Direct detection and automated sequencing of individual alleles after electrophoretic strand separation: identification of a common nonsense mutation in exon 9 of the human lipoprotein lipase gene

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ABSTRACT

Large-scale screening by direct sequencing of DNA to detect molecular variants remains a laborious endeavor whose difficulty is compounded by heterozygosity. We show that mobility shifts of single-stranded DNA electrophoresed under nondenaturing conditions can be used not only to detect variants (Orita,M. et al., 1989, Genomics, 5, 874–879), but also to separate and sequence directly individual alleles. In this manner, we have identified a common variant of human lipoprotein lipase resulting from a nonsense mutation in exon 9 of the gene. Whether this variant is of functional significance remains to be determined.

INTRODUCTION

A variety of methods have been proposed to identify directly mutations in genomic DNA, whether enzymatic (1,2), chemical (3–5) or physical (6–8). Their scope is further enhanced when they are used to analyze enzymatically amplified DNA (9). These methods can complement direct sequencing in large-scale screening of subjects, by identifying that subset of DNA segments to be sequenced.

The method proposed by Orita et al. (7,8), referred to by the authors as 'single-strand conformation polymorphism (SSCP) analysis', has the merit of great experimental simplicity. It relies on the differential electrophoretic mobility of single-stranded DNA under nondenaturing conditions, as even a single nucleotide substitution may induce conformational changes leading to detectable mobility shifts (10). Because the detection of point mutations by direct sequencing of enzymatically amplified DNA from heterozygous individuals can present experimental difficulties, we investigated whether the method proposed by Orita et al. (7,8) could also be used to purify single-stranded molecules harboring mutations, which could then be submitted to sequencing. We report that separation of single-stranded DNA in nondenaturing polyacrylamide gels, followed by elution, enzymatic amplification, and direct sequencing according to a thermocycling protocol on an automated sequencer, led to the identification and characterization of a common variant of human lipoprotein lipase (LPL), a key enzyme in the hydrolysis of dietary and endogenous fat (11).

MATERIALS AND METHODS

Direct sequencing of asymmetrically amplified single-stranded DNA

In all instances, genomic DNA was prepared from peripheral blood leukocytes following standard procedures. A 248 bp segment of exon 29 of the apolipoprotein B gene (apo B) was amplified by the polymerase chain reaction using oligonucleotide primers (5′-GAGTGGAAGGACAAGGCCCAG-3′, 5′-CCTA-CCTCCCTTATGAACATAGT-3′) derived from the normal apoB sequence (12,13), and the resulting product was purified on a Centricon 100 (Amicon, Danvers, MA). An aliquot (1 μl) was used to generate single-stranded DNA by asymmetric amplification (14), one primer being augmented with sequence complementary to the universal M13 priming site (5′-TGTAAAACGACGGCCAGT-3′). Sequencing was performed by the dideoxy chain termination method (15) with *Taq* polymerase by means of an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA).

Direct search for mutations in genomic DNA

For each of the 9 translated exons of human LPL (16), 0.1 μg genomic DNA was enzymatically amplified (17) on a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) basically following the method of Orita et al. (1). The reaction mixture contained 10 pmol of each primer, 2 nmol of each dNTP, 0.1 μg of genomic DNA, 10 μCi of [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml, NEN) and 0.25 units of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT) in 10 μl of amplification buffer. The oligonucleotides used as primers and the cycling parameters are given elsewhere (18). The reaction mixture was diluted with 50 μl of 0.1% $NaDodSO_4$ and 10 mM EDTA. An aliquot was

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withdrawn and mixed with equal volume of 95% formamide dye, boiled for 2 min. and applied (1 μl/lane) to a 5% polyacrylamide gel containing 1×TBE (90 mM Tris-borate 2 mM EDTA) and 10% glycerol. Electrophoresis was performed at room temperature. The gel was dried and autoradiographed without intensifying screen for one to three hours.

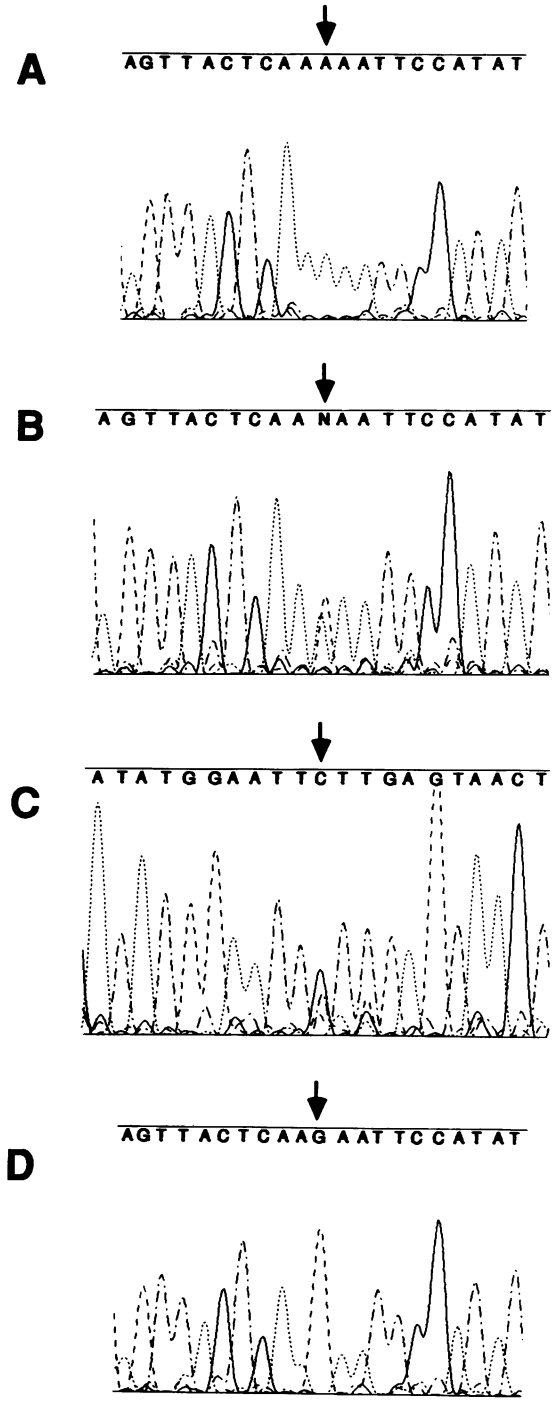

Figure 1. Direct sequencing of genomic DNA in the vicinity of an *Eco*RI site (GAATTC) polymorphism of the apolipoprotein B gene. Representative results of six independent replicates: subject homozygous for absence of the site (A); heterozygous subject, sense (B) and anti-sense strand (C); subject heterozygous for preserved site (D).

Direct sequencing of DNA fragments recovered from a dried gel

Individual bands revealed by electrophoresis of amplified DNA fragments spanning exon 9 of human LPL were cut from the dried gel, suspended in 100 μl of H_2O and vortexed. After incubation at 37°C for 1 hr, 1 μl of each aliquot was subjected to enzymatic amplification. The sense- and antisense-primers included at their 5′-end a sequence complementary to M13 universal (5′-TGTAAAACGACGGCCAGT/TATTCACATCC-ATTTTCTTCCAC-3′) and reverse (5′-CAGGAAACAGCTA-TGACC/GTCAGCTTTAGCCCAGAATGCTC-3′) priming sites respectively. The reaction mixture was submitted to 30 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Amplified products were spin dialyzed with Centricon 100 (Amicon, Danvers, MA).

Direct sequencing of double-stranded DNA was performed on an ABI 373A DNA sequencer, using fluorescent M13 primers, *Taq* polymerase, and a thermocycling protocol supplied by the manufacturer (19). In brief, 12 μl of a 40 μl volume of amplified product were submitted to iterative extensions on a DNA thermocycler. Ten cycles were applied, defined by the following conditions: denaturation at 95°C for 30 sec, annealing at 60°C for 1 sec and extension at 70°C for 1 min. This was followed by ten additional cycles of denaturation (95°C for 30 sec) and extension (70°C for 1 min). The repeated application of several rounds of denaturation, reannealing and extension should increase the frequency with which termination occurs with incorporation of ddNTP rather than dNTP as would occur when template reannealing prevents further extension.

Allele-specific oligonucleotide hybridization

To verify the presence and test for the zygosity of the mutation identified by sequencing, genomic DNA from the subjects

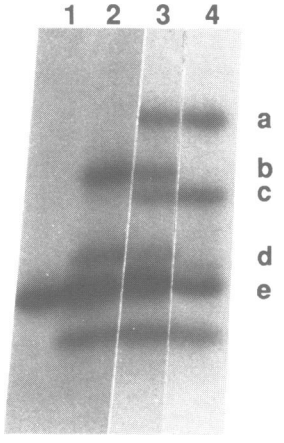

Figure 2. Single strand conformation polymorphism analysis in exon 9 of the human lipoprotein lipase gene. Lane 1: double-stranded DNA from subject in lane 2; lane 2: common homozygous pattern; lane 3: presumptive heterozygote; lane 4: presumptive homozygous variant. Individual bands submitted to sequencing are identified by lower-case characters.

analyzed was submitted to enzymatic amplification as described above. For each amplification product, 5 µl (1/20th) were spotted in duplicate on nylon membrane (BioTrace™ RP, Gelman Sciences, Ann Arbor, MI), and each membrane was hybridized with ^{32}P-end labeled oligonucleotide probes. The wild type and mutant probes were defined as 5′-TAAGAAGTCAGGCTG GT-3′ and 5′-TAAGAAGTGAGGCTGGT-3′. The membranes were washed in 6×SSC (1×SSC = 0.15 M NaCl/15 mM Na citrate, pH 7.0), at 49°C for both probes, and submitted to autoradiography.

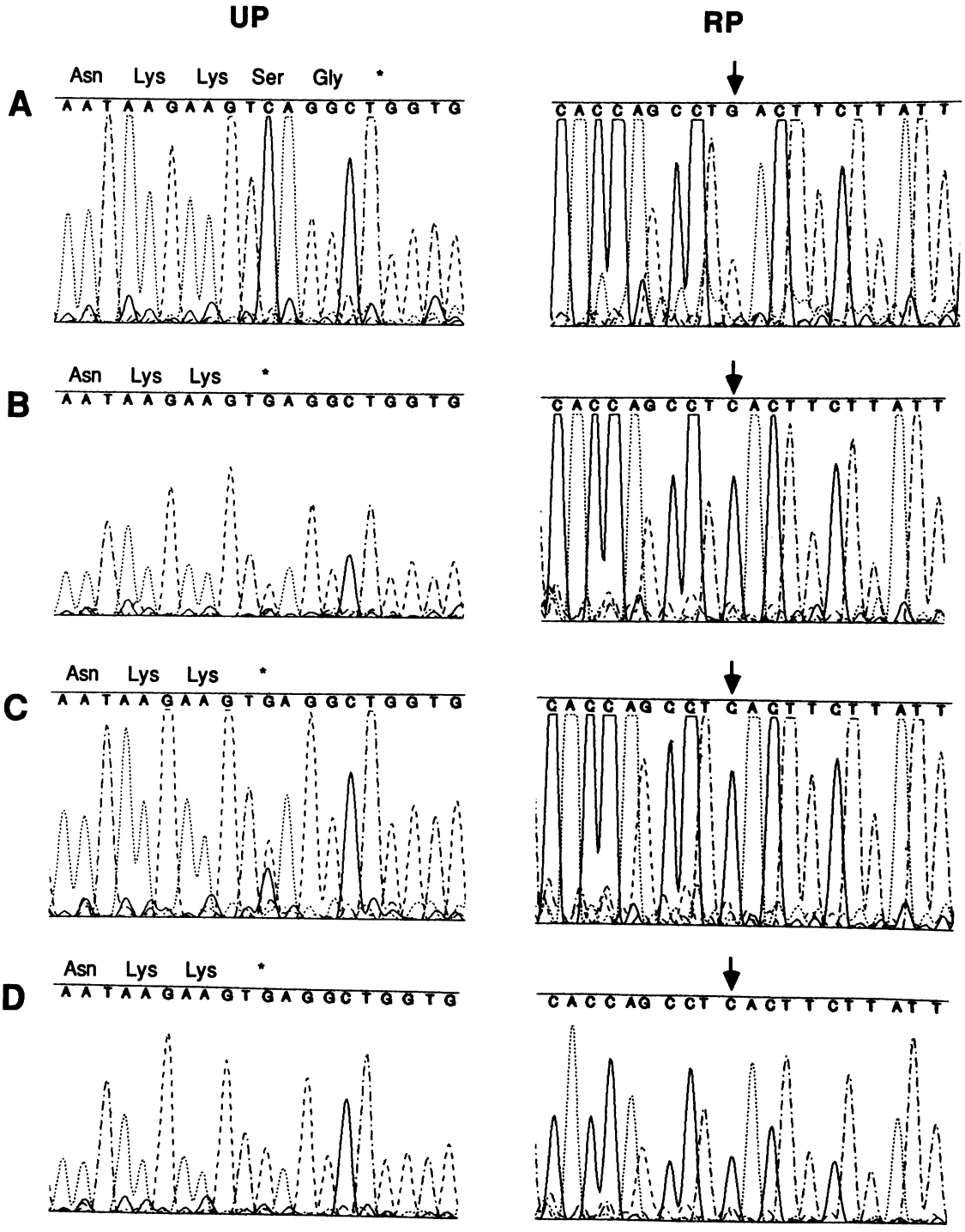

Figure 3. Direct sequencing of double-stranded DNA generated from bands excised from the gel displayed in Figure 2. UP: sequence of the sense strand using the M13 universal primer; RP: sequence of the anti-sense strand obtained by extension from the M13 reverse primer. Panel A: sequence originated from band *b* in lane 2; panel B: from band *c* in lane 3; panel C: from band *a* in lane 3; panel D: from band *c* in lane 4. Note that the last nucleotide of the termination codon (TGA) is encoded by exon 10.

RESULTS AND DISCUSSION

Direct sequencing of heterozygous variant

To test our ability to identify a nucleotide substitution in the heterozygous state by direct sequencing on an automated instrument, three subjects presenting all three possible genotypes at an *Eco*RI polymorphic site of the apolipoprotein B gene (20) were analyzed. Single-stranded DNA was generated by asymmetric enzymatic amplification (14) of genomic DNA as described above, using specific primers augmented with a sequence complementary to a universal M13 priming site. The latter were used to initiate polymerase extension with fluorescent primers in a chain termination sequencing protocol, and the resulting material was analyzed by means of an ABI 373A DNA sequencer.

Six independent sequencing experiments were performed for each individual, each in direct and reverse orientations. Representative results are presented in Figure 1. In all cases, homozygotes yielded the expected sequences (Figure 1A, 1D). For the heterozygous subject, sequencing of the sense strand revealed a double peak of adenine and guanine, leading to an ambiguity in base-calling by the instrument (Figure 1B). In the reverse orientation, however, all six clones yielded a weaker signal at the corresponding position, and defied interpretation (Figure 1C). Inspection of the sequences generated revealed other positions which were equally ambiguous. From these experiments we concluded that the identification of nucleotide substitutions in the heterozygous state by direct sequencing of enzymatically amplified products was not reliable, at least under automated conditions.

Electrophoretic separation of single-stranded DNA

While screening for mutations of the human LPL gene in a series of hypertriglyceridemic subjects by analysis of single-stranded DNA fragments in nondenaturing polyacrylamide gels (8), we identified bands with electrophoretic mobilities distinct from wild-type in several individuals, including controls, for a fragment encompassing exon 9 of LPL. Figure 2 illustrates the three electrophoretic patterns observed among such subjects. The pattern seen in lane 2 was the most common one among controls. The pattern observed in lane 3 suggested heterozygosity for a molecular variant; under this assumption, the pattern observed in lane 4 could reflect homozygosity for this variant.

Sequencing of electrophoretic fragments

In order to identify this molecular variant and confirm our tentative interpretation, individual bands in lanes 2, 3 and 4 were excised from the gel. Each excised fragment was submitted to 30 cycles of enzymatic amplification, using primers which included intron sequences flanking exon 9 of LPL as well as sequences complementary to M13 universal or reverse primers. Automated DNA sequencing of the double-stranded DNA so produced was performed using M13 universal or reverse fluorescent primers, following a thermocycling protocol (19). Representative results are summarized in Figure 3. A single transversion was identified (C → G_{1595}). The presence of this mutation in genomic DNA, the genotypes of the individuals examined in Figure 2, and the molecular nature of the DNA prepared from each excised fragment were characterized by allele-specific oligonucleotide hybridization. The reality of the (C → G_{1595}) transversion was confirmed, and the genotypes of the individuals examined in Figure 2 were indeed as postulated (Figure 4).

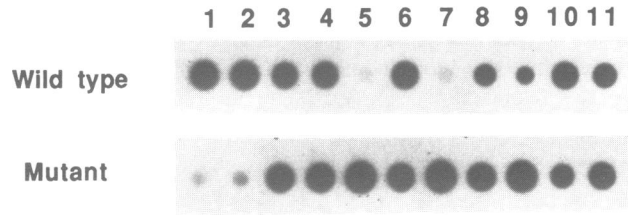

Figure 4. Allele-specific oligonucleotide hybridization after enzymatic amplification of a segment of exon 9 of the LPL gene. Columns 1 to 6 correspond to total genomic DNA from wild-type homozygotes (1,2), heterozygotes (3,4,6) and homozygote (5) for (C → G_{1595}) transversion. Columns 7 to 11 identify amplification products of DNA excised from the gel displayed in figure 2; 7: band *c* in lane 4; 8: band *c* in lane 3; 9: band *a* in lane 3; 10: band *b* in lane 3; 11: band *e* in lane 3.

Sequencing of double-stranded DNA by the thermocycling protocol yielded greatly improved signal-to-noise ratio when compared to results obtained with conventional sequencing protocols. Although exponential amplification and signal saturation prevent accurate quantitation, the degree of allelic purification achieved by excising bands from the gel was documented by oligonucleotide hybridization: band *a* yields high purification (Figure 4, column 9), while bands *b* and *c* are only modestly resolved (Figure 4, columns 8 and 10). The mutation was correctly identified in all sequencing experiments, however, establishing that even moderate purification of an allele is sufficient to resolve the sequencing ambiguities generated by heterozygosity.

Frequency of exon 9 variant in hypertriglyceridemic and randomly ascertained subjects

The mutation so identified introduces of a stop codon which must lead to the synthesis of a truncated protein lacking the last two amino acids at the carboxy-terminus of the normal enzyme (21). Previously, we have shown that an amino acid substitution (Glu^{188}) in exon 5 of the gene led to the synthesis of a functionally deficient enzyme (22) accounting for delayed expression of hypertriglyceridemia in heterozygous individuals (23). Others (24) have reported a significant association between restriction site polymorphisms of the LPL gene and hypertriglyceridemia in a case-control comparison (24). By hybridization with allele-specific oligonucleotides, we established individual genotypes for the exon 9 mutation in two samples. The first sample consisted of 35 unrelated hypertriglyceridemic subjects ascertained through the Cardiovascular Genetics Research Clinic of Dr Roger Williams at the University of Utah; they exhibited plasma triglyceride concentrations in excess of the 99th percentile and plasma cholesterol concentrations in high density lipoproteins below the 5th percentile of age and sex reference values. The second sample included 86 random, unrelated population controls. This analysis revealed that the (C → G_{1595}) transversion was present in 28 (33%) controls, including two homozygotes, and in 3 (9%) hypertriglyceridemic cases; this difference was statistically significant at a 5% nominal level when Fisher's exact test was applied ($p=.037$). Such comparisons, however, can be affected by cryptic stratification or sampling bias.

Some experimental data suggest that LPL might be anchored to the surface of adipocytes by a phosphatidylinositol anchor and that insulin release of the enzyme may result from the activation of a glycosyl-PI-specific phospholipase C (25). This would imply post-translational processing of LPL at its carboxy-terminal end. No evidence of such processing was found when LPL from bovine milk was submitted to protein sequencing (26).

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