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Genetics

Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100

(genetic disease/cholesterol metabolism)

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ABSTRACT **Familial defective apolipoprotein (apo) B-100 is a genetic disease that leads to hypercholesterolemia and to an increased serum concentration of low density lipoproteins that bind defectively to the apoB,E(LDL) receptor. The disorder appears to result from a mutation in the gene for apoB-100. Extensive sequence analysis of the two alleles of one subject heterozygous for the disorder has revealed a previously unreported mutation in the codon for amino acid 3500 that results in the substitution of glutamine for arginine. This same mutant allele occurs in six other, unrelated subjects and in eight affected relatives in two of these families. A partial haplotype of this mutant apoB-100 allele was constructed by sequence analysis and restriction enzyme digestion at positions where variations in the apoB-100 are known to occur. This haplotype is the same in three probands and four affected members of one family and lacks a polymorphic *Xba* I site whose presence has been correlated with high cholesterol levels. Thus, it appears that the mutation in the codon for amino acid 3500 (CGG → CAG), a CG mutational "hot spot," defines a minor apoB-100 allele associated with defective low density lipoproteins and hypercholesterolemia.**

The level of cholesterol in human plasma is to a large extent controlled by the interaction of low density lipoproteins (LDLs) with apoB,E(LDL) (or LDL) receptors. LDLs transport about two-thirds of the plasma cholesterol and are removed from the circulation by the apoB,E(LDL) receptor as a result of binding to apolipoprotein B-100 (apoB-100), the protein moiety of LDL. Individuals with high LDL levels are often predisposed to premature coronary disease. In principle, elevated LDL concentrations that result from inefficient clearance of LDL particles by the receptor may derive either from defects in the receptor or from defects in the ligand, apoB-100. The former class of genetic disorder is well established: familial hypercholesterolemia (1). Individuals with this genetic disease may lack the apoB,E(LDL) receptor or possess defective variants of it. However, because this genetic defect is relatively rare and most hypercholesterolemic individuals possess apoB,E(LDL) receptors that lack detectable defects, attention has been focused on other possible causes of increased LDL levels. One possibility is the presence of a mutant form of apoB-100.

A group of moderately hypercholesterolemic patients has been shown (2) to have a reduced fractional clearance rate of autologous LDL as compared with normal LDL. This finding suggested that the retarded clearance resulted from a defective binding of LDL to its receptor, thereby accounting for the high levels of LDL cholesterol. LDLs from one of these patients exhibited defective binding to the apoB,E(LDL) receptor on fibroblasts in an *in vitro* assay (3). Studies of the kindred of this patient indicated that the proband and affected family members are heterozygotes and that the defect is transmitted in a co-dominant fashion. This pattern of inheritance is consistent with the fact that each LDL particle contains but one apoB-100 molecule (4). Thus, affected individuals contain two LDL populations, one with defective receptor binding capacity and the other with normal properties.

The present study concerns the molecular basis of this disease, termed familial defective apoB-100 (3). The DNA of the mutant allele contains a mutation that segregates with the defective binding and the moderate cholesterolemia in the kindred referred to above. The genomes of six unrelated subjects with moderate hypercholesterolemia contain the same mutation in the apoB gene. The relationship of this mutation to other genetic markers that have been used to trace defective apoB alleles in the population is discussed.

MATERIALS AND METHODS

Patient Material. Plasma and leukocytes were obtained from individuals of three families. The proband of the R. family, G.R., had been shown to have a reduced clearance of autologous LDL compared with homozygous normal LDL (2). Of eight R. family members tested, four have LDLs that bind abnormally to the apoB,E(LDL) receptor in normal human fibroblasts (3). Two other patients (W.S. and R.N.) were identified as probands of the S. and N. families, respectively. These patients were found to have delayed *in vivo* clearance of autologous LDL from plasma (5). Likewise, W.S. and 4 of 15 family members tested have LDLs that exhibit abnormal binding to normal human fibroblast apoB,E(LDL) receptors. R.N. (who was recruited into the study by Margo Denke, Dallas, TX) also possesses LDLs that have abnormal receptor binding. Techniques to define abnormal binding of LDL to receptors in normal human fibroblasts have been presented in detail elsewhere (3). DNA was prepared by phenol extraction and ethanol precipitation.

Genomic Libraries. Two libraries were prepared from G.R. DNA in phage λ. The first contained DNA partially digested with *Mbo* I and ligated to arms of λ FIX (Stratagene) (4×10^6 recombinants with inserts of between 9 and 22 kilobases). The second contained DNA digested to completion with *Eco*RI and inserted into λ ZAP (Stratagene) (7×10^6 recombinants with inserts of between 0.1 and 10 kilobases).

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Abbreviations: apo (prefix), apolipoprotein; LDL, low density lipoprotein; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide; RFLP, restriction fragment length polymorphism.

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Table 1. Sequence and cDNA position of oligonucleotides used

Primer or probe	Sequence	cDNA fragment
PCR1	AAATAACCTTAATCATCAATTGGT	bp 7,591–7,614
PCR2	GGTTCTTAGCAGCAAGAGTCC	bp 7,809–7,789
PCR3	GTGTGCCTTCATACACAT	bp 10,000–10,017
PCR4	TCTCGGGAATATTCAGGAACTATTG	bp 10,644–10,668
PCR5	GTGCTCCCAGAGGGAATATATGCGT	bp 10,823–10,799
PCR6	CTAAGGATCCTGCAATGTCAAGGT	bp 11,124–11,101
ASO1	CTCTTCCACATTTCAAGG	bp 10,075–10,092
ASO2	CTCTTCCAGATTTCAAGG	bp 10,075–10,092
ASO3	AGCACACGGTCTTCA	bp 10,701–10,715
ASO4	AGCACACAGTCTTCA	bp 10,701–10,715
ASO5	AGAAGCCACACTCCA	bp 10,784–10,798

The libraries were screened with various human apoB cDNA probes.

DNA Sequencing. DNA from λ clones was subcloned into M13 vectors and sequenced by the Sanger dideoxynucleotide chain-termination method (6).

Oligonucleotides. All oligonucleotides were synthesized on an Applied Biosystems synthesizer model 380B. Those used as primers for the polymerase chain reaction (PCR) or as allele-specific oligonucleotide probes (ASOs) are listed in Table 1. Probes were end-labeled with T4 polynucleotide kinase and [^{32}P]ATP to 10^6 cpm/ng.

PCR. The PCR was used for local amplification of sections of the human apoB gene. Reactions were conducted in an automated Perkin–Elmer/Cetus thermal cycler by using thermostable *Thermus aquaticus* DNA polymerase obtained from Cetus (7).

***Xba* I Polymorphism.** The presence or absence of the *Xba* I site at position 7673 was determined by amplifying a segment of exon 26 from position 7591 to position 7809 by using primers PCR1 and PCR2. Complete digestion of the amplified band with *Xba* I yielded two fragments, of 136 and 82 base pairs (bp).

***Msp* I Polymorphism.** The presence or absence of the *Msp* I site at position 11,039 was determined by amplifying a 480-bp segment of exon 26 from position 10,644 to position 11,124 by using primers PCR4 and PCR6. Complete digestion of the amplified band with *Msp* I yielded two fragments, of 395 and 85 bp.

Plasmids. Plasmid p46a, containing an *Eco*RI–*Eco*RI 1549-bp fragment from exon 26 subcloned from the G.R. λ ZAP DNA library, represents the wild-type allele. Plasmid p43, containing an *Eco*RI–*Eco*RI 1549-bp fragment from exon 26 subcloned from the G.R. λ ZAP library, represents the mutant allele (i.e., possessing the G → A substitution at the codon for amino acid 3500).

RESULTS

A Mutation in the Human ApoB Gene. To identify a mutation that could be responsible for a defect in the ability of LDL to bind to its receptor, we examined the part of the gene corresponding to the carboxyl-terminal half of apoB-100. Lipoproteins containing apoB-48, the amino-terminal half of apo-B100, do not bind to the apoB,E(LDL) receptor (8); also, the epitopes of antibodies that block LDL binding to the receptor are localized to the carboxyl-terminal one-third of the protein in a region encoded by the large 7572-bp exon 26 (9). Since G.R. is heterozygous for the defect, only substitutions occurring in one but not both of the patient's two apoB alleles can be causally related to defective LDL.

Fig. 1 illustrates the positions at which differences in the apoB-100 sequence have been reported. This information was assembled from known DNA polymorphisms (10, 11) together with four complete cDNA sequences (12–15), a gene sequence (10), and several partial sequences (16, 17). All reported variations are included, even though some may have resulted from cloning or sequencing errors. Codons and amino acids reported in the majority of the publications are termed "consensus" in Fig. 1, even though the appropriateness of this designation could be contested. Likewise, codons and amino acid residues depicted in the lower half of the figure are referred to as variants only because they have been reported less often. These two sequences do not represent established haplotypes, and their relative frequencies in the population have not been ascertained (10).

Two libraries of G.R. DNA were constructed in λ vectors. ApoB-containing clones covering the regions illustrated in Fig. 1 were isolated. Several of the 13 subclones were screened for differences by DNA sequencing. After extensive sequencing of multiple isolates, only one change was detected. This alteration reflected a CGG → CAG change in the codon for amino acid 3500, causing a substitution of glutamine for arginine (Fig. 2). To the best of our knowledge, this variant has not been reported. No other previously unreported nucleotide changes were observed. Furthermore, none of the variant positions illustrated in Fig. 1 were shown to be heterozygous in G.R. DNA except position 7673, the *Xba* I site, which is a third-position substitution that does not change the amino acid. An extensive sequence analysis of the mutant allele yielded the results that are summarized in Fig.

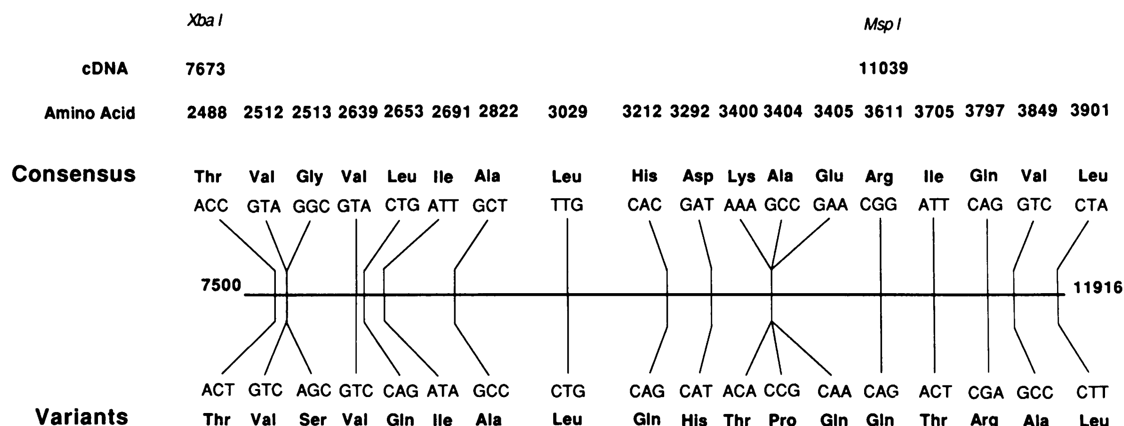


FIG. 1. Variations in base sequence of the gene and in amino acid sequence of the protein for human apoB as determined by restriction fragment length polymorphisms (RFLPs) with *Xba* I and *Msp* I. The region in exon 26 from bp 7500 to bp 11,916 on the cDNA map is shown. The consensus sequence is defined as the nucleotide and amino acid sequence determined by a majority of investigators reporting the sequence; the variants represent alternative residues reported by a minority of investigators.

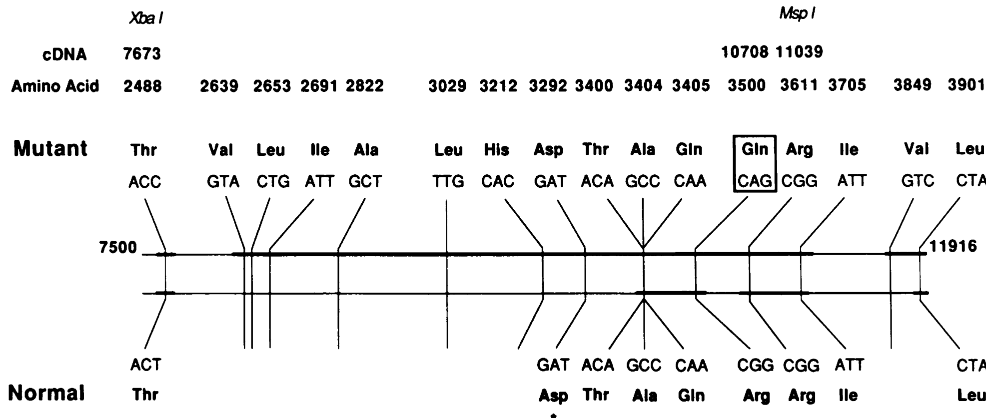


FIG. 2. Representation of the base and amino acid sequence of the mutant and normal alleles of patient G.R. in the region between bp 7500 and bp 11,916 of the human apoB gene. The thickened portion of the two horizontal lines indicates the directly sequenced portions of the two alleles. The asterisk indicates the base sequence deduced from probing with ASO1 and ASO2. The mutation in the codon for amino acid 3500 is boxed for emphasis.

2 in terms of the variant positions of Fig. 1. Sequencing of the other G.R. allele has been less extensive, but the sequences established at several of the variant positions conform to published sequences. The sequence of the codon for amino acid 3292, deduced by annealing an amplified region encompassed by PCR3 and PCR5 with oligonucleotides ASO1 and ASO2, proved to encode aspartic acid in both alleles.

Inheritance of the Arginine/Glutamine Polymorphism. To test whether the CGG → CAG mutation in the codon for amino acid 3500 segregated with defective LDLs, a family study was undertaken. A region of the apoB gene of the R. and S. kindreds encompassing the mutation was amplified, and the amplified DNA was screened with ASOs. To this end, a pair of 15-mer oligonucleotides representing the mutant and normal alleles were synthesized: ASO3 and ASO4 (see Table 1). A third oligonucleotide probe (ASO5) within the amplified region was used, representing a region whose sequence was identical in both G.R. alleles. These three oligonucleotides were used to probe the amplified DNAs in a slot blot (Fig. 3). Two plasmid DNAs were included to evaluate the discrimination afforded by the two allele-specific probes. The results demonstrate that the mutation segregates in members of both families with defective LDLs and moderate hypercholesterolemia. Only affected family members carry the mutant allele. The extent of reaction with the third oligonucleotide complementary to positions 10,784–10,798 controls for differential amplification of each DNA. Analysis of the DNA of a third proband, R.N. (Fig. 3 *Left*), indicates that this subject is also heterozygous for the same mutation. Four other individuals with defective LDLs, who were unrelated to any of the aforementioned, were also heterozygous for the mutation.

Haplotype of the Mutant Allele. We wished to establish a partial haplotype of the mutant allele, in part to determine the extent to which other markers (e.g., the *Xba* I polymorphism at position 7673) correlate with this allele. This question was of interest because earlier studies established that the X2 allele(i.e., the allele containing the *Xba* I site) is associated with higher levels of plasma cholesterol (18, 19). For this purpose, we determined the genotype of each member of the R. kindred and of two other hypercholesterolemic patients at the *Xba* I site (the 5′ side of the Arg → Gln change) and the *Msp* I site (the 3′ side). For both RFLPs the genotype was determined by digesting amplified DNA representing these two regions with *Xba* I and *Msp* I. The results are summarized in Fig. 4. With respect to the *Xba* I genotype, it is clear that the mutant allele must be X1. For example, the proband and his two brothers are X1/X2, and the affected granddaughter is X1/X1, but the unaffected son of G.R. is X2/X2. The genotypes of the unrelated patients R.N. and W.S. are consistent with this view, as is the fact that the G.R. mutant allele has the codon ACC for amino acid 2488 (i.e., X1). In contrast, the normal allele of G.R. is X2. Although the *Msp* I polymorphism proved to be less informative, the data are

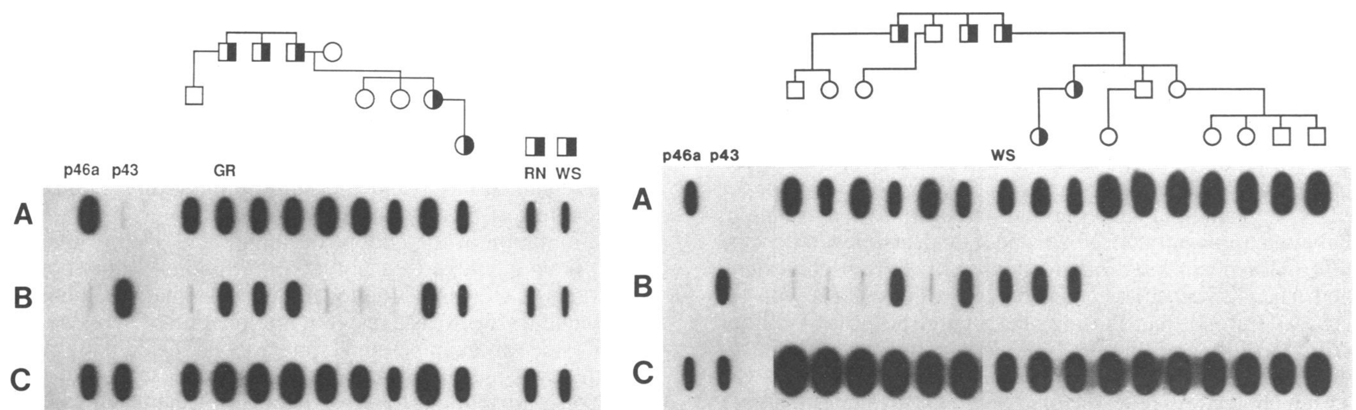
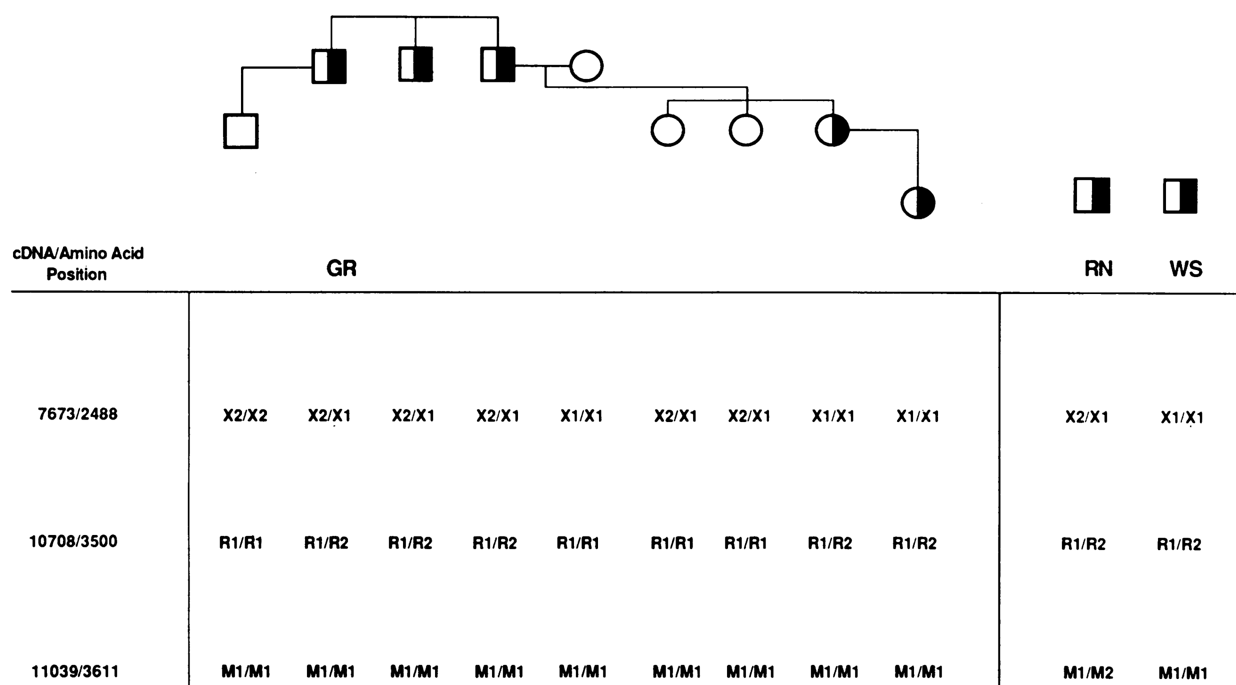


FIG. 3. Inheritance of the mutation in the codon for amino acid 3500 of the human apoB-100 gene. (*Left*) The pedigree of the proband G.R., his kindred, and two unrelated subjects (R.N. and W.S.). (*Right*) The pedigree of proband W.S. and his kindred. Half-solid circles and squares represent individuals whose LDLs bound defectively to apoB,E(LDL) receptors, and open symbols represent subjects whose LDLs bound normally (5). Rows A, B, and C, respectively, are autoradiograms of slot blots probed with ^{32}P-labeled oligonucleotides ASO3, ASO4, and ASO5. DNA from each subject was subjected to the PCR using oligonucleotide primers PCR5 and PCR6. Conditions for the amplification were 30 cycles of denaturation for 1 min at 94°C, annealing for 1 s at 37°C, and extension at 58°C. Also included are DNA of plasmids p46a and p43, representing the wild-type and mutant allele sequences, respectively. The slot blot was probed with ^{32}P-labeled oligonucleotides ASO3, ASO4, and ASO5. After overnight annealing at 42°C in a solution of 0.9 M NaCl, 0.09 M EDTA, and 0.1% $NaDodSO_4$ with 250 μg of tRNA per ml, the filter was washed twice for 5 min in 0.15 M NaCl/0.015 M sodium citrate at room temperature, and 2 min in 0.15 M NaCl/0.15 M sodium citrate at 46°C for ASO3, at 44°C for ASO4, and at 46°C for ASO5.



cDNA/Amino Acid Position	GR									RN	WS
7673/2488	X2/X2	X2/X1	X2/X1	X2/X1	X1/X1	X2/X1	X2/X1	X1/X1	X1/X1	X2/X1	X1/X1
10708/3500	R1/R1	R1/R2	R1/R2	R1/R2	R1/R1	R1/R1	R1/R1	R1/R2	R1/R2	R1/R2	R1/R2
11039/3611	M1/M1	M1/M1	M1/M1	M1/M1	M1/M1	M1/M1	M1/M1	M1/M1	M1/M1	M1/M2	M1/M1

FIG. 4. Genotypes of members of the R. family (G.R. is the proband). Below the pedigree are listed genotypes at three positions: the *Xba* I RFLP (X1 and X2), amino acid 3500 (R1 and R2), and the *Msp* I RFLP (M1 and M2). Also listed are genotypes of two other probands, R.N. and W.S. The *Xba* I RFLP was determined by subjecting the DNA of each subject to the PCR using primer oligonucleotides PCR1 and PCR2. Conditions for the amplification were 30 cycles of denaturation for 1 min at 94°C, annealing at 37°C for 2 min, extension for 3 min at 58°C, and digestion with excess *Xba* I for 3 hr at 37°C, followed by acrylamide gel analysis. For the *Msp* I polymorphism, amplification was for 30 cycles by using oligonucleotide primers PCR4 and PCR6 under the same conditions, except that extension was for 5 min. The data for the codon 3500 polymorphism R1/R2 are from Fig. 3.

consistent with the view that the mutant allele has the partial haplotype X1-Gln^{3500}-M1 and contains no other undescribed variations between bp 7673 and bp 11,912.

DISCUSSION

A high degree of genetic polymorphism in human LDL has been recognized since 1976, when it was established that allotypic antibodies in the serum of multiply transfused patients are capable of distinguishing allelic variants of LDL (20). These allotypic variants have been shown to be closely linked to DNA polymorphisms in the apoB gene (21). Furthermore, comparison of five complete and several partial published human apoB gene sequences revealed more than 60 nucleotide substitutions, the majority of which affect the amino acid sequence of the protein (10). Our intention in the present study was to determine whether any of these polymorphisms correlated with familial defective apoB-100.

As a result of many previous studies, two correlations between apoB polymorphisms and abnormalities in lipoprotein metabolism have been established. Serum cholesterol and triacylglycerol levels are higher in individuals with the Ag(x$^-$) epitope than in individuals with the Ag(x$^+$) epitope (22). Furthermore, the *Xba* I RFLP, which is in strong linkage disequilibrium with the Ag(x) epitope, is also correlated with serum cholesterol levels. Homozygotes with alleles that lack the *Xba* I site (i.e., X1/X1) tend to have lower serum cholesterol levels than X2/X2 homozygotes or X1/X2 heterozygotes (18, 19). This effect seems to be attributable to a difference in the level of LDL and apoB (23). Other authors have noted an association between the *Xba* I polymorphism and myocardial infarction (24). Other RFLPs in the apoB gene fail to correlate with abnormalities in lipid metabolism (19).

The mutation reported here changes the codon for amino acid 3500 and correlates with hypercholesterolemia and with defective LDL binding to the receptor. All three familial defective apoB-100 probands described here and all affected members in two kindreds have this same mutation. Our most recent data define a total of seven unrelated probands heterozygous for this mutation, all of whom have defective LDLs. Furthermore, all of their relatives shown to carry the mutant allele also have LDLs that are defective in binding to the receptor (data not shown). We have failed to detect any other differences between the two alleles of the original proband G.R., except for the fact that he is heterozygous for the *Xba* I polymorphism at nucleotide 7673 that changes the third position of a threonine codon. However, we have not yet determined the complete sequence of this mutant apoB allele. Therefore, the argument that the substitution of glutamine for arginine at amino acid 3500 is responsible for the defective binding of LDL to the receptor remains circumstantial. Attempts to express full-length apoB gene constructs in cultured cells that differ only in this codon are a logical test of this proposition.

The mutation in the codon for amino acid 3500, CGG $\rightarrow$ CAG, is an example of a potential mutational "hot spot." Mutations at CG dinucleotides in higher animals are unusually frequent (25, 26) as a result of methylation of cytosine on the 5′ side of guanine and spontaneous deamination of 5-methylcytosine to thymine (27). In the present case the mutation is attributable to a C $\rightarrow$ T transition in the noncoding strand. Such mutational hot spots have been reported in the human gene for factor VIII (28). It has been estimated that the extent of hypermutability at CG dinucleotides in the factor VIII gene is 10- to 20-fold greater than the rate at other positions (29). That CG dinucleotides in general are potential mutational hot spots is suggested by the unusually high frequency of polymorphism at a number of loci in human DNA where CG occurs in the recognition site for certain restriction enzymes such as *Msp* I and *Taq* I (29). In the case of the human apoB gene, two other polymorphisms have

been reported (11, 30) that result from mutations at CG dinucleotides, both at *Msp* I sites. Again by analogy to the factor VIII gene (28), these three CG dinucleotides may be hot spots where recurrent mutations have taken place in the human population.

The amino acid substitution at position 3500 occurs in a region implicated in LDL binding to the apoB,E(LDL) receptor. Although the receptor binding domain of apoB-100 has yet to be precisely defined, several lines of evidence point to this region of the protein encoded by exon 26. Two sequences in this region encode stretches of basic amino acids beginning at positions 3147 and 3359, the second of which displays some similarity to the receptor binding domain of apoE (14). Furthermore, the epitopes of several antibodies that inhibit LDL binding to its receptor also map to this region (31). Of particular relevance is the fact that one of these monoclonal antibodies, MB47, can distinguish mutant from normal LDL in terms of affinity (5). The epitope of this antibody has been localized between residues 3350 and 3506 (12). Thus, the difference in affinity is presumably attributable to the substitution of glutamine for arginine. The fact that this antibody can recognize a change on the surface of LDL and also inhibit receptor binding is consistent with a role for arginine-3500 in receptor binding. This amino acid is located within a cluster of basic residues in apoB-100, although its sequence does not resemble that of the receptor binding domain of apoE.

In addition to establishing the presence of the mutation in the codon for arginine-3500 in hypercholesterolemic subjects and their relatives, the haplotype of the mutant allele must be defined. This is important because the human apoB gene is so polymorphic: at least 14 haplotypes exist at different frequencies in various populations (32). At present, our information is limited to the region of exon 26 corresponding to amino acids 2488–3611 [i.e., between the polymorphic *Xba* I site (18, 19) and a polymorphic *Msp* I site (11)]. Of particular interest is the fact that the mutant alleles of all three probands examined contain the X1 variant of the *Xba* I polymorphism. Thus, although the association between the mutation at position 3500 and the *Xba* I polymorphism has yet to be studied in a large number of subjects, it would appear unlikely that previous studies reporting association between the X2 genotype and high cholesterol levels simply reflect the mutation discussed here. On the contrary, the mutation at position 3500 appears to define a minor X1 allele that segregates with defective LDL and moderate hypercholesterolemia.

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