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Nonsynonymous Polymorphic Sites in the Apolipoprotein (apo) A-IV Gene Are Associated with Changes in the Concentration of apo B– and apo A-I–containing Lipoproteins in a Normal Population

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Summary

The aims of this study were to detect polymorphic sites in the apolipoprotein (apo) A-IV gene, to establish their frequencies, to determine potential haplotypes, and to investigate the role of these polymorphisms in lipid metabolism. A sequencing study of four individuals led to the identification of two synonymous mutations (codons 9 and 54) and three nonsynonymous mutations (Val_{-8}→Met, Gln_{360}→His, and Thr_{347}→Ser) and of a VNTR polymorphism within a series of three or four CTGT repeats in the noncoding region of exon 3. Frequencies of these polymorphisms were determined in 291 students by using naturally occurring (*Bst*EII for the synonymous mutation in codon 54, *Hin*fI for Thr_{347}→Ser, and *Fnu*4HI for Gln_{360}→His) or artificially introduced restriction-enzyme cutting sites (*Bst*EII for the synonymous mutation in codon 9 and *Mam*I for Val_{-8}→Met), subsequent to PCR amplification. The four-base deletion/insertion polymorphism and its localization cis or trans to the mutations in codons 347 and 360 were studied by direct sequencing of PCR-amplified DNA from 87 students. Frequencies of the rarer alleles were .007 for apo $A\text{-}IV_{-8:Met}$, .04 for the synonymous mutation in codon 9, .14 for the synonymous mutation in codon 54, .16 for apo $A\text{-}IV_{347:Ser}$, .07 for apo $A\text{-}IV_{360:His}$, and .39 for the four-base of insertion. Apo $A\text{-}IV_{360:His}$ in all cases was cis-localized to the $(CTGT)_3$ repeat and apo $A\text{-}IV_{347:Thr}$; and apo $A\text{-}IV_{347:Ser}$ was cis-localized to the $(CTGT)_4$ repeat and apo $A\text{-}IV_{360:Gln}$. Four haplotypes formed from these three polymorphic sites were thus found. The apo $A\text{-}IV_{347:Ser}$ allele was associated both with significantly lower plasma apo B concentrations in both sexes and with significantly lower LDL-cholesterol concentrations in men. Heterozygous carriers of apo $A\text{-}IV_{360:His}$ exhibited significantly higher concentrations of LDL-cholesterol and lower Lp(a) concentrations, compared with apo $A\text{-}IV_{360:Gln}$ homozygotes. We could not confirm the previously reported association of apo $A\text{-}IV_{360:His}$ with elevated HDL-cholesterol concentrations. In the population, the Val_{-8}→Met polymorphism was not associated with significantly different lipid concentrations, but in a family study the Met_{-8} allele was associated with lower HDL-cholesterol and higher LDL-cholesterol concentrations. In conclusion, our results indicate an important role of the apo A-IV gene locus in the metabolism of apo B and, to a lesser extent, apo A-I containing lipoproteins.

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Introduction

Apolipoprotein (apo) A-IV, a plasma glycoprotein of known amino acid sequence, is synthesized and secreted by enterocytes after cotranslational cleavage of a 20-amino-acid prepeptide (Elshourbagy et al. 1986; Karathanasis et al. 1986). The protein's function is not

well understood. It is found in lymph chylomicrons and in plasma high-density lipoproteins (HDL) (Weisgraber et al. 1978; Beisiegel and Utermann 1979; Utermann and Beisiegel 1979; Green et al. 1980; Weinberg and Scanu 1983; Bisgaier et al. 1985). In vitro, apo A-IV activates lecithin:cholesterol acyltransferase (Chen and Albers 1985; Steinmetz and Utermann 1985) and specifically interacts with HDL-binding sites on various cells, thereby promoting cholesterol efflux (Ghiselli et al. 1985; Dvorin et al. 1986; Stein et al. 1986; Savion and Gamliel 1988; Steinmetz et al. 1990; Weinberg and Patton 1990). These reported functions suggest a possible role by apo A-IV in reverse cholesterol transport, a metabolic pathway that is thought to be responsible for the transport of excess cellular cholesterol to the liver, for degradation or excretion.

On isoelectric focusing (IEF), apo A-IV exhibits a genetic polymorphism with two frequent isoforms, termed "apo A-IV-1" and "apo A-IV-2" (Menzel et al. 1982). Reported allele frequencies are .91–.93 and .07–.09, respectively (reviewed in Lohse and Brewer 1991). apo A-IV-2 differs from apo A-IV-1 by a histidine-for-glutamine replacement in position 360 of the mature protein (Boerwinkle et al. 1990; Lohse et al. 1990*a;* Tenkanen et al. 1991). In addition, a Thr→Ser replacement in codon 347 of mature apo A-IV has been identified (Boerwinkle et al. 1990; Lohse et al. 1990*c*). The allele frequencies of the latter two electrophoretically indistinguishable polymorphs are .784 (Thr) and .216 (Ser) (Boerwinkle et al. 1990). Besides these frequent apo A-IV polymorphs, rare apo A-IV variants have been identified by IEF and have been structurally analyzed (Lohse et al. 1990*b,* 1991).

The data summarizing the impact that the apo A-IV polymorphism has on lipid metabolism are inconsistent. In three screening studies using IEF and immunoblotting, apo A-IV-1/2 heterozygotes were found to exhibit significantly higher levels of HDL-cholesterol (Menzel et al. 1988, 1990) or triglycerides (Eichner et al. 1988), compared with apo A-IV-1/1 homozygotes. Other studies failed to establish a significant association when different lipid or apolipoprotein plasma concentrations were used (De Knijff et al. 1988; Hanis et al. 1991). The functional consequences of the Gln→His replacement are also a matter of controversy: in one study, apo A-IV-2 was a better activator of LCAT (Weinberg et al. 1990); in another study, apo A-IV-1 was (Tenkanen et al. 1991). Since all previous screening studies have been performed using protein isoforms as phenotypic markers, the conflicting results may have originated from a variety of defects causing identical net charge differences, either from the presence of additional electrically neutral amino acid substitutions or from linkage of one or both of the electrophoretically detectable mutations to other polymorphic sites in the apo A-IV gene.

We therefore sequenced the apo A-IV gene from an apo A-IV-1/2 heterozygote and from three individuals with different forms of familial HDL deficiency. This study led to the detection of six polymorphic sites. Allele frequencies, possible associations among polymorphic sites, and impact of the polymorphisms on plasma lipid metabolism were subsequently analyzed in 291 students. Furthermore, we were interested in the question of whether one of the apo A-IV polymorphisms is linked to an *XmnI* polymorphism, 20 kb 5′ of the apo A-IV gene, which was recently shown to be associated with familial combined hyperlipidemia (Hayden et al. 1987; Wojciechowski et al. 1991).

Subjects and Methods

Subjects

Sequencing of the apo A-IV gene was performed on DNA samples from four unrelated individuals. One was selected by the diagnosis of apo A-IV-1/2 heterozygosity by using IEF (fig. 1). The three other individuals were patients with different forms of HDL deficiency, namely, apo A-I deficiency (Funke et al. 1991*b*), fish-eye disease (Funke et al. 1991*a*), and Tangier disease (Assmann et al. 1989). For the screening study, blood samples were taken from 291 20–25 year-old medical student volunteers after overnight fasting and were analyzed for serum concentrations of lipids, lipoprotein(a) [Lp(a)], apo A-I, and apo B. The impact of the Val_{-8}→Met polymorphism in apo A-IV was studied in a family with fish-eye disease (Funke et al. 1991*a*).

Quantitative Analyses

Serum concentrations of triglycerides and cholesterol were quantified with an autoanalyzer (Hitachi/Boehringer, Mannheim). HDL-cholesterol concentrations were measured after precipitation with phosphotungstic acid/$MgCl_2$ (Boehringer, Mannheim). LDL-cholesterol was calculated using the Friedewald formula (Friedewald et al. 1972). Concentrations of apo A-I and of apo B were determined with a modified commercially available turbidimetric assay (Boehringer, Mannheim) (Sandkamp et al. 1988). Lp(a) was

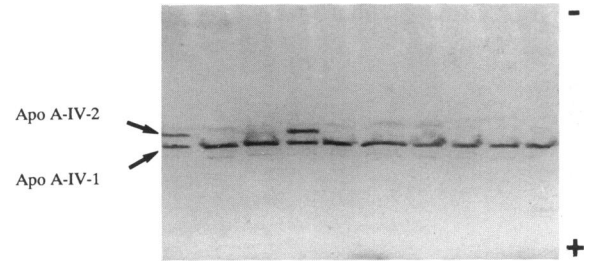


Figure 1 Demonstration of the apo A-IV protein polymorphism by IEF and subsequent immunoblotting. apo A-IV-1 and apo A-IV-2 denote the two genetic isoforms.

quantified by electroimmunodiffusion (Sandkamp et al. 1990).

Gene Sequencing

Blood for leukocyte isolation and subsequent DNA preparation was collected in EDTA-containing tubes at a final concentration of 50 mM. In the four subjects described above, we sequenced the exons, the splice donor and acceptor sites of the apo A-IV gene, and 65–76 bases of upstream sequence. Eight 21–23-base-long oligonucleotides were used as primers for PCR amplification (Saiki et al. 1985) (fig. 2): primer 1, 5′-ATGTGTCACCTTCCAGCGTGGAC-3′ (781→803); primer 2, 3′-CTCCATCCTGCACTACTCAGAGC-5′ (1117→1095); primer 3, 5′-GCAGAGGGGCCACACAGGAGACG-3′ (1356→1378); primer 4, 3′-CCTTGCCAGTACATTGCATGGCC-5′ (1605→1583); primer 5, 5′-TCTTGCCGTGTAAATGCCAAAT-3′ (2262→2284); primer 6, 3′-TCAGGTTGCCACGCACGTCCTCG-5′ (2974–2952); primer 7, 5′-TCCAGATGAAGAAGAACGCCGAG-3′ (2867→2889); and primer 8, 3′-CAGCTGCAAGGAGGATTCATCC-5′ (3528→3506) (Karathanasis et al. 1986). Primers were synthesized using the Gene Assembler (Pharmacia, Freiburg). Amplification primers were also used as sequencing primers. The amplification reaction was carried out in 100 μl of the buffer recommended by the supplier of the *Taq* polymerase (Cetus), 0.5–1 μg DNA, and final concentrations of 200 μM of each dNTP, and 0.1 μM of each primer. Initial denaturation at 100°C for 10 min was followed by the addition of 2–5 U *Taq* polymerase and 30 cycles of incubation at 96°C for 2 min, 60°C for 1 min; and 70°C for 1 min. Product purification was done by electrophoresis in 4% NuSieve GTG agarose (FMC). DNA of the expected size was cut out of the gel and electroeluted in .5 × TAE for 1.25–1.5 h at 10 V/cm. DNA was desalted and concentrated to 70 μl by

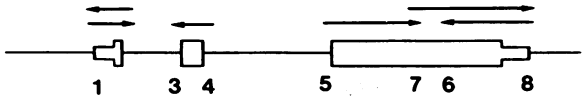


Figure 2 Structure of the apo A-IV gene. Arrows indicate the sequencing strategy; numbers refer to the primers described in the Subjects and Methods section. Odd numbers are upper-strand primers; and even numbers are lower-strand primers.

ultrafiltration centrifuging three times, at 5 krpm for 20 min each time, in Centricon X-100 tubes (Amicon) that refilled to 2 ml with water between the runs. Single-strand production was done by the method of Gyllensten and Erlich (1988), by using the same reaction and purification scheme as above. Primer concentration was 0.1 μM; a second primer was not used. Sequencing was done from a single strand after phenol extraction and LiCl/ethanol precipitation, by using T7 polymerase (Pharmacia) and following the protocol of the polymerase supplier. Labeling times were 0.5–5 min. After termination, the sample volume was reduced to 6 μl in an evaporation centrifuge; half of it was applied to the sequencing gel (6% acrylamide gel with 29:1 cross-linking, in 135 mM Tris, 45 mM boric acid, 2.5 mM EDTA).

RFLP analyses of polymorphic sites.—In a population study, the presence or the absence of five single-base substitutions in the apo A-IV gene were analyzed using either naturally occurring (*Bst*EII for the synonymous mutation in codon 54, *Hin*fI for Thr_{347}→Ser, and *Fnu*4HI for Gln_{360}→His) or artificially created polymorphic enzyme cutting sites (*Mam*I for Val_{-8}→Met, and *Bst*EII for the synonymous mutation in codon 9). RFLPs were determined from PCR-amplified DNA. Reaction conditions for the PCR were as described above. Primers 5 and 6 were used for the *Bst*EII polymorphism in codon 54, and primers 7 and 8 were used for the *Hin*fI and *Fnu*4HI polymorphisms. In the other cases, the enzyme cutting sites were specifically introduced into the mutant alleles by the use of site-directed mutagenic PCR primers (mismatched nucleotides are underlined) (Kumar and Barbacid 1988; Kumar and Dunn 1989). Mutagenic primers were 5′-AGCTTCTACTCACCGGCGACAGCCA-3′ (nucleotides 1067→1043) for the *Mam*I polymorphism and 5′-ACTGGCTGAAGTAGTCCCACATCAC-3′ for the *Bst*EII polymorphism in codon 9. Oligonucleotide 1 (described above) was used as the lower-strand primer in both cases.

*Xmn*I *polymorphism.*—The X1 and X2 alleles of the *Xmn*I polymorphism 5′ of the apo A-I gene were iden-

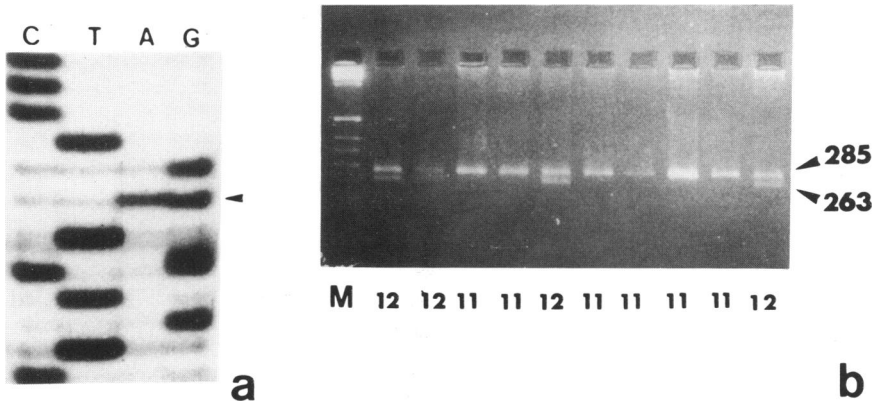


Figure 3 *a*, G→A substitution in codon −8, leading to a methionine-for-valine replacement in position 13 of the apo A-IV prepeptide. This Val_{-8}→Thr polymorphism was analyzed using a *Mam*I polymorphism which was artificially introduced by mutagenic primers. *b*, Met_{-8} allele, the presence of which led to the formation of 263-bp (M*2) instead of 285-bp (M*2) DNA fragments. Numbers below the lanes denote genotypes. Lane M, Molecular-weight marker.

tified using mutagenic PCR primers 5′-TGCCAG-ACCTAGCAGGGGACA-3′ and 5′-GCATCTTAC-CAAGCAGGCAGTTTC-3′. PCR amplification was done using 500 ng genomic DNA and 100-nM and 200-μM concentrations of primers and dNTPs, respectively. After an initial denaturation step of 5 min at 100°C, PCR was done by 30 cycles, each consisting of 1 min at 96°C, 1 min at 60°C, and 1 min at 70°C. Final extension was done at 70°C for 5 min. When the *Xmn*I site was present, the 522 bp–long PCR fragment was cut into fragments of 409 bp and 113 bp.

Statistical analyses.—Statistical analyses were done using the SPSS package. All concentrations represent mean values ± SD, except those for Lp(a) and triglycerides, which are expressed as geometric means with $-1\ SD < x < +\ 1\ SD$ confidence intervals.

Results

Identification of Polymorphic Sites in the apo A-IV Gene

Direct sequence analysis of the apo A-IV gene from one apo A-IV-1/2 heterozygote and from three HDL-deficient patients provided information on both alleles and revealed six polymorphic sites in the apo A-IV gene: (1) A G→A substitution in codon −8 leads to a methionine-for-valine replacement in position 13 of the apo A-IV prepeptide (fig. 3*a*); (2 and 3) Two A→G and T→C replacements, in codons 9 and 54, respec-

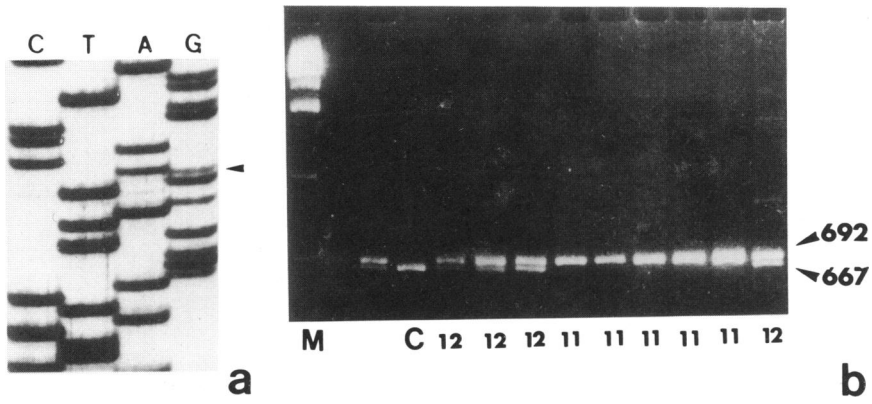


Figure 4 *a*, A→G replacement in codon 9, does not change the apo A-IV primary structure. This polymorphism was analyzed by an artificially created *Bst*EII RFLP (see Subjects and Methods). *b*, 667-bp (instead of 692-bp) DNA fragments identified in the presence of the rare allele (B*2). Numbers below the lanes denote genotypes. Lane M, Molecular-weight marker. Lane C, Uncut (left) and cut (right) controls.

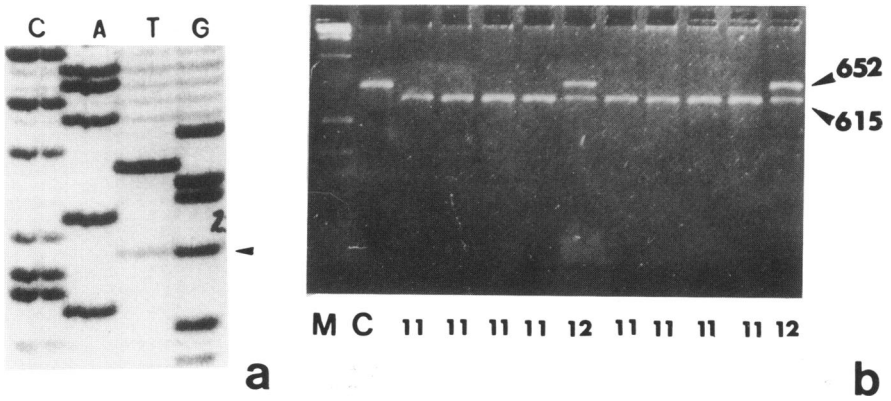


Figure 5 *a*, Synonymous T→C substitution in codon 54. *b*, Loss of a naturally occurring *Bst*EII restriction site, caused by synonymous T→C substitution in codon 54. Numbers below the lanes denote genotypes. Lane M, Molecular-weight marker. Lane C, Uncut control.

tively, did not change the apo A-IV primary structure (figs. 4*a* and 5*a*); (4 and 5) The transversions A→T in codon 347 and G→T in codon 360, leading to Thr→Ser and Gln→His substitutions, respectively (fig. 6), have already been described by other investigators (Boerwinkle et al. 1990; Lohse et al. 1990*a*, 1990*c*; Tenkanen et al. 1991); (6) Finally, we identified in exon 3 a VNTR polymorphism which showed either three or four CTGT motifs (= $[CTGT]_4$ and $[CTGT]_3$) (fig. 6). Heterozygosity for the VNTR polymorphism was determined by the relatively decreased density of the upper CTGT motif and by the appearance of characteristic frameshift patterns with doublets upstream from the VNTR.

Determination of Allele Frequencies

Frequency determination of the various dimorphic sites was done as follows: first, the Val_{-8}→Thr polymorphism was analyzed using an artificially created *Mam*I polymorphism. In the presence of the Met_{-8} allele, mutagenic PCR primers introduced a *Mam*I restriction site into the amplification product. Thus, after *Mam*I incubation, 263-bp fragments (mutant allele M*2) instead of 285-bp DNA fragments (wild-type allele M*1) were obtained (fig. 3*b*). Second, the synonymous mutation in codon 9 was analyzed by an artificially created *Bst*EII RFLP. The resulting 667-bp and 692-bp DNA fragments were indicative of the presence of the mutant (B*2) and the wild-type allele (B*1), respectively (fig. 4*b*). Third, the synonymous mutation in codon 54 resulted in the loss of a naturally occurring *Bst*EII restriction site and in the generation of 652-bp (B2) instead of 615-bp (B1) DNA fragments (fig. 5*b*). Fourth, the mutation underlying Thr_{347}→Ser forms a *Hin*fI restriction site and was identified by the loss of a 617-bp DNA fragment (H1) and by the formation of two DNA fragments (H2), one of 370 bp and one of 247 bp (fig. 7*a*). Fifth, the mutation underlying Gln_{360}→His results in the loss of a *Fnu*4HI restriction site and was identified by the presence of 189-bp (F2) instead of 180-bp DNA fragments (F1) (fig. 7*b*). Sixth, the VNTR polymorphism was analyzed by direct sequencing of the 3′ region of exon 3 in 87 students (fig. 6). This analysis also gave information on the cis/trans-localization of this polymorphism with the point mutations in codons 347 and 360 (see below). Allele frequencies of the dimorphic sites were determined in 291 students. All frequency data are presented in table 1.

Determination of Haplotypes

The cis- or trans-localization of the point mutations underlying Thr_{347}→Ser and Gln_{360}→His with the $(CTGT)_3/(CTGT)_4$ polymorphism was analyzed by direct sequencing of 23 DNA samples from apo A-IV(His/Gln_{360}) heterozygotes and of 64 DNA samples from apo A-IV(Gln_{360}) homozygotes, which were selected on the basis of the results from the *Fnu*4HI RFLP analysis. Sequence analysis was limited to the T and G lanes, since the two point mutations both add thymidines to the sequence and since the VNTR polymorphism causes a characteristic frameshift also detectable in the T lane (fig. 6).

In figure 6, the VNTR alleles are easily identified by the occurrence of either four or three –TGT motifs. In the case of heterozygosity the upper –TGT motif is less dark than the three below. The G→T transversion causing the Gln→His substitution is characterized by the presence of a T within a sequence region that otherwise does not contain a T. Linkage of either the

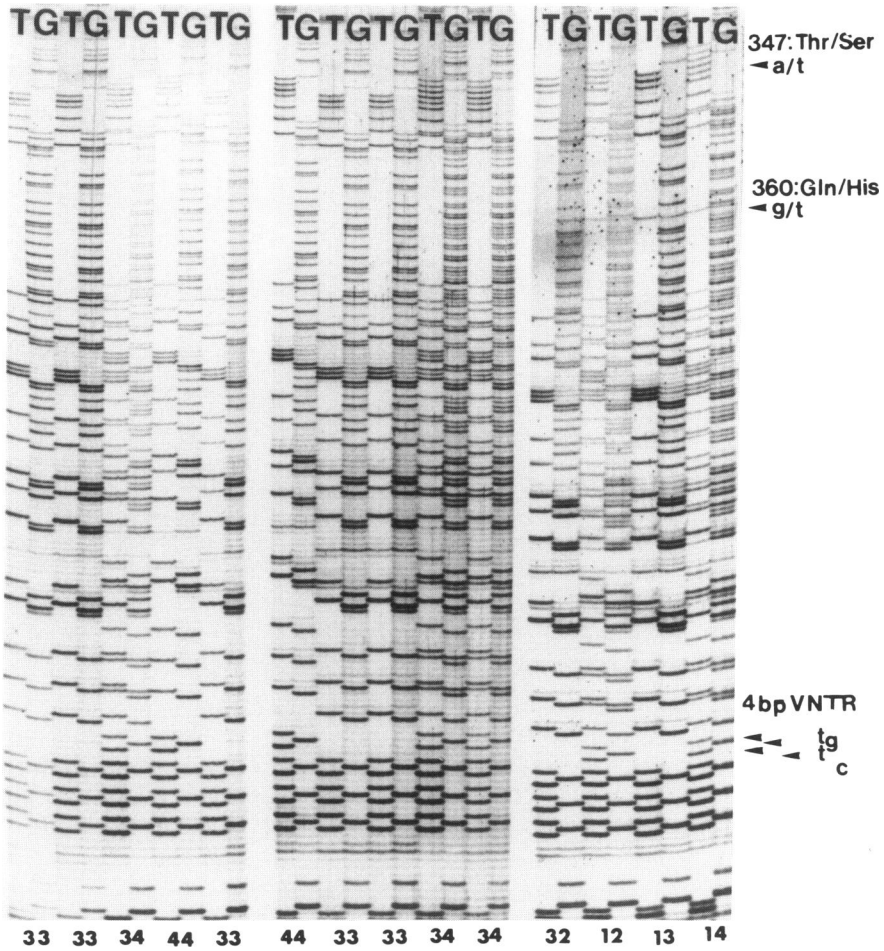


Figure 6 Sequencing analysis of the 3′ region of exon 3 of the apo A-IV gene. Only T and G lanes are demonstrated. The transversions A→T in codon 347 and G→T in codon 360, leading to Thr→Ser and Gln→His substitutions, respectively, have been described by other investigators (Boerwinkle et al. 1990; Lohse et al. 1990*a*, 1990*b*; Tenkanen et al. 1991). The VNTR polymorphism represents a difference between the sequence reported by Karathanasis et al. (1986) and that reported by Elshourbagy et al. (1986). Sequence analysis of this region in 87 students also gave information on the cis/trans-localization of the VNTR polymorphism with the point mutations underlying Gln_{360}→His and Thr_{347}→Ser (for details, see text). Numbers below the lanes denote the genotypes deduced for the haplotypes thus constructed (table 2). Arrows indicate the positions of mutated nucleotides.

$(CGTG)_4$ or the $(CTGT)_3$ allele with this single-base substitution leads to the occurrence of the T band in two different positions separated by four bases. The T band was exclusively found in the lower of the two possible positions, and, therefore, the point mutation in apo A-IV(His_{360}) is cis-localized with the $(CTGT)_3$ allele. The wild-type apo-A-IV(Gln_{360}) occurs with both three and four CTGT repeats.

The T-for-A substitution in apo A-IV(Ser_{347}) adds another T to a series of three subsequently occurring T's in the sequence analysis system used. Depending on the cis-localization with either the $(CTGT)_3$ or the $(CTGT)_4$ allele, the mutation can cause (1) a series of either four T's shifted toward longer DNA fragment sizes (in $[CTGT]_4/[CTGT]_4$ homozygotes) or six T's (in $[CTGT)_3/[CTGT]_4$ heterozygotes) in the case of linkage with the $(CTGT)_4$ polymorphic site and (2) a series of either four unshifted T's (in $[CTGT]_3/[CTGT]_3$ homozygotes) or five T's (in $[CTGT]_4/[CTGT]_3$ heterozygotes) in the case of linkage to the $(CTGT)_3$ allele. Actually, only case (1) was assessed. Thus, apo A-IV(Ser_{347}) has only occurred together with the $(CTGT)_4$ polymorphic site on a common allele. Apo A-IV(Thr_{347}) is present on both VNTR alleles.

The strict association of Ser_{347} with $(CTGT)_4$ and of His_{360} with $(CTGT)_3$ shows that the two nonsynonymous point mutations are located on different alleles.

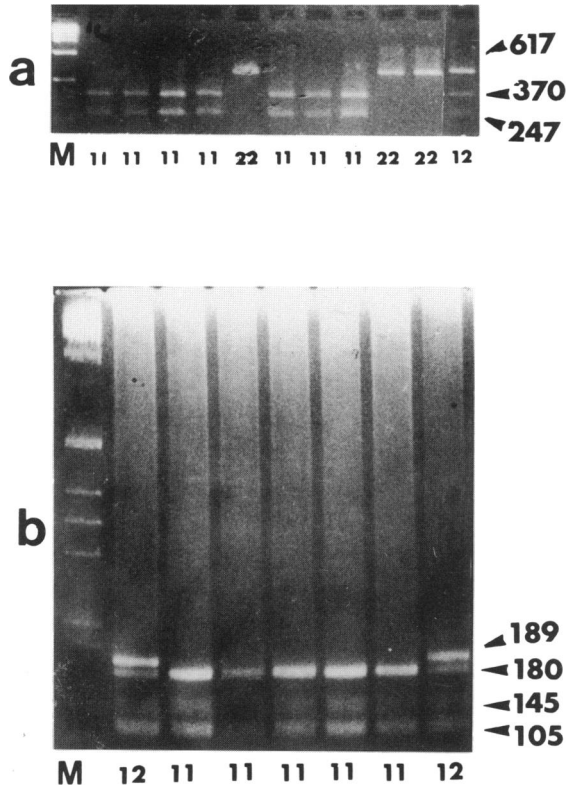


Figure 7 RFLP analyses of the point mutations underlying Thr_{347}→Ser and Gln_{360}→His. *a*, Mutation underlying Thr_{347}→Ser. This mutation forms a *Hin*fI restriction site and was identified by the loss of a 617-bp DNA fragment and the formation of one 370-bp fragment and one 247-bp fragment. *b*, Mutation underlying Gln_{360}→His. This mutation results in the loss of a *Fnu*4HI restriction site and was identified by the finding of a 189-bp (instead of 180-bp) DNA fragment. Numbers below the lanes denote genotypes. Lane M, Molecular-weight marker.

Thus, only four of eight possible haplotypes were identified (table 2). Frequencies for allele 1 (Thr_{347}-His_{360}-[CTGT]$_3$) and allele 4 (Ser_{347}-Gln_{360}-[CTGT]$_4$) were determined on the basis of the results obtained by

Table 2

Definition and Frequency of Haplotypes in 3′-Region of Apo A-IV Gene

Codon 347/Codon 360	VNTR	Haplotype Frequencies	
		Observed	Expected
Thr/His	(CTGT)$_3$	.070	.02
Thr/Gln	(CTGT)$_4$	.235	.47
Thr/Gln	(CTGT)$_3$	.535	.31
Ser/Gln	(CTGT)$_4$	.160	.09
Thr/His	(CTGT)$_4$	0	.04
Ser/His	(CTGT)$_4$	0	.007
Ser/Gln	(CTGT)$_3$	0	.06
Ser/His	(CTGT)$_3$	0	.004

Note.—Allele frequencies were determined by *Hin*fI and *Fnu*4HI RFLP analysis of DNA samples from 291 students (table 1) and by direct sequencing of 23 DNA samples from apo A-IV(Gln/His_{360}) heterozygotes and 64 DNA samples from apo A-IV(Gln_{360}) homozygotes (fig. 6).

*Fnu*4HI (allele 1) and *Hin*fI (allele 4) RFLP analyses, as the Thr_{347} mutation was not associated with (CTGT)$_4$ and the His_{360} mutation was not associated with (CTGT)$_3$. Frequencies of allele 2 (Thr_{347}-Gln_{360}-[CTGT]$_4$) and allele 3 (Thr_{347}-Gln_{360}-[CTGT]$_3$) were calculated from a comparison of their frequencies in 64 apo A-IV(Gln_{360}) homozygotes in whom the 3′-region of the apo A-IV gene was sequenced.

Impact of Polymorphic Sites in the apo A-IV Gene on Serum Concentrations of Lipids, Lipoproteins, and Apolipoproteins

To assess the possible impact of polymorphic sites in the apo A-IV gene on lipid metabolism, we compared serum concentrations of cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, Lp(a), apo A-I, and apo B with the different genotypes at the six dimorphic sites. Haplotypes formed by Thr_{347}→Ser,

Table 1

Frequencies of Apo A-IV Alleles in Students

Polymorphic Site	Polymorphism	Detection Method	No. of Subjects	Frequency of	
				Allele 1	Allele 2
Codon −8	G→A, Val→Met (M*1, M*2)	*Mam*I*	376	.993	.007
Codon 9	A→G, synonymous (B*1, B*2)	*Bst*EII*	94	.968	.032
Codon 54	T→C, synonymous (B1, B2)	*Bst*EII	94	.855	.145
Codon 347	A→T, Thr→Ser (H1, H2)	*Hin*fI	289	.840	.160
Codon 360	G→T, Gln→His (F1, F2)	*Fnu*4HI	291	.930	.070
VNTR exon 3	(CTGT)$_3$ and (CTGT)$_4$	Sequencing	87	.607	.393

Note.—An asterisk (*) denotes the detection by an artificially created restriction-enzyme cutting site.

Table 3

Apo A-IV(Val$_{-8}$→Met): Frequency and Impact on Lipid Values

Lipid	Lipid Concentration in (mg/dl)	
	Normal Subjects (n = 370)	Heterozygotic Subjects (n = 6)
Cholesterol	190.3 ± 33.4	204.8 ± 20.8
Triglycerides[a]	87.1 ± 49.4	110.4 ± 53.5
	49.6 < 77.9 < 122.5	58.3 < 99.2 < 168.9
HDL cholesterol	57.3 ± 15.5	55.4 ± 16.6
LDL cholesterol	114.6 ± 29.1	127.4 ± 27.0
Lp(a)[a]	3.8 < 9.9 < 47.6	8.7 < 9.4 < 11.8
apo A-I	153.0 ± 28.7	139.8 ± 33.9
apo B	78.6 ± 20.4	87.8 ± 12.0

[a] Serum concentrations of triglycerides and Lp(a) do not exhibit a Gaussian frequency distribution; therefore mean values for them are presented as $-1\ SD < x < +1\ SD$ ranges as obtained by retransformation of ln(triglycerides) and ln[Lp(a)], respectively.

Gln_{360}→His, and the VNTR polymorphism were also analyzed for these associations.

MamI (Val$_{-8}$→Met).—No significant differences in lipid, lipoprotein, and apolipoprotein concentrations were found between 6 heterozygotes and 370 normals (table 3). However, because of the low frequency of the variant allele, our association study may have overlooked a possible impact on this mutation on lipid metabolism. Among six heterozygotes, we identified three individuals with HDL-cholesterol and apo A-I concentrations below 30 mg/dl and 110 mg/dl, respectively, which are the 10th percentiles in the Prospective Cardiovascular Münster (PROCAM) population (Assmann and Schulte 1986). Furthermore, we studied the impact of the Val$_{-8}$→Met substitution on lipid metabolism in a family with homozygous, heterozygous, and unaffected individuals (table 4). This family is also affected with fish-eye disease, which is caused by a mutation in the LCAT gene and which, among other effects, causes decreased HDL-cholesterol concentrations (Funke et al. 1991*a*). Therefore, we differentiated the family members into two groups, one with heterozygotes for the fish-eye disease defect and another with family members unaffected by this defect. In both groups the mutant apo A-IV(Met$_{-8}$) allele was found to (*a*) decrease serum concentrations

Table 4

Apo A-IV(Val$_{-8}$→Met): Impact on Lipid Values in a Family with Fish-Eye Disease

Lipid	Lipid Concentrations in				
	Fish-Eye Disease Heterozygotes			Normals	
	(Val/Val)[a]	(Val/Met)[b]	(Met/Met)[c]	(Val/Val)[d]	(Val/Met)[e]
Cholesterol	137.8 ± 6.9	169.2 ± 34.1	194	207.4 ± 33.1	185 ± 26
Triglycerides	115.5 ± 64.3	79.4 ± 16.8	120	126.2 ± 48.0	92 ± 10
HDL cholesterol	46.5 ± 11.0	38.2 ± 2.4	31	58.4 ± 6.5	56 ± 11
LDL cholesterol	70.0 ± 15.9	114.8 ± 34.2	140	123.2 ± 25.2	113 ± 12
apo A-I	121.0 ± 16.7	116.6 ± 8.2	111	150.4 ± 11.5	144 ± 12
apo B	50.5 ± 2.9	70.8 ± 21.0	77	77.4 ± 11.1	65 ± 11

Note.—Detailed family data of the kindred with fish-eye disease have been reported elsewhere (Funke et al. 1991*b*).

[a] Three males and one female; mean age 22.8 ± 12.3 years.

[b] Three males and two females; mean age 18.4 ± 13.4 years.

[c] One male and no females; age 25 yr.

[d] Two males and three females; mean age 25.4 ± 13.1 years.

[e] One male and one female; ages 51 and 4 years, respectively.

of both HDL-cholesterol and apo A-I by at least 4%, and (*b*) increase LDL-cholesterol by at least 9%. The homozygous carrier of the apo A-IV(Met$_{-8}$), who is a heterozygote for fish-eye disease, exhibited the lowest concentrations of both apo A-I and HDL-cholesterol and the highest LDL-cholesterol concentration among the fish-eye-disease heterozygotes (table 4).

BstEII polymorphisms in codons 9 and 54.—The two synonymous mutations in codon 9 and 54 were analyzed in 94 students and were not associated with any significant changes in lipid, lipoprotein, and apolipoprotein levels.

HinfI (Thr$_{347}$→Ser).—Comparisons of serum concentrations of lipids, lipoproteins, apo A-I, and apo B are summarized in the left column of figure 8. The presence of one or two Ser$_{347}$ alleles led to increases in the HDL-cholesterol plasma concentration (Thr/Thr, 55.8 ± 14.8 mg/dl [n = 188]; Thr/Ser, 57.2 ± 16.2 mg/dl [n = 74]; and Ser/Ser, 67.0 ± 15.5 mg/dl [n = 6] and to decreases in both LDL-cholesterol (Thr/Thr, 115.6 ± 28.4 mg/dl [n = 184]; Thr/Ser, 113.0 ± 29.7 mg/dl [n = 69]; and Ser/Ser, 108.3 ± 36.5 mg/dl [n = 6]) and apo B concentrations (Thr/Thr, 77.7 ± 19.7 mg/dl [n = 157]; Thr/Ser, 74.5 ± 19.5 mg/dl [n = 61]; and Ser/Ser, 52.0 ± 0.8 mg/dl [n = 4]), all showing gene-dosage effects. Differences between the alleles were significant for apo B concentrations in the entire population ($P < .01$, Kruskal-Wallace test; $P < .01$, Mann-Whitney tests for both Thr/Thr vs. Ser/Ser and Thr/Thr vs. Thr/Ser + Ser/Ser), as well as in both the female subgroup and the male subgroup ($P < .05$). Differences for LDL-cholesterol were not significant in the entire population, except in men ($P < .05$, Kruskal-Wallace test; $P < .05$, Mann-Whitney tests for both Thr/Thr vs. Ser/Ser and Thr/Thr vs. Thr/Ser + Ser/Ser). Differences for HDL-cholesterol were not significant, but the unidirectional trends assessed in the entire population, as well as in the sex-differentiated subpopulations, suggest that the *Hin*fI polymorphism also affects this parameter.

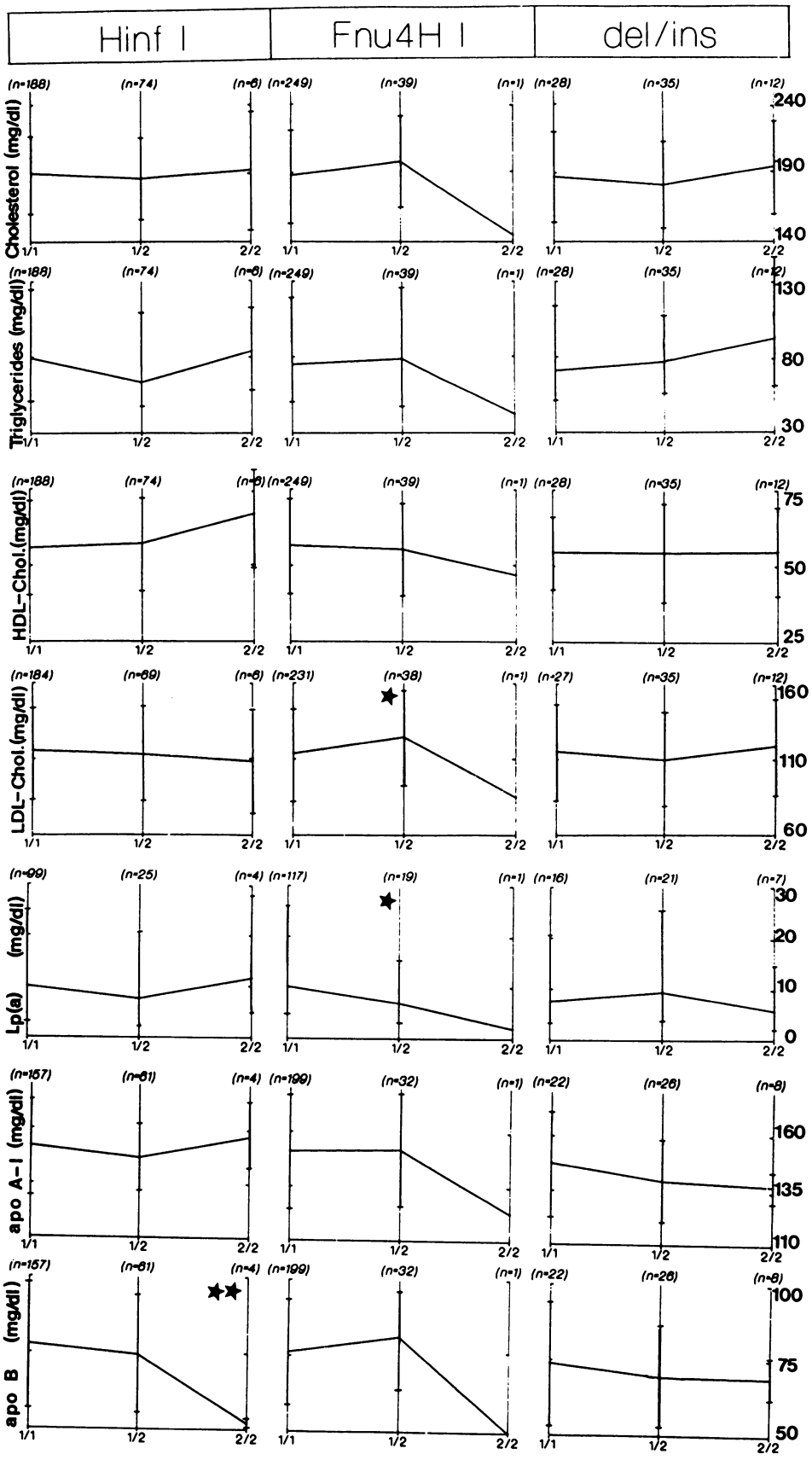
Fnu4HI (Gln$_{360}$→His).—Since only one apo A-IV-2/2 homozygote was found among 291 students, only data from apo A-IV-1/1 homozygotes and apo A-IV-1/2 heterozygotes were used to estimate the impact of the *Fnu*4HI polymorphism on lipid metabolism (fig. 8, *middle column*). apo A-IV-1/1 homozygotes had significantly lower serum concentrations of LDL-cholesterol (113.7 ± 28.9 mg/dl [n = 231] versus 124.5 ± 29.6 mg/dl [n = 38]; $P < .05$, Mann-Whitney) and higher concentrations of Lp(a) (3.9 < 10.2 < 26.5 [n = 117] vs. 3.1 < 6.9 < 15.2 mg/dl [n = 19]; $P < .05$, Whitney-Mann). By differentiation on sex, these differences were not significant but had the same tendency: LDL-cholesterol was 116.4 ± 27.9 mg/dl (n = 119) versus 123.6 ± 30.3 mg/dl (n = 24) in men, and 110.5 ± 29.9 mg/dl (n = 112) versus 126.0 ± 29.5 mg/dl (n = 14) in women. Lp(a) concentrations were 3.9 < 10.7 < 29.5 mg/dl (n = 66) versus 4.8 < 7.5 < 11.5 mg/dl (n = 14) in man and 3.9 < 9.6 < 24.0 mg/dl (n = 49) versus 2.6 < 6.4 < 16.1 mg/dl (n = 5) in women. Male and female apo A-IV-1/1 homozygotes also had lower apo B concentrations than did apo A-IV-1/2 heterozygotes, but these differences were not significant. Previously published differences in serum concentrations of triglycerides and HDL-cholesterol, between apo A-IV-1/1 and apo A-IV-1/2 (Eichner et al. 1988; Menzel et al. 1988, 1990), were not confirmed. In contrast to previous studies, HDL-cholesterol was slightly lower in apo A-IV-1/2 heterozygotes. On sex differentiation, HDL-cholesterol levels were higher in male apo A-IV(Gln$_{360}$→His) heterozygotes (not significant) but were lower in females compared with apo A-IV$_{360:Gln}$ homozygotes of the same sex (not significant).

VNTR in the 3′ region of exon 3.—Unidirectional changes were observed for serum concentrations of triglycerides, apo A-I, and apo B (fig. 8, *right column*). However, no parameter was characterized by parallel changes in both sexes. Furthermore, no difference was significant. Thus, the VNTR polymorphism does not appear to be associated with quantitative changes in lipid metabolism.

Haplotypes.—Among the three haplotypes containing the apo A-IV(Gln$_{360}$) mutation, no significant differences in lipid, lipoprotein, and apolipoprotein concentrations were observed. An identical finding was made for the three haplotypes containing the apo A-IV(Thr$_{347}$) mutation.

Linkage to XmnI Polymorphic Sites in apo A-I

Because of a recent report on an association between an *Xmn*I RFLP within the apo A-I/C-III/A-IV gene cluster and familial combined hyperlipidermia (Hayden et al. 1987; Wojciechowski et al. 1991), we analyzed whether one of the two nonsynonymous mutations in codons 347 and 360, which both are associated with changes in the plasma concentration of apo B–containing lipoproteins, are linked to the rarer X2 allele. Neither the Thr/Ser polymorphism nor the Gln/His polymorphism is linked with the *Xmn*I polymorphism in APOLP1 (data not shown).


Hinf I
Fnu4H I
del/ins
Cholesterol (mg/dl)
Triglycerides (mg/dl)
HDL-Chol.(mg/dl)
LDL-Chol.(mg/dl)
Lp(a) (mg/dl)
apo A-I (mg/dl)
apo B (mg/dl)
1/1
1/2
2/2

Discussion

apo A-IV exhibits an electrophoretically detectable genetic polymorphism with two frequent allele products that were previously shown to differ by the amino acids glutamine (apo A-IV-1) and histidine (apo A-IV-2) in position 360 (Boerwinkle et al. 1990; Lohse et al. 1990*a*; Tenkanen et al. 1991). Another polymorphism in the apo A-IV gene leads to an electrophoretically undetectable Thr→Ser exchange in position 347 (Boerwinkle et al. 1990); Lohse et al. 1990*c*). Besides these two previously reported mutations in the apo A-IV gene, our sequencing study in four unrelated individuals revealed the presence of four other electrophoretically undetectable mutations in the apo A-IV gene: a G→A transition in codon −8 leads to a Val→Met replacement in position 13 of the apo A-IV prepeptide; and two mutations in codons 9 and 54, as well as VNTR polymorphism in the 3′ region of exon 3, do not change the apo A-IV primary structure. Thus, the human apo A-IV gene appears to be highly polymorphic, as it has been shown to be in baboon, dog, and horse (Juneja et al. 1989; Ferrell et al. 1990).

In a normal population, the three nonsynonymous mutations were associated with significant changes in lipid metabolism. In both men and women the heterozygous presence of the Ser_{347} allele (frequency .16) was associated with 15% lower apo B, 3% lower LDL-cholesterol, and 10% higher HDL-cholesterol. The effects were doubled in homozygotes, thus indicating a gene-dosage effect for the Thr_{347}→Ser mutation. Differences were significant for apo B in both sexes and for LDL-cholesterol in men. Heterozygosity for apo A-IV(His_{360}) (frequency .07) was associated with 10% higher LDL-cholesterol and 30% lower Lp(a) concentrations, compared with apo A-IV(Gln_{360}) homozygotes. Another study investigating the apo A-IV polymorphism by IEF, however, did not assess the differences in LDL-cholesterol (Hanis et al. 1991). Differences in HDL-cholesterol concentrations that other investigators found in Tyroleans and Icelanders (Menzel et al. 1988, 1990) were not confirmed in the present study. In the population study, apo A-IV (Met_{-8}) was associated with 3% lower HDL-cholesterol, 9% lower apo A-I, 11% higher LDL-cholesterol, and 12% higher apo B. Presumably because of the low frequency (.007) of the apo A-IV(Met_{-8}) allele, these differences were not significant. However, three of six apo A-IV(Met_{-8}) heterozygotes had HDL-cholesterol and apo A-I concentrations below the 10th percentiles of sex-matched controls from the PROCAM population. Also, results from a family study indicate HDL-lowering and LDL-increasing effects of this mutation: in a family with fish-eye disease (Funke et al. 1991*a*) heterozygotes for apo A-IV (Met_{-8}) had 4% lower concentrations of both HDL-cholesterol and apo A-I and had 9% higher concentrations of HDL-cholesterol than did unaffected family members, irrespective of the presence or absence of the fish-eye-disease defect. Among all fish-eye-disease heterozygotes, the homozygote for apo A-IV(Met_{-8}) had the lowest HDL-cholesterol and the highest LDL-cholesterol concentrations. Our finding that only nonsynonymous (but not synonymous) mutations quantitatively affect lipid metabolism is a feature that has also been observed for other apolipoproteins. For instance, population and family studies using polymorphisms in the genes coding for apolipoproteins A-I, B, or E that do not change the primary structure of the encoded gene products yielded controversial results regarding these apolipoproteins' impact on lipid metabolism. By contrast, several nonsynonymous mutations in these genes are associated with changes in lipid metabolism (reviewed in Assmann et al. 1990; McCarthy 1991; Mahley et al. 1990). We thus believe that the observed lipid changes associated with nonsynonymous mutations in the apo A-IV gene are causative and are mediated through an altered primary structure in the encoded proteins—and not through genetic linkage of these polymorphisms to mutations in other genes. This hypothesis is further supported by results from our haplotype studies. Neither the three apo A-IV(Gln_{360})–containing alleles nor the three apo A-IV(Thr_{347})–containing alleles differed by mean values for associated lipid, lipoprotein, or apolipoprotein concentrations. Moreover, the antagonistic effects of the Thr_{347}→Ser and Gln_{360}→His mutations on the concentrations of apo B–containing lipoproteins also does not support the assumption of genetic

Figure 8 Graphic demonstration of mean serum concentrations of lipid, lipoproteins, and apolipoproteins, as a function of the allelic variation at three polymorphic sites in the apo A-IV gene. *Hin*fI refers to the Thr/Ser polymorphism in position 347, *Fnu*4HI to the Gln/His polymorphism in position 360, and del/ins to the four-base VNTR polymorphism in exon 3. Values for triglycerides and Lp(a) are geometric means. $*P < .01$ (Whitney-Mann and Kruskal-Wallace tests), and $**P < .05$ (Whitney-Mann test).

linkage between these polymorphic sites and metabolically relevant mutations in other genes.

The three amino acid substitutions in apo A-IV differ by their localization. The Val_{-8}→Met mutation affects the primary structure of the apo A-IV prepeptide that is intracellularly removed before apo A-IV is secreted from enterocytes as a structural component of chylomicrons or nascent HDL particles (Elshourbagy et al. 1986; Karathanasis et al. 1986). Therefore, it is reasonable to hypothesize that the Val_{-8}→Met substitution in pre-apo A-IV affects the processing of this zymogen to the mature apolipoprotein. Whether this interference affects apo A-IV plasma concentration and/or the composition of apo A-IV–containing particles will be an interesting question. The other two substituted amino acids, Thr_{347} and Gln_{360}, are localized in two adjacent alpha-helices. Alike apo A-I, alpha-helices in apo A-IV are assumed to be involved in lipid binding and in LCAT activation (Anantharamaiah et al. 1990; Weinberg and Jordan 1990). In computer models the Gln_{360}→His substitution in apo A-IV increases the hydrophobicity and the alpha-helical content of apo A-IV (Lohse et al. 1990*a*; Weinberg et al. 1990). However, presently available experimental data on the LCAT-activating properties of apo A-IV(Gln_{360}) and apo A-IV(His_{360}) are controversial. In one study, apo A-IV(His_{360}) was the better LCAT activator (Weinberg et al. 1990); in another study apo A-IV(Gln_{360}) was (Tenkanen et al. 1991). Since apo A-IV(Gln_{360}) alleles can contain either a threonine or a serine in position 347, the two studies may have been biased by the presence of this additional mutation. This explanation and the data presented in the present paper suggest that the Thr_{347}→Ser substitution is of functional relevance.

Effects of the Thr_{347}→Ser substitution on HDL-cholesterol serum concentration were paralleled by effects on the concentration of apo B and LDL. Also, the Val_{-8}→Met and Gln_{360}→His substitutions affected the serum concentration of apo B–containing lipoproteins. At first sight, this finding is somewhat unexpected, because apo A-IV is not a structural component of apo B–containing lipoproteins. However, with two of its properties, apo A-IV may also interact with the metabolism of apo B–containing lipoproteins: (1) Elsewhere apo A-IV has been shown to modulate the activity of lipoprotein lipase, which hydrolyzes VLDL and eventually generates LDL (Goldberg et al. 1990). Structural differences of the apo A-IV isoproteins may thus vary the conversion of VLDL and thereby affect the concentration of LDL. (2) Another interpretation refers to a distinct, apo A-IV–containing HDL particle that has recently been isolated by immunoaffinity chromatography (Steinmetz et al. 1990) and by isotachophoresis (Nowicka et al. 1990). Apo A-IV–containing particles have been shown to promote cholesterol efflux from adipocytes and macrophages (Nowicka et al. 1990; Steinmetz et al. 1990). The mechanism of cholesterol efflux promotion has been controversially discussed. However, several recently published studies indicate that the cholesterol-enriched particle contains apo A-IV, apo A-I, apo D, LCAT, and cholesterol-ester transfer protein and that this particle participates in the transfer of cholesterol esters from HDL to apo B–containing lipoproteins (Francone et al. 1989; Lefevre et al. 1989; Nowicka et al. 1990). The antagonistic effect of the Thr_{347}→Ser substitution on serum concentrations of both LDL-cholesterol and apo B, on the one hand, and on serum concentrations of HDL-cholesterol, on the other hand, could therefore be explained by the hypothesis that the structural variation in apo A-IV affects the regular function and metabolism of this cholesterol-ester transfer complex.

The recently reported association between the rarer allele (i.e., X2) of an *Xmn*I polymorphism that is located approximately 20 kb 5′ of the apo A-IV gene (Karathanasis 1985; Coleman et al. 1986), and familial combined hyperlipidemia (Hayden et al. 1987), as well as the linkage of this polymorphism with familial combined hyperlipidemia in seven Scottish families (Wojciechowski et al. 1991), also point to a role of the apo A-I/C-III/A-IV gene cluster in the metabolism of apo B–containing lipoproteins. Different mechanisms for this association and the ones described in the present paper are likely, because of the absence of linkage between the rare alleles of the nonsynonymous mutations in apo A-IV and the X2 allele. On the basis of our current findings, however, the speculation that the X2 allele may be linked to an as yet unidentified defect in the apo A-IV gene (Wojciechowski et al. 1991) is further supported.

In summary, our results indicate that apo A-IV is a highly polymorphic protein. Genetic variation in this protein contributes to alterations in serum concentrations of both apo A-I– and apo B–containing lipoproteins. Future studies need to unravel the pathomechanisms whereby structural alterations of this apolipoprotein affect lipoprotein metabolism.

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