequency of this genetic disorder in the

Abbreviations: HL, hepatic lipase; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; LPL, lipoprotein lipase; PCR, polymerase chain reaction; RT, reverse transcription; CMV, cytomegalovirus early promoter; SV40 polyA, simian virus 40 polyadenylation signal.

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general population may be underestimated. Patients with a deficiency of HL may present with features characteristic of type III hyperlipoproteinemia, including hypercholesterolemia, hypertriglyceridemia, accumulation of triglyceride-rich lipoproteins, and premature cardiovascular disease (17, 21–25). However, unlike patients with type III hyperlipoproteinemia, HL-deficient patients have triglyceride-enriched lipoproteins and a normal VLDL-cholesterol/total triglyceride ratio of <0.3, a finding that is useful in distinguishing the two genetic syndromes (17, 21, 24).

The effect of HL deficiency on the metabolism of lipoproteins has been investigated in several HL-deficient patients. Large buoyant LDL-like particles with a greater proportion of triglyceride compared to the control subjects' LDL have been identified in the patient presented in this study (17). Furthermore, HL deficiency leads to a reduction of the fractional clearance rate of apolipoprotein B from VLDL and IDL and delayed transfer of this apolipoprotein to both IDL and LDL (26). Thus, particles in the density range of VLDL and IDL accumulate whereas the LDL concentration is reduced (17, 25, 26). HDL appears to be almost exclusively present as large triglyceride-rich particles corresponding in size to the HDL_2 density fraction (21, 25).

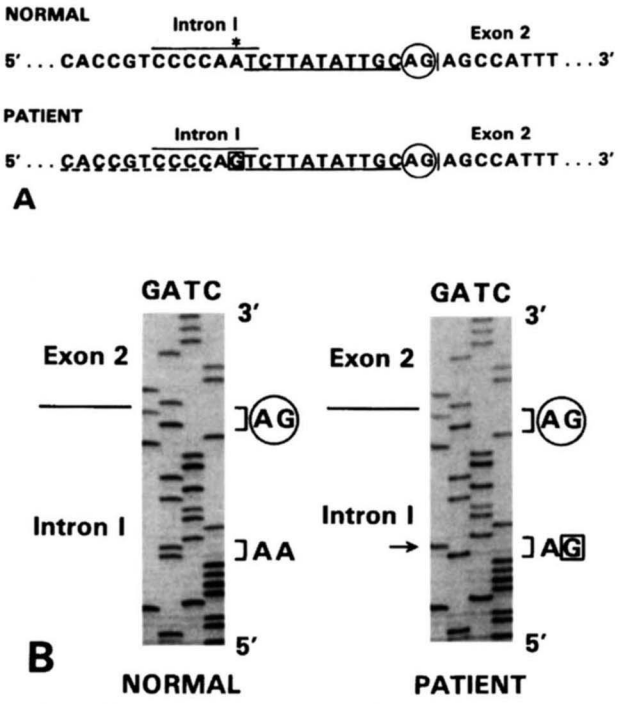

Fig. 1. DNA sequence analysis of the HL gene. Panel A illustrates the sequence surrounding the acceptor splice site present in intron I of the normal and patient's HL gene. The identified mutation is boxed and its location indicated by an asterisk. The native AG site is circled. The polypyrimidine strings preceding the normal AG site (solid line) and the newly created AG motif (dashed line) are underlined. A potential branch lariat signal is indicated by a solid line over the sequence and the core A of this consensus sequence is indicated by an asterisk. Panel B shows the autoradiogram of DNA sequencing gels of the HL gene of a normal individual and the HL-deficient patient.

The underlying molecular defects that lead to HL deficiency have been investigated in two independent kindreds (16, 19, 20). Two mutations resulting in the replacement of Ser^{267} by Phe and Thr^{383} by Met have been described in the affected proband from the Ontario kindred. In the kindred from Quebec, a mutation that leads to the substitution of Thr^{383} by Met was identified in only one of two alleles. The authors thus speculated that the deficiency imparted by this mutation might be modulated by other factors such as age, gender, or by the effect of variation at other genes (19, 20). Additional sequence variants of the HL gene that have been identified in normolipidemic subjects with normal HL activity involve mutations in exon 3 ($Val^{73} \rightarrow$ Met) (27) and in exon 5 ($Asn^{193} \rightarrow$ Ser) (16, 19).

In the present report we describe the underlying molecular defect in the HL gene of a patient presenting with HL deficiency, hypertriglyceridemia, and premature cardiovascular disease (17, 18). The patient is homozygous for a single A → G substitution in intron I of the HL gene. The functional significance of this gene defect was established by in vitro expression of normal and mutant minigene constructs that demonstrated that the A → G substitution leads to alternative splicing, resulting in premature termination of translation and the formation of a truncated non-functional enzyme.

MATERIAL AND METHODS

Study subject

The proband is a 55-year-old male who presented at age 27 with hypertriglyceridemia and recurrent episodes of pancreatitis. At age 41, the patient suffered a myocardial infarction. A detailed description of his clinical presentation, lipid values, and lipoprotein profile has been published (17, 18). Absent HL activity in post-heparin plasma established the diagnosis of HL deficiency in this patient.

DNA sequence analysis of genomic DNA

DNA was extracted from leukocytes as described earlier (18). Synthetic oligonucleotide primers based on the published HL gene sequence (10, 11) were prepared on a DNA synthesizer (Applied Biosystems, Inc., model 380B, Foster City, CA) using the phosphoramidite method. Genomic DNA (100 ng) was amplified by the polymerase chain reaction (28) using an automated DNA Thermal Cycler (Perkin-Elmer Cetus, Emeryville, CA) and 330 ng each of two HL-specific primers. Amplification was performed in a reaction mixture of 50 mM

KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and 125 μM each dGTP, dATP, dTTP, and dCTP with 5 units of Taq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA). Cycles included denaturation at 95°C for 1 min, annealing at 55°C, and polymerization at 72°C for 2 min for 30 cycles. Amplified DNA was subjected to electrophoresis in 1.5% low melting point agarose (Bethesda Research Laboratories, Gaithersburg, MD) gels. The DNA fragments were isolated and subcloned using the TA-Cloning system (Invitrogen, La Jolla, CA). Sequencing was performed with T7 DNA polymerase (Sequenase, United States Biochemical Corp., Cleveland, Ohio) using the dideoxynucleotide chain termination method (29).

Restriction enzyme digestion with BsrI

Genomic DNA was amplified using the primers 5′-GGCTTGTGCTTGTAGAAGCAGC-3′ and 5′-AACGGCATGTCAGCATTTCC-3′. The amplified fragment contained 93 bp of intron I, the entire exon 2, and 23 bp of intron II. The amplified fragment was isolated using a 1.5% low-melting agarose gel and digested with 8 units of the restriction enzyme BsrI (New England Biolabs, Beverly, MA) for 4 h at 65°C. The digested fragments were electrophoresed in a minigel containing 1% SEAKEM ME agarose and 2% NUSIEVE GTG agarose (FMC Bioproducts, Rockland, ME).

Synthesis of expression vector

Two minigene constructs designated pSN (normal) and pSP (mutant) consisting of exons 1 and 2 as well as 192 bp of intron I of HL were generated from genomic DNA of a normal subject and the patient by the overlap extension method (30) (see Fig. 3). The truncated 192-bp intron consisted of 100 bp of the 5′ end joined to 92 bp of the 3′ terminus of HL intron I. PCR was performed using DNA polymerase from *Pyrococcus furiosus* (Pfu; Stratagene Inc., La Jolla, CA) and 30 cycles with 1-min denaturation at 95°C, 1-min annealing at 50°C, and 2-min extensions at 72°C (31). An XbaI restriction site in the 5′ untranslated region was created using the primer 5′-CTTCAAAAATTAT↓CTAGAAAGCCTGGACCC-3′. A termination codon (TGA) and an HpaI site were introduced at the end of exon 2 with the antisense primer 5′-GAGGAGTT↓AACGCCTCACTCCTGTAACGTGTC-3′. The constructs were then subcloned into the XbaI and HpaI restriction sites of the pCMV expression vector which is a pUC-18-derived plasmid containing the cytomegalovirus (CMV) early promoter and the simian virus (SV40) polyadenylation signal as described previously (32). The constructs, pSN and pSP, were identical in structure except for the base substitution in question as confirmed by DNA sequence analysis (29).

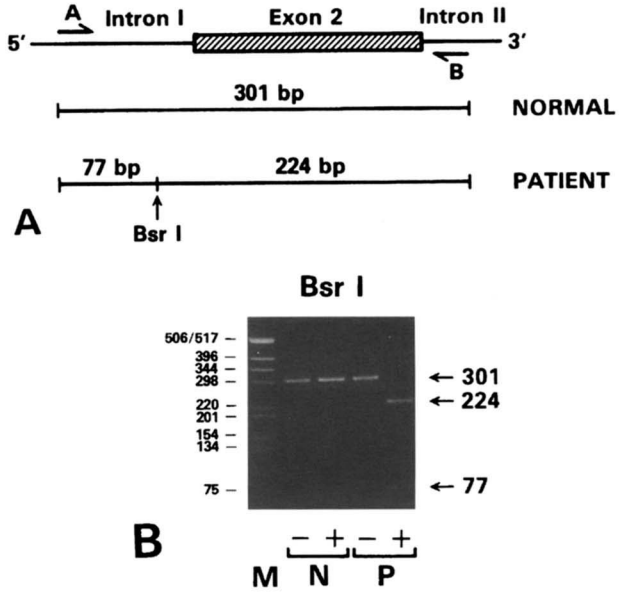

Fig. 2. Restriction enzyme analysis of PCR amplified DNA. Panel A illustrates the PCR product of normal or patient HL DNA after amplification using primers A and B. The location of the unique BsrI restriction enzyme site identified in the patient's HL gene is indicated. Panel B shows the agarose gel electrophoresis analysis of undigested (-) and BsrI-digested (+) PCR-amplified DNA from a normal subject (N) and the patient (P). DNA molecular size is indicated in bp. DNA molecular size standards are shown in lane M.

In vitro expression in human embryonic 293 cells

Plasmids used for transfection were purified by the double banding cesium chloride method (33). Each plasmid was transfected in triplicate in two separate transfections by calcium phosphate co-precipitation (34). Forty μg of plasmid DNA was added to each 100-mm plate of 293 cells. These 293 cells were used because they contain the E1 region of adenovirus (35) making them a very efficient expression system using the CMV promoter (36). Total RNA was harvested 48 h after the addition of DNA using guanidinium thiocyanate as described earlier (37). To determine transfection efficiencies, cells were transfected with previously described plasmids, including pRSV-luciferase and pCMV-LPL (38).

Reverse transcription–polymerase chain reaction (RT–PCR)

RT–PCR was performed as described earlier (39). To prepare cDNA, RNA samples were incubated with the antisense primer 5′-CTGGTCTTCATCTCATGCAGC-3′ at 70°C for 15 min in the presence of 0.1 M dithiothreitol, 80 units RNAsin (Promega, Madison, WI), 150 mM deoxynucleotide 5′ triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and water to a final volume of 100 μl. The hybridization

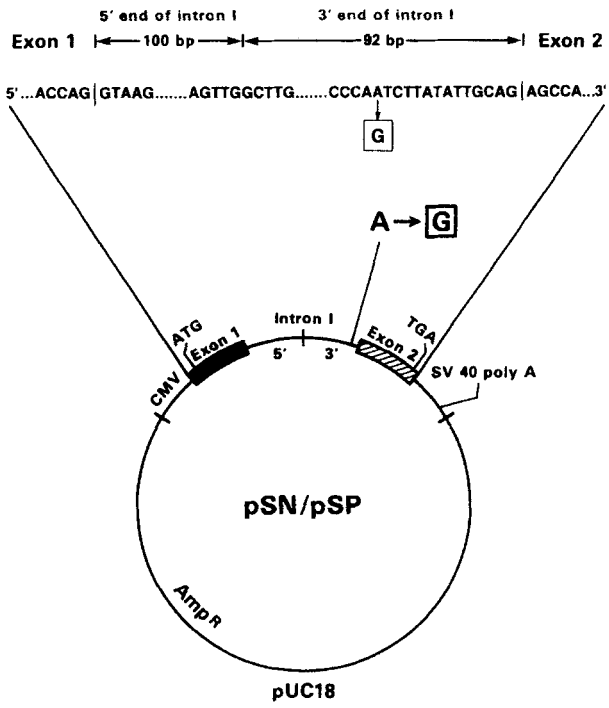

Fig. 3. Schematic representation of normal (pSN) and mutant (pSP) splicing constructs. The HL minigene insert containing exons 1 and 2 as well as 192 bp of intron I was cloned into a pUC18 parent vector containing the CMV immediate early promoter and SV 40 polyadenylation signal. The 192-bp intron I was synthesized by ligating 100 bp of the 5′ end with 92 bp of the 3′ end of HL intron I. The A to G mutation is indicated by a box.

mixture was allowed to cool to 37°C. Forty units of Moloney murine leukemia virus reverse transcriptase (Pharmacia, Piscataway, NJ) was added and the extension was performed at 37°C for 1 h. The resultant first-strand cDNA was amplified by PCR (28) using Taq DNA polymerase at the conditions described for genomic DNA after the sense primer 5′-GTGTTTCTCCATTCTGTTGG-3′ was added. The PCR products were analyzed on a 1.5% low melting point agarose gel, subcloned, and sequenced as described above. To quantitate the ratios of the various in vitro splicing products of the patient, the RT–PCR products were Southern blotted as previously described (40) and probed with a hepatic lipase-specific DNA probe. The blots were scanned and single bands were quantitated with the Betascope 603 Blot Analyzer from Betagen, Waltham, MA.

Western blotting

Western blotting was performed as previously described (41). Post heparin plasma was partially purified by heparin-Sepharose chromatography. The fractions were run on 10% SDS-PAGE and a polyclonal goat anti-hepatic lipase antibody that does not recognize LPL (kindly provided by Dr. Ira Goldberg, Columbia University, New York, NY) was used as first antibody. In addition, transfection medium from cells transfected with human hepatic lipase and grown without fetal calf serum was run as a positive control.

RESULTS

Sequence analysis of the 9 HL-exons, exon-intron splice junctions, as well as 1.5 kb of the 5′-flanking region and 156 bp of the 3′ flanking region of the HL gene was performed after PCR amplification of patient genomic DNA. At least six independent clones were sequenced for each amplified fragment. Our studies identified a single-base substitution, A → G, in intron I located 14 bp upstream of exon 2, that was present in all six sequenced clones (**Fig. 1**). The A → G substitution introduces an additional AG dinucleotide in close proximity to the native 3′ splice signal (42–44) thus providing an alternative 3′ splice site. In addition, this mutation destroys the core A of a branch lariat signal potentially utilized for splicing at the native 3′ splice site. A second mutation resulting in the substitution of Asn^{193} → Ser in the fifth exon of all six clones was also identified by our sequencing studies (data not shown). However, this A → G mutation results in the synthesis of a previously described isoprotein variant that is not associated with HL deficiency (19).

The identified A → G mutation introduces a unique BsrI restriction enzyme site in intron I of the patient's HL gene. In order to establish the homozygous nature of the defect, a 301 bp DNA fragment containing the region of the mutation was PCR-amplified from genomic DNA of a normal subject and the HL-deficient patient (**Fig. 2A**). Digestion of patient but not of normal amplified DNA with BsrI should result in the formation of two fragments, 224 and 77 bp in length (Fig. 2A). Figure 2B illustrates restriction fragments generated after PCR-amplified normal and patient DNA was digested with BsrI. Complete digestion of the proband's DNA established homozygosity for the intron I, A → G, substitution.

In order to evaluate whether the newly introduced AG motif disrupts the normal splicing process and/or functions as an alternative splice site, we generated two minigene constructs, pSN (normal) and pSP (mutant) consisting of exons 1 and 2 as well as 192 bp of intron I of the HL gene (**Fig. 3**). The two constructs were utilized to transfect human embryonal 293 cells. The truncated 192 bp intron I consists of 100 and 92 bp of the 5′ and 3′ ends of intron I, respectively. Previous studies have indicated that the minimal size of a functional intron is 66 to 70 nucleotides (44, 45).

Human 293 cells were transfected with the normal (pSN) and mutant (pSP) constructs, respectively, and total RNA isolated from transfected cells was analyzed by RT–PCR followed by separation of the DNA by agarose gel electrophoresis. Using the primers indicated in **Fig. 4A,** the predicted size of a correctly spliced product is 135 bp whereas that of unspliced RT–PCR DNA is 327 bp. RT–PCR of total RNA derived from cells transfected with the normal construct, pSN, amplified the expected 135 bp fragment (Fig. 4B).

In contrast, the splicing pattern of RNA isolated from cells transfected with the mutant construct (pSP) included DNA fragments that were 327, 213, and 148 bp in length (Fig. 4B). No correctly spliced products were identified. The size of the larger 327 bp product coincides with the expected fragment length of unspliced mRNA. In addition to the new AG dinucleotide introduced 13 bp upstream of the native splice site by the identified mutation, an additional AG motif, 78 bp upstream of exon 2, is present. If these two alternative sites are utilized for splicing the expected products would be 213 and 148 bp in length which is consistent with the sizes of the two other DNA fragments identified in Fig. 4B. Thus, it appears that RT–PCR of RNA isolated from cells transfected with the mutant construct resulted in the formation of either unspliced or alternatively spliced DNA fragments. The ratio of unspliced products to products spliced at the newly created AG site 13 bp upstream to products spliced at the cryptic splice site 78 bp upstream was 1:2.9:0.8 as determined by Southern blotting of RT–PCR fragments (data not shown). No correctly spliced product was present in the cells transfected with the mutant construct. No RT–PCR product was detected in the absence of RNA (lane C_1, Fig. 4B), in the absence of reverse transcriptase (lane C_2), or when RNA from cells transfected with the control plasmid was used (lane C_3).

In order to further characterize the most prominent RT–PCR product (148 bp in length) we digested this fragment with BsrI. Alternative splicing at the newly created AG site results in the insertion of 13 bp and formation of a new BsrI restriction site. Thus, digestion

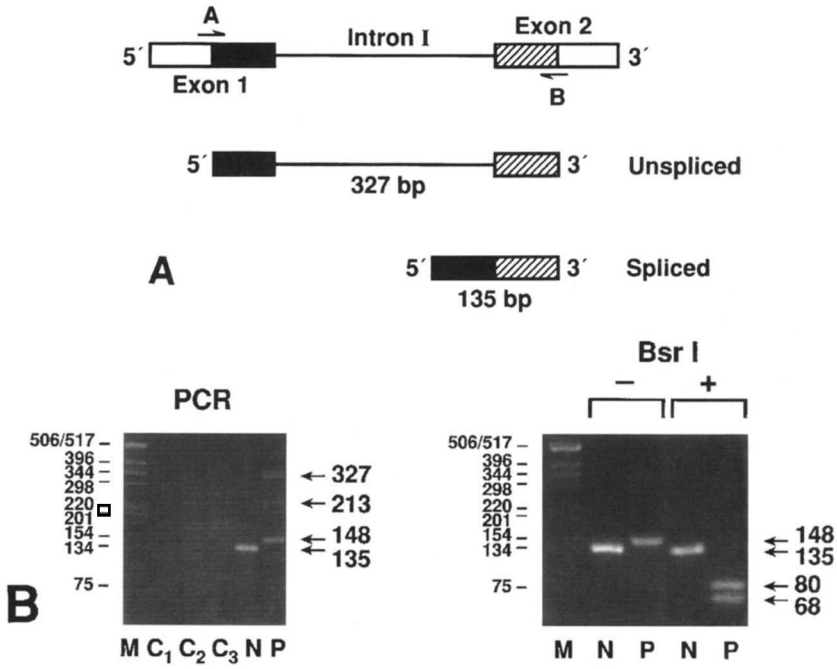

Fig. 4. Restriction enzyme analysis of RT-PCR products. Total RNA isolated from cells transfected with normal and mutant constructs was analyzed by RT-PCR and restriction digestion with BsrI. Panel A is a schematic representation of expected RT-PCR-amplified DNA using primers A and B. The amplified unspliced product has a size of 327 bp and contains the complete 192-bp sequence of intron I present in the mutant expression vector. Correct splicing removes the 192 bp of intron I leading to the formation of a 135-bp fragment. Panel B, left, shows the products obtained after PCR-amplification of cDNA obtained from reverse transcription of total RNA isolated from cells transfected with normal (N) and mutant (P) constructs. In addition, the absence of specific bands in reactions without RNA (lane C_1), without reverse transcriptase (lane C_2) or with RNA from cells transfected with the vector plasmid (lane C_3) is illustrated. Panel B, right, illustrates the 135 bp (N) and the 148 bp (P) fragments before (-) and after (+) digestion with BsrI. Digestion of the 148 bp product leads to the formation of fragments 68 and 80 bp in length. DNA molecular size is indicated in bp. DNA molecular size standards are shown in lane M.

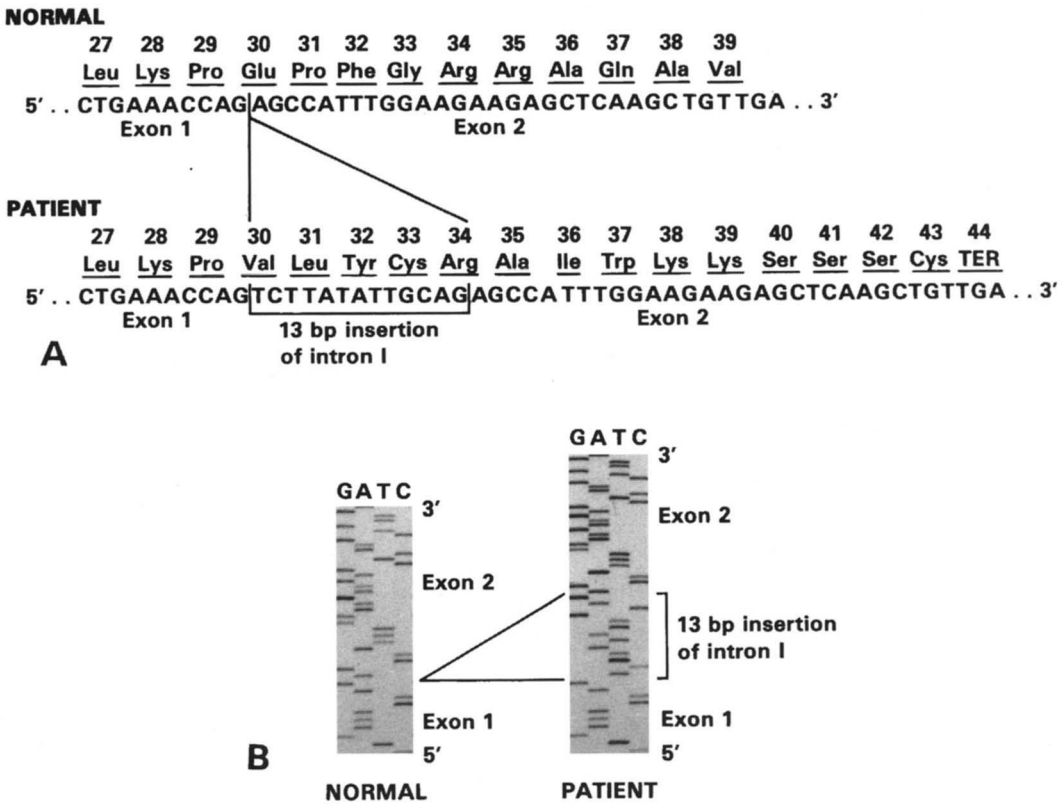

Fig. 5. DNA sequence analysis of RT-PCR amplified splice products. The 135-bp (Normal) and 148-bp (Patient) fragments shown in Fig. 4B were isolated, subcloned and sequenced. Panel A illustrates the HL gene sequence of a normal individual and the HL-deficient patient. The 13 bp insertion of intron I as well as the introduction of a premature termination codon in the patient's DNA sequence is indicated. Panel B shows the autoradiogram of DNA sequencing gels for normal and patient DNA.

of the 148 bp RT–PCR product with BsrI should lead to the formation of fragments 68 and 80 bp in length. In Fig. 4B, incubation with BsrI resulted in complete digestion of the 148 bp product to fragments 68 and 80 bp in length indicating that alternative splicing at the newly introduced AG mutation site had occurred.

This observation was definitively established by DNA sequence analysis of all three RT–PCR DNA fragments. **Figure 5** illustrates the autoradiogram of sequencing gels from the normally spliced 135 bp and alternatively spliced 148 bp products. The insertion of 13 bp corresponding to the intronic sequence between the A → G mutation and exon 2 is demonstrated. In similar studies, sequence analysis of 327 and 213 bp products established that these fragments represented unspliced as well as alternatively spliced DNA, respectively (data not shown). Based on these data we conclude that the A → G mutation leads to the production of little or no normally spliced mRNA. Our studies thus demonstrate the preferential use of the mutant AG motif as a 3′ acceptor splice site. Translation of this abnormally spliced mRNA leads to a shift in the reading frame resulting in the introduction of a premature termination codon (TGA) in exon 2 (Fig. 5A). Premature termination of translation would occur at codon 44 of the mutant HL mRNA leading to the synthesis of a truncated non-functional HL enzyme. Splicing at a second alternative acceptor splice site, (TTGGCTTGTGCTTGTAG/A), located 78 bp upstream of exon 2 (**Fig. 6B**) does not shift the reading frame but leads to the introduction of a stop codon (TGA) in intron I and premature termination of translation at codon 41 of the abnormal mRNA.

To demonstrate that the splice mutation leads to a deficiency of HL in vivo, we performed Western blot hybridization of patient and control post-heparin plasma after chromatography on heparin-Sepharose as described in Materials and Methods (**Fig. 7**). Using a monospecific, goat anti-HL polyclonal antibody, an immunoreactive band of approximately 60 kD was detected in the lane containing pooled post-plasma from normal individuals (Fig. 7, lane 2) but not in lane 3, which contained our patient's post-heparin plasma, thus establishing HL deficiency, in vivo. This approach can provide qualitative information regarding the presence or absence of HL in post-heparin plasma, and is thus,

useful to evaluate complete HL deficiency. However, it cannot be efficiently utilized to accurately identify heterozygote family members that are expected to have half-normal post-heparin plasma levels of HL as enzyme losses that occur during partial purification of HL by heparin-Sepharose affinity chromatography do not permit accurate quantitation of the plasma enzyme concentrations. Thus, we attempted to quantitate the post-heparin plasma HL concentrations in immediate family members directly, without prior purification by heparin-Sepharose. However, the large amounts of plasma albumin, which co-migrate on SDS-PAGE with HL, precluded our visualization of the HL immunoreactive band, even in control plasma (data not shown).

Analysis of plasma post-heparin HL activities in immediate family members, however, indicated that the heterozygous state indeed correlated with lower plasma HL activities. **Figure 8** illustrates analysis of the restriction fragments obtained after digestion of PCR-amplified DNA from the patient as well as immediate family members with BsrI. As expected, the patient's offsprings were heterozygous for the gene defect. The heterozygous state was associated with half-normal plasma post-heparin HL activities of 86–94 nmol/min per ml, compared to 0 and 162 nmol/min per ml, for the patient and his normolipidemic wife, respectively.

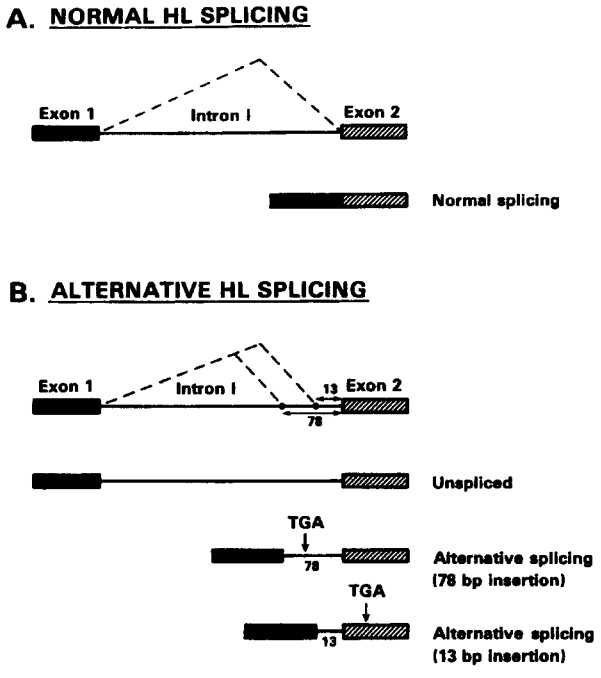

Fig. 6. Overview of splicing pattern of normal and patient HL mRNA. The different splice products identified by analysis of total RNA isolated from cells transfected with normal (panel A) and mutant (panel B) constructs are illustrated. Termination codons (TGA) are indicated.

DISCUSSION

HL deficiency is a genetic syndrome characterized by hypertriglyceridemia, hyperlipidemia, and the accumulation of triglyceride-rich remnant lipoproteins in plasma (17, 21–25). Patients with HL deficiency may present phenotypically with features similar to Type III hyperlipoproteinemia including palmar xanthomas and premature cardiovascular disease. Although, to date, very few patients with HL deficiency have been described (16–21), the frequency of this syndrome in the general population may be underestimated due to the difficulty in identifying patients with HL deficiency (16). Thus, the role of HL deficiency as an underlying defect in various hyperlipidemic disorders present in the general population remains to be established.

Our present understanding of the underlying molecular defects that lead to HL deficiency is limited. Several mutations have been identified in the HL gene of affected individuals from two different kindreds with HL deficiency (16, 19, 20). Only recently has the functional significance of two of these mutations been established by expression studies (46). In the present report, we describe a novel A → G substitution in intron I of the HL gene of a patient with absent HL activity in post-heparin plasma (17, 18). This mutation creates an alternative splice site 13 bp upstream of the native acceptor 3′ splice site, as well as disrupts a potential branch lariat sequence utilized in normal splicing. We propose that this defect results in abnormal splicing of HL mRNA and HL deficiency in this patient. This concept is supported by one of the currently suggested models for mRNA splicing involving spliceosome scanning (42–44, 47). In this model, splicing is initiated by cleavage within the splice donor sequence. This is followed by the formation of a branch lariat involving a 2′–5′ phosphodiester linkage between the 5′ terminal G residue of the intron and the core A residue present within a branch lariat signal sequence in the intron upstream of the splice acceptor. The spliceosome then identifies the splice acceptor by scanning 5′ to 3′ along the intron and locating the first AG dinucleotide and associated polypyrimidine tract that follows the 2′–5′ branch point. The A → G transition described in this report creates an AG dinucleotide that follows an 8/10 polypyrimidine tract and therefore satisfies sequence requirements for a new acceptor site.

In vitro studies emphasize the importance of the sequences surrounding the native AG acceptor splice site. Thus, additional AG signals are usually not found in close vicinity upstream of an acceptor site and the first AG is predominantly used when a series of closely spaced AG sequences occurs (48). Furthermore, deletion experiments have demonstrated that the closer a cryptic splice site is brought to a normal acceptor site,

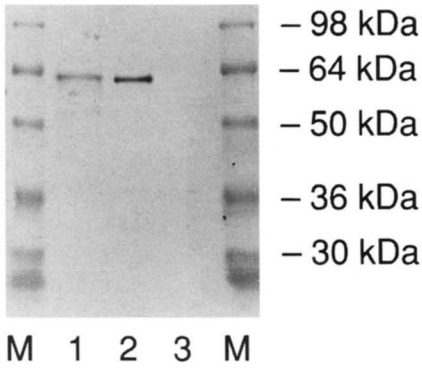

Fig. 7. Anti hepatic lipase Western blot of normal and patient post heparin plasma. Post heparin plasma was partially purified by heparin Sepharose chromatography and separated by 10% SDS-PAGE. After transfer, the blot was developed with a polyclonal goat antibody specific for hepatic lipase. Lane 1, medium from 293 cells transfected with normal HL cDNA and grown without fetal calf serum, dialyzed and lyophilized. Lane 2, post heparin plasma pooled from normal controls. Lane 3, post heparin plasma from the patient. M, molecular weight marker.

the more often the alternative site is utilized (45). In fact, the cryptic 3′ splice signal is used exclusively if it is moved to less than 21 bp upstream of the native acceptor site (45). In this context it is interesting that the mutation identified in our patient creates a new AG motif that is located only 13 nucleotides upstream of the native acceptor site and thus should function as an efficient alternative 3′ acceptor sequence.

In addition to introducing a potential alternative acceptor splice site, the A → G substitution identified in our study also destroys sequence information necessary for the utilization of the native 3′ splice acceptor sequence. The identified A → G transition mutates the core A of a potential branch lariat signal (CCCCA[A → G]T) located 13 nucleotides upstream of the normal intron I/exon 2 junction that matches 6 of the 7 residues in the loosely defined mammalian branch point consensus sequence (YNYTRAY, Y:pyrimidine, R:purine, N:purine or pyrimidine) (44, 49, 50). Thus, the normal 2′–5′ branch formation may be blocked and the scanning spliceosome may fail to recognize the native AG motif. There is some evidence that deletion or mutation of the normal mammalian branch signal typically does not completely abolish splicing but leads instead to the use of nearby cryptic branch signals (44). We found additional putative branch signals located 1 base (GGCTAAG, 5 matches) and 21 bases (TTCAGAT, 6 matches) upstream of the polypyrimidine tract associated with the AG dinucleotide formed by the A → G transition. Either of these branch signals could be potentially used for splicing at this alternative acceptor sequence.

In order to evaluate the functional significance of this defect, the role of this A → G mutation in disrupting branch lariat formation and/or creating an alternative acceptor splice site was investigated by in vitro transfection. Two minigene constructs containing normal and mutant HL gene sequences were transfected in human 293 cells. Analysis of the spliced products by RT–PCR of total RNA isolated from transfected cells established that the A → G mutation leads to alternative splicing with the use of the newly created 3′ splice site. Thus, sequence analysis of the major RT–PCR product (148 bp in length) established splicing at the new AG site. In addition, our studies identified two other minor RT–PCR products 213 and 327 bp in length. The 213 bp fragment resulted from alternative splicing at an AG site 78 bp upstream of the native 3′ splice signal while the 327 bp product represented unspliced mRNA. The identification of the two other alternatively spliced fragments together with the fact that no normally spliced sequence was detected is consistent with our proposal that the mutation destroys sequence information necessary for normal splicing, namely the branch lariat signal. Stop codons in both alternative splice products lead to premature termination of translation resulting in the formation of a non-functional truncated enzyme.

There are only six established splice mutations in five genes reported to date that create a novel AG site upstream of an acceptor site. Only a small number of these systems, most notably β-globin, have been examined in any depth (51–57). A potential explanation is that these mutations are difficult to detect and characterize using currently available techniques. In all six cases, the disruption of normal splicing and the preferential use of the newly created 3′ splice site was observed. It is intriguing that five of these six described mutations lead to the formation of the A-nucleotide in position

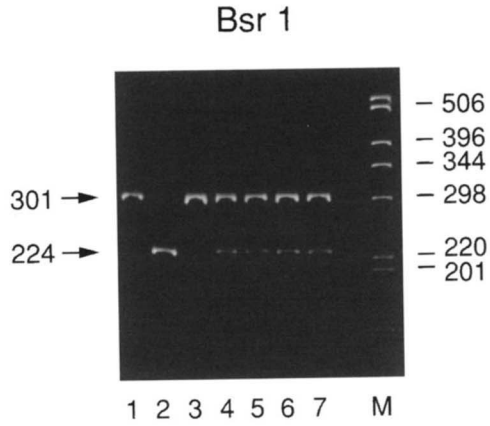

Fig. 8. Family analysis with restriction enzyme analysis of PCR amplified DNA. Genomic DNA was amplified and digested with BsrI as described above. DNA molecular size is indicated in bp. DNA molecular size standards are shown in lane M; lane 1, undigested DNA from patient; lane 2, patient DNA digested with BsrI; lane 3, DNA from patient's wife digested with BsrI; lanes 4–7, DNA from patient's children digested with BsrI.

-2 of the newly created AG splice motif. To our knowledge, the A → G transition identified in this study is the first mutation that destroys the core of a potential branch lariat signal leading to the generation of an alternative AG acceptor site by introducing the G nucleotide in position -1.

It should be mentioned that we cannot exclude any additional splicing events in the presence of the mutation identified in the present study. As we constructed minigenes, we cannot rule out, for instance, that exon 3 or other exons modulate the splicing events in vivo. However, the data from all six patients in the literature (51–57), which are consistent with the spliceosome scanning model described above, are in agreement with our results that demonstrate the disruption of normal splicing and preferential use of the newly created AG site located 5′ of the wild type acceptor site. In addition, the absence of hepatic lipase activity as well as immunodetectable hepatic lipase protein in the post heparin plasma of the patient corroborates the in vitro findings of abnormal splicing indicating that the splice site defect observed in vitro is indeed the cause of hepatic lipase deficiency in this patient.

In conclusion, our study identifies a novel mutation in the HL gene of a patient with absent HL activity and protein in post heparin plasma and establishes alternative splicing as the molecular basis underlying this enzyme deficiency. The unique restriction site for the enzyme BsrI created by the mutation will be a useful addition for future screening techniques to determine the frequency of HL deficiency and to establish its role in the expression of hyperlipidemia as well as development of premature atherosclerosis.

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