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Partial Gene Duplication Involving Exon-Alu Interchange Results in Lipoprotein Lipase Deficiency

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Summary

Major structural rearrangements are uncommon causes of mutation in human genetic diseases. We have previously described that a significant proportion of unrelated patients of western European descent who are deficient in lipoprotein lipase (LPL) activity have a major structural rearrangement in the LPL gene. Here we report the detailed characterization of this mutation. We show that this rearrangement is due to a duplication of approximately 2 kb which results from juxtaposition of intron 6 to a partially duplicated exon 6. We have sequenced both the junction fragment of this duplication and the corresponding wild-type regions and have found that the breakpoint in intron 6 is associated with the simple repeat found at the 3′ end of an Alu element. The breakpoint within exon 6 shows no homology to this simple repeat. This result both suggests that this interchange arose as a nonhomologous recombination event and shows that such events resulting in duplication which occur in normal gene evolution may also lead to genetic disease. Cloning of the junction fragment has allowed synthesis of appropriate primers for rapid screening for this rearrangement in other families with LPL deficiency.

Introduction

In humans, major rearrangements detected by Southern analysis are generally an uncommon cause of mutation resulting in inherited disease. For example, approximately 3.5% of mutations in the low-density-lipoprotein (LDL) receptor gene (Horsthemke et al. 1987; Langlois et al. 1988), 5% of mutations in the factor VIII gene (Gitschier 1988), and approximately 15% of mutations in the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene (Yang et al. 1984) are caused by major structural rearrangements. In contrast, rearrangements are a common cause of mutation in Duchenne and Becker muscular dystrophy (Monaco et al. 1985; Kunkel et al. 1986; Thomas et al. 1986). The reasons for the differences in frequency of major rearrangements causing genetic disease are unknown but may be related to the size and structure of the gene and/or its chromosomal location.

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We have recently described that two major rearrangements account for a significant proportion of mutations in the lipoprotein lipase (LPL) gene (Langlois et al. 1989) causing LPL deficiency. Of 19 mutant LPL alleles studied, major rearrangements were seen in four unrelated probands. In one of the rearrangements, a normal 2.2-kb *Stu*I genomic fragment appeared as 4.2 kb in four probands. A 6-kb deletion also caused LPL deficiency in one of these affected persons.

The structure and partial restriction map of the human LPL gene have recently been determined (Deeb and Peng 1989). The gene is approximately 30 kb in length and consists of 10 exons. This has allowed assignment of the *Stu*I fragment involving the above rearrangement to the exon 6–intron 6 region of the gene.

LPL is an enzyme which plays a primary role in triacylglycerol metabolism (Borensztajn 1987; Brunzell 1989). Specifically, LPL hydrolyses the triacylglycerol moieties located within the lipid core of very-low-density lipoproteins and of chylomicrons and facilitates the transport of cholesterol from triacylglycerol-rich particles to LDL and high-density lipoprotein (HDL). This activity occurs at the luminal surface of the vascular endothelium and results in the transport of free fatty

acid into these tissues for storage or oxidation. Absence of this activity results in elevated levels of chylomicrons and VLDL in the blood. This deficiency is inherited as an autosomal recessive trait.

Here we report the molecular basis for the more common rearrangement in the LPL gene. We have found that this mutation consists of a 2-kb direct tandem duplication of DNA extending from within exon 6 to the 3′ end of an Alu element located within intron 6.

Material and Methods

Southern Analysis

Genomic DNA was prepared according to a method described by Hayden et al. (1987). Five micrograms of digested genomic DNA or 30 pg digested cloned DNA was run on 0.7% agarose gels, depurinated, denatured, and immediately transferred to nylon filters (Nytran; Schleicher & Schuell). Prehybridization and hybridization were performed in the solutions described by Church and Gilbert (1984), with final wash conditions at 65°C in 0.1 × SSC.

LPL Fragment Isolation

Two DNA probes were used to refine the localization of the rearrangement; S219 is a 219-bp internal *Stu*I probe isolated from the LPL cDNA as described by Langlois et al. (1989). This probe detects both wild-type and mutant *Eco*RI fragments and was used to screen the library constructed from the proband. The other probe used, C480, is a 480-bp *Stu*I-*Hin*cII fragment derived from the junction between exon 6 and intron 6 (see fig. 2). DNA probes were labeled with ^{32}P by the method of Feinberg and Vogelstein (1983).

Library Construction and Screening: Characterization of the mutant LPL gene

To characterize this mutation further, a library was constructed from 4–8-kb size-selected, *Eco*RI-digested genomic DNA carrying this rearrangement, and appropriate recombinant phage were selected by hybridization with the 219-bp *Stu*I LPL cDNA probe.

Genomic DNA (100 μg) from one of the probands (in family D) carrying the major rearrangement (causing an increased DNA fragment size) was digested with *Eco*RI for 2 h and then size fractionated on a 0.5% agarose gel in TBE buffer. DNA (4–8 kb) was electroeluted, extracted with phenol:chloroform:isoamyl alcohol (50:50:1), and precipitated with ethanol. One microgram purified insert was ligated to 0.5 μg of *Eco*RI-digested, dephosphorylated lambda gt10 vector. This ligation mix was packaged in vitro (Stratagene) and plated on the *Escherichia coli* strain C600 hfl. Recombinant phage (1×10^6) were screened by standard procedures (Maniatis et al. 1983) with the S219 probe described above. Purified positive phage were amplified, and minipreparations of DNA were screened for the presence of the 5.8-kb *Eco*RI band expected to be derived from the rearrangement. Restriction maps were derived either from multiple enzyme digests or from partial digests of end-labeled DNA (Smith and Bernstiel 1976).

Sequence Analysis

The duplication junction fragment was identified as a 190-bp *Sau*3AI restriction fragment that was found in mutant but not in wild-type DNA. Fragments were subcloned into Bluescribe or Bluescript vectors (Stratagene), and sequence was obtained from either single-stranded templates or double-stranded templates (Gauterman et al. 1988) by using the Sequenase kit supplied by United States Biochemical.

Polymerase Chain Reactions

Genomic DNA was obtained from LPL-deficient probands and their family members for analysis by the polymerase chain reaction (PCR). The reactions were performed in a Perkin-Elmer/Cetus Corporation DNA thermocycler using the recommended buffer (Saiki et al. 1988), 200 μM dNTPs, 1 μM each primer, 250 ng genomic DNA, and 1 μ thermostable DNA polymerase from thermus aquaticus (Cetus) in 50-μl reactions. The reaction mixtures were denatured at 94°C for 1 min, annealed at 62°C for 2 min, and extended at 72°C for 2 min for a total of 30 cycles. The primers used were LPL-3, 5′-AATCTGACCAAGGATAGTGG-3′, and LPL-4, 5′-GCATGATGAAATAGGACTCC-3′. Sequence of PCR-amplified products was determined from single-stranded templates produced by amplification with a single primer from 2 ng double-stranded PCR fragments that were purified from agarose gels using GeneClean (Bio101).

Results

Southern Blot Analysis

Southern blot analysis revealed the presence in the proband of a genomic LPL fragment 2 kb longer than its normal counterpart (Langlois et al. 1989). For example, *Eco*RI digests of patient DNA probed with C480 (an LPL gene fragment from the exon 6–intron 6 junction) reveal, in addition to constant bands seen both

in mutant and in wild type, a mutant band at 5.8 kb relative to the wild-type band seen at 3.8 kb (fig. 1). Further analysis showed that the 3.8-kb and a 5.8-kb fragments could be specifically detected by an internal subfragment of the LPL cDNA, a 219-bp Stu-I fragment (data not shown).

Characterization of the Mutant LPL Gene

Seven positive phage clones were detected using the 219-bp *Stu*I LPL cDNA probe. Purified phage were isolated and digested with *Eco*RI, revealing two classes of recombinant clones. One contained only the mutant 3.8-kb band of wild-type size. The other class of positive phage possessed two bands, one of 5.8 kb and the other 3.8 kb.

The following two experiments suggested that these latter positives did not contain a double insert of 3.8-kb and 5.8-kb fragments but in fact consisted of two distinct populations of phage, each possessing only one of the two bands. First, partial digests of DNA from phage stocks containing both bands did not reveal a larger band of 9.6 kb, which would be expected if each individual phage contained both fragments. Second, single-purified plaques were capable of giving rise either to stocks containing only the 3.8-kb band or to stocks containing both the 5.8-kb and 3.8-kb fragments. Both of these results are consistent with the idea that recombinant phage containing the mutant 5.8-kb fragments are capable of reverting to the wild-type form, with the resulting loss of the 2-kb duplication.

This reversion to wild type only occurred in a recombination-competent bacterial host (c600) and was not observed when the clone was transferred to bacteria that were defective in recombination (DH5α). A recombination-mediated reversion of this rearrangement from mutant to wild type is consistent with a structure that contains a direct tandem duplication of adjacent DNA sequences. A duplicated structure was also suggested by the double intensity of the mutant 5.8-kb band relative to the normal 3.8-kb band seen in the Southern blot of patient DNA shown in figure 1 (lane 2).

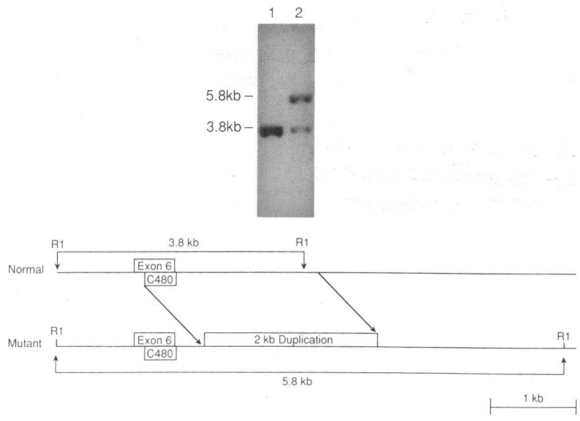

Figure 1 Southern analysis of mutant and wild-type DNA probed with the C480 fragment from the exon 6–intron 6 junction. *A*, Genomic DNA digested with *Eco*RI: 5 μg wild type (lane 1) and 5 μg patient KR (lane 2). A partial restriction map of the normal and mutant gene is shown.

Restriction maps of the mutant and wild-type clones are shown in figure 2. From digestions with multiple enzymes, it was clear that the mutant clone did not contain 2 kb of foreign DNA relative to wild type. In fact, digests with several different restriction enzymes revealed in each case a single unique restriction fragment present in the mutant and absent in the wild type. In addition, in the mutant clone several bands were present at twice the intensity. These results strongly suggested that the aberration was caused by a direct tandem duplication of adjacent DNA and that the unique fragments generated from digests of the mutant DNA arose from the breakpoint of the rearrangement. The restriction map shown in figure 2 was confirmed by partial digestion mapping of end-labeled DNA (not shown).

Sequence Analysis

We have sequenced the wild-type regions around the duplication breakpoints, as well as a 190-bp *Sau*3AI junction fragment from the rearrangement itself (fig. 2). Sequencing was done in both directions, except for a small segment of DNA as shown in figure 2. The sequence of exon 6 corresponded exactly to that portion of the cDNA sequence reported by Wion et al. (1987). In addition, we identified two Alu elements (Sawada et al. 1985) within this intronic region (shown in fig. 2), one of which is located in the vicinity of the 3′-most breakpoint of the rearrangement. The exact sequences of the duplication breakpoint and of the corresponding wild-type regions are shown in figure 3.

This analysis has shown that sequences extending 3′ from nucleotide 1072 (based on the cDNA sequence of Wion et al. [1987]) within exon 6 (Deeb and Peng 1989) have been juxtaposed to a position farther downstream within intron 6. The breakpoint within the intron has occurred within the TAAA repeats located in the right arm of the 3′-most Alu element. No insertions, deletions, or mismatches were observed between the rearrangement junction fragment and the corresponding wild-type regions. Further, no significant homology exists between the regions of exchange, and no sequences with significant secondary structure are evident.

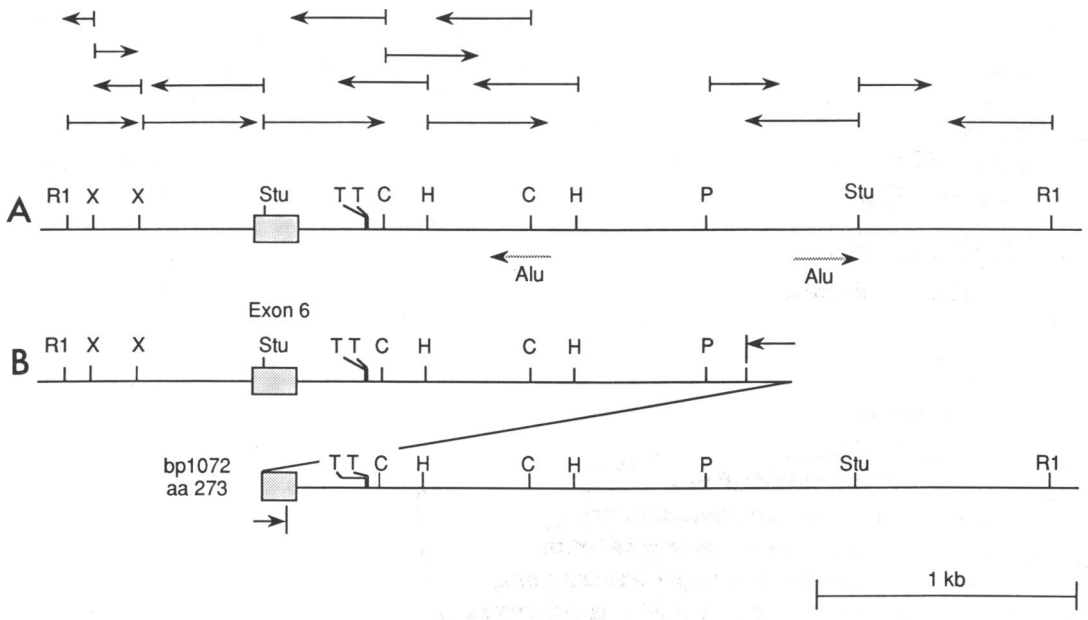

Figure 2 Restriction map and sequencing strategy of wild-type and mutant DNA at the exon 6–intron 6 junctions. *A*, Restriction map of wild-type DNA, showing the location of exon 6 (shaded box). The sequencing strategy is shown above the restriction map. Two Alu elements present in opposite orientation are shown. *B*, Restriction map of cloned mutant DNA, showing region duplicated in a direct tandem fashion. Ends of horizontal lines which are connected by a diagonal line indicate duplication breakpoints. RI = *Eco*RI; X = *Xbo*I; T = *Sst*I; C = *Hin*cII; H = *Hin*dIII; P = *Pst*I. Arrows and bar line indicate *Sau*3AI fragment sequenced in both directions.

The Use of PCR to Identify the Rearrangement Junction Fragment

The structure of this rearrangement allows the design of oligonucleotide primers which should specifically amplify only the junction region in the PCR. Since sequences that are normally 5′ to the breakpoint have been moved 3′ to this position, oligonucleotides shown in figure 3 that would normally prime and extend away from each other in wildtype will result in the amplification of a 261-bp fragment in the mutant. This was the case both for cloned DNA (not shown) and for genomic DNA of patients bearing this rearrangement. Figure 4 shows a pedigree of family A segregating the rearrangement, as analyzed by PCR diagnosis. Perfect concordance of results with previous Southern blot analyses was observed. In addition, this amplified fragment could be detected in other patients (families B–D) suspected, on the basis of Southern analysis, of carrying similar rearrangements (fig. 4). Sequence derived from these PCR-amplified fragments showed that each had junctions identical to that shown for the cloned rearrangement breakpoint (fig. 3). DNA from nine LPL-deficient patients with origins outside of Europe, including India, Pakistan, Malaysia, Vietnam, Turkey, and Colombia, failed to show evidence for this major duplication.

Discussion

Major duplications have rarely been described as a mechanism for mutagenesis causing inherited human disease. A few patients with familial hypercholesterolemia (FH) (Lehrman et al. 1987*a*), hemophilia (Gitschier 1988), Lesch-Nyhan syndrome (Yang et al. 1984), and muscular dystrophy (Hu et al. 1989) have been shown to have a major duplication associated with their illness. Recently it has been shown that 2.5% (3/120) of patients with Duchenne and Becker muscular dystrophy have a duplication underlying their disease (Hu et al. 1988).

The current findings on the probands with a common duplication causing LPL deficiency are of interest from different points of view. The different ancestries of these patients suggest that the mutation occurred many centuries ago somewhere in northwestern Europe. The backgrounds of the families of the probands differ, representing British, French, German, and Polish descent for many generations. We, so far, have been unable to show any genetic relationship among the progenitors of these families. DNA haplotyping using known RFLPs within the LPL gene has previously demonstrated that all the mutations occurred on the same DNA haplotype (Langlois et al. 1989). The failure to demonstrate a similar mutation in nine probands

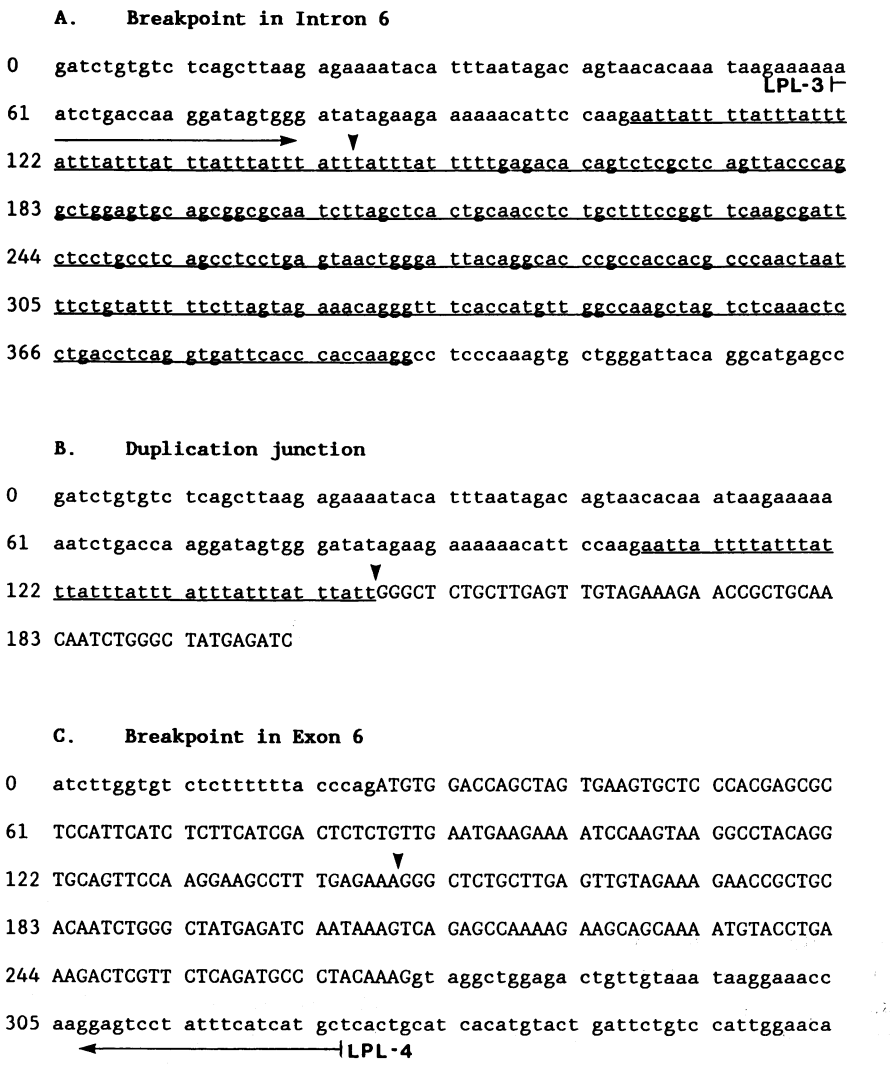

Figure 3 Sequence of duplication breakpoints and junction fragment. Underlined bases correspond to Alu sequences, and horizontal arrows indicate oligonucleotides used in PCR analysis. Vertical arrows show breakpoints in the sequence that are associated with the duplication. *A*, Sequence in intron 6 around breakpoint. *B*, Duplication junction fragment, *C*, Exon 6 and associated flanking intron sequences. The exon sequences are capitalized, and the intron sequences are denoted by small letters.

with origins outside of Europe is compatible with this hypothesis. In the present paper we have shown that the duplication is identical in all four probands. This rearrangement is unusual in that no other common duplication has yet been described in families which accounts for a significant proportion of mutations underlying any human genetic disorder.

Gene duplication events are considered to be important in the evolution of new genes. For example, nonhomologous recombination has contributed to genetic variation at the haptoglobin locus (Maeda et al. 1984). Unequal recombination events can also cause genetic disease and have been shown to be a mechanism underlying the duplication causing dysfunction of the LDL receptor gene. This mutation occurred by duplication of an internal region of the LDL receptor gene as a result of homologous exchange between two Alu elements located within different introns (Lehrman et al. 1987*a*). Examples of homologous exchange causing gene deletion have also been described (Efstratiadis et al. 1980; Hobbs et al. 1986; Lehrman et al. 1986, 1987).

We show here that nonhomologous interchange can also result in a duplication event causing human genetic disease. In sequences around the breakpoints we have

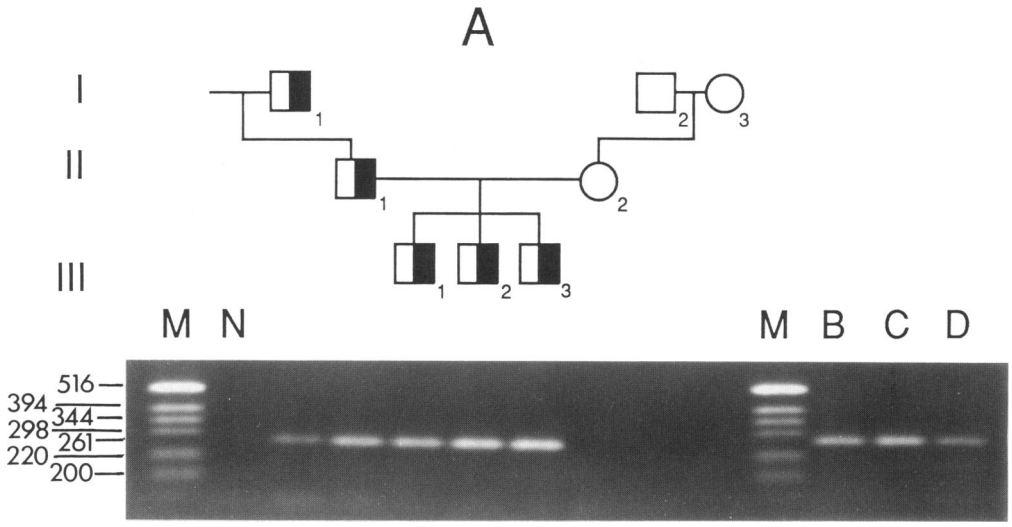

Figure 4 PCR analysis of the duplication. *A*, Family segregating the duplication. Half-filled symbols indicate carriers of the rearrangement. *B–D*, Individuals from other unrelated families who also carry the duplication. N = a normal unaffected control; M = marker DNA (BRL).

looked for any common features which might account for the disruption and duplication at this particular junction. No possible double-stem-loop structure could be postulated to account for this interchange event. Sequence analysis around the junction fragment did not reveal any obvious homology which might have caused the duplication at this site. It is of note that the LPL duplication involves the AT-rich region at the end of the right arm of the consensus Alu repeat, which is a distinctly uncommon location for rearrangements involving this sequence (Lehrman et al. 1987*b*).

It is noteworthy that during cloning of the mutant allele there were spontaneous reversions to the wild-type allele. This phenomenon has been reported previously in phage continuing α-globin genes (Lauer et al. 1980). The locations of the breakpoints which occur in the cloned DNA containing LPL or α-globin are indistinguishable from those associated with LPL deficiency or α-thalassemia, respectively. However, in contrast to the LPL DNA, regions of homology around α-globin predisposed to unequal crossing-over during propagation of the recombinant phage (Lauer et al. 1980). No similar regions of homology were found around the breakpoints of the duplication underlying LPL deficiency. It is conceivable that the breakpoints are adjacent to several sites which promote recombination and which are currently undefined.

It is of interest that approximately 120 bp downstream of the breakpoint there is a stretch of 13 bp which spans the 3′ exon/intron boundary of exon 6 and which is similar to the consensus sequence which detects a variable-number-of-tandem-repeat (VNTR) polymorphism (Nakamura et al. 1987). VNTRs have recently been shown to be more frequent in the telomeric regions of the chromosomes, where recombination also occurs at a rate greater than that in other areas of the chromosome (Chandley and Mitchell 1988; Royle et al. 1988). The significance of the VNTR's in close proximity to the junction fragment remains uncertain, however.

This duplication event occurred by a nonhomologous exchange event occurring between either homologous chromosomes, sister chromatids, or a single chromosome. The data that we have observed for this mutation are consistent with both exchange and nonexchange models of interchange. For example, a duplication such as this could be caused by an intrachromosomal event, involving DNA nicking, strand separation and resynthesis of both single-stranded regions, and subsequent ligation to produce the direct tandem duplication. Such duplication events could conceivably occur during DNA replication or by repair mechanisms that normally operate to correct DNA damage.

We have previously reported that this mutation does not produce detectable levels of protein. Functional protein could be anticipated if, during RNA splicing, the normal donor site of the first exon 6 was joined to the normal acceptor of exon 7. The absence of any detectable protein, suggests, however, that alternate splicing events may well have occurred. This could possibly involve ligation of the 5′ end of the partially duplicated

exon 6 as the acceptor site to the donor of the first exon 6, or it may have in fact left an incompletely processed mRNA (bearing a portion of intron 6) which was rapidly degraded, resulting in aberrant and/or reduced levels of protein.

The cloning of the junction fragment has allowed us to develop a very rapid method of screening for this rearrangement in other families with LPL deficiency. LPL deficiency is a rare recessive metabolic disorder in most parts of the world. However, it has recently been shown that certain families with familial combined hyperlipidemias have LPL mass and activity which are compatible with the carrier status for LPL deficiency (Babirak et al. 1989). Characterization of common defects underlying human LPL deficiency will allow, in suitable families, rapid testing for certain mutations causing LPL heterozygosity. Furthermore, more-detailed characterization of the other mutations underlying human LPL deficiency will help to determine the relationship between the structure and function of the LPL protein.

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