note on methodology

Restriction isotyping of human apolipoprotein A-IV: rapid typing of known isoforms and detection of a new isoform that deletes a conserved repeat

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Summary Genetic polymorphisms of apolipoprotein A-IV (apoA-IV) have been detected by isoelectric focusing of serum proteins. Because genetic variation in apoA-IV has significant effects on lipid risk factors, we used restriction enzyme isoform genotyping (restriction isotyping) to determine apoA-IV isoform genotypes at the DNA level for a large population (n = 509). In contrast to isoelectric focusing methods, restriction isotyping relies on nucleotide differences, enabling unambiguous typing of known isoforms and detection of new alleles that mimic other isoforms with shared charge properties. To determine genotypes for the common A-IV-1 isoform (Gln at aa position 360) and A-IV-2 isoform (360His), we used a mismatched primer for polymerase chain reaction (PCR) to introduce a restriction site (*Pvu*II) that distinguishes each isoform. Using a portion of the same PCR reaction, we used *Hinf*I to distinguish isoforms with Thr at position 347 (347Thr) versus Ser (347Ser). In surveys for these common genotypes, we detected heterozygotes for an allele with an insertion of 12 bp. Nucleotide sequencing showed that this allele is identical to the A-IV-0 isoform that inserts a hydrophilic repeat (Glu Gln Gln Gln) in a conserved region near the carboxy terminus. In addition, we discovered a new allele with a 12 bp deletion that removes a repeat (Glu Gln Gln Gln) from the same region. Nucleotide sequencing showed that this allele removes an acidic charge relative to A-IV-1, so we have named this isoform A-IV-2*. This isoform has not been discovered at the protein level, perhaps due to shared charge properties with A-IV-2 isoforms. —**Hixson, J. E., and P. K. Powers.** Restriction isotyping of human apolipoprotein A-IV: rapid typing of known isoforms and detection of a new isoform that deletes a conserved repeat. *J. Lipid Res.* 1991. **32:** 1529–1535.

Supplementary key words gene amplification • polymerase chain reaction

Human apolipoprotein A-IV (apoA-IV) is produced by intestinal epithelial cells and is a protein constituent of intestinal-derived triglyceride-rich chylomicrons (1). In plasma, apoA-IV is also found in high density lipoproteins (HDL) and as "free" apoA-IV that is not associated with lipoproteins. While its precise function in lipid metabolism is not yet known, several properties of apoA-IV suggest an important role in reverse cholesterol metabolism. ApoA-IV is an activator of lecithin:cholesterol acyltransferase (LCAT) (2), and may affect activities of lipoprotein lipase (LPL) (3) by interaction with apoC-II (4). In vitro studies have shown that apoA-IV promotes cholesterol efflux from cultured fibroblasts (5) and adipose cells (6) via cellular receptors (7).

ApoA-IV is synthesized with a 20 amino acid signal peptide that is cotranslationally cleaved to produce a mature protein consisting mainly of 22 amino acid (aa) tandem repeats that form amphipathic α-helices (8, 9). Several charge polymorphisms (isoforms) for apoA-IV have been detected and subsequently sequenced after amplification of genomic sequences (10, 11). The two major isoforms are A-IV-1 and A-IV-2 (12, 13) with a G to T nucleotide change that substitutes His for Gln at position 360 (10). Other rare isoforms have been detected including A-IV-0 with an insertion of 4 aa at position 361 and A-IV-3 with a Glu to Lys substitution at position 230 (11). In addition, nucleotide sequence comparisons detected an A to T change that substitutes Ser for Thr at position 347 (14).

Studies of human populations have detected significant effects of the A-IV-2 isoform on lipid risk factors of atherosclerosis (15, 16). In vitro studies of purified isoforms indicate that the A-IV-2 isoform has distinct structural properties associated with higher affinities for phospholipid surfaces and increased efficiency of LCAT activation (17). In addition, a recent study has detected linkage of familial combined hyperlipidemia with a DNA polymorphism in the apoA-I-C-III-A-IV gene cluster, perhaps reflecting effects of apoA-IV genotype on this disorder (18). We have been motivated by these reports to develop rapid and accurate methods of typing apoA-IV polymorphisms for large-scale studies of human populations. We recently described a technique called restriction isotyping (restriction enzyme isoform genotyping) to distinguish isoforms at the DNA level (19). In this report, we describe the use of restriction isotyping to type apoA-IV isoforms in hepatic DNA from a population-based study entitled "Pathobiological Determinants of Atherosclerosis in Youth" (PDAY) (20).

MATERIALS AND METHODS

Extraction of DNA from liver samples

Liver samples were collected at autopsy from male victims of violent death (accidents, homicide, suicide; 15–34 years of age) as part of the PDAY multicenter study (n = 509). The tissue samples were immediately frozen at −80°C, and shipped on dry ice for further processing.

Abbreviations: apoA-IV, apolipoprotein A-IV; bp, base pairs; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LPL, lipoprotein lipase; aa, amino acid.

High molecular weight DNA was isolated from human liver samples using a modification of a simple salting out procedure (21). Briefly, 0.5 g of minced tissue was Dounce-homogenized and centrifuged to collect the nuclear pellet. The pellet was resuspended in 1 ml of lysis buffer (5% SDS, 50 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl) containing 100 μg/ml proteinase K, and digested overnight at 55°C. A saturated solution of NaCl was then added to precipitate the cellular proteins, and nuclear DNA was precipitated from the supernatant by addition of ethanol. The DNA was collected, washed with 70% ethanol, lyophilized, and resuspended in 1 ml H_2O for subsequent analyses.

Restriction isotyping of apoA-IV isoforms

ApoA-IV sequences were amplified from hepatic DNA samples using PCR (22) and oligonucleotide primers constructed from published nucleotide sequences in exon 3 (23, 24). The forward primer used for restriction isotyping of A-IV-1, A-IV-2, 347Thr, and 347Ser was F1 (5'-GCCCTGGTGCAGCAGATGGAACAGCTCAGG-3') and the reverse primer with a mismatch (underlined) was R1 (5'-CATCTGCACCTGCTCCTGCTGCTGCTC-CAG-3'). The forward primer used for amplification of insertion/deletion alleles (A-IV-0 and A-IV-2*) was F2 (5'-CCTGGAGAAGGACCTGAGGGACAAGG-3') and the reverse primer was R2 (5'-CAGCTCTCCAAAGG-GGCCAGCATCTGCAC-3'). In addition to the buffer and nucleotide components described by the supplier of *Taq* polymerase (Perkin Elmer Cetus), each amplification reaction contained 0.5 μg of hepatic DNA, 1 pmol/μl of each primer 10% dimethyl sulfoxide, and 0.025 units/μl of *Taq* polymerase in a final volume of 20 μl. Each reaction mixture was heated at 95°C for denaturation (5 min), and subjected to 30 cycles of amplification by primer annealing (65°C for 1 min), extension (70°C for 2 min), and denaturation (95°C for 1 min).

After amplification, each PCR reaction was split into two samples for digestion with restriction enzymes. In one sample, 5 units of PvuII (Bethesda Research Laboratories) were added to distinguish A-IV-1 and A-IV-2 alleles. In the other sample, 5 units of HinfI (New England Biolabs) were added to distinguish 347Ser and 347Thr alleles. In both cases, the enzymes were added directly to each reaction mixture for digestion (>3 h at 37°C). Each reaction mixture was loaded onto 8% polyacrylamide non-denaturing gels (1 mm thick × 10 cm long) and electrophoresed for 3 h under constant current (45 mA). After electrophoresis, the gel was treated with ethidium bromide (0.2 mg/l) for 10 min and DNA fragments were visualized by UV illumination. The sizes of restriction fragments were estimated by comparison with PstI-digested lambda bacteriophage DNA.

Asymmetric amplification and direct nucleotide sequencing of apoA-IV isoforms

Double-stranded PCR reactions were performed as described above with equimolar amounts of primers F1 and R2 in 100 μl reaction volumes, and the amplified products were eluted from 8% polyacrylamide gels to remove excess primers and dNTPs. Double-stranded PCR products (50 ng) were used for asymmetric PCR to produce single-stranded DNA with primer R2 (1 pmol/μl) and limiting amounts of primer F1 (0.01 pmol/μl) in a total reaction volume of 100 μl (25). PCR conditions were identical to those for double-stranded PCR, except the number of cycles was increased to 35. Prior to sequencing of single-stranded products, excess primers and dNTPs were removed by filtration with Centricon-30 microfilters. Single-stranded products of asymmetric PCR were sequenced by the chain termination method with modified T7 polymerase (US Biochemicals) and [^{35}S]dATP (26). The internal primer used for sequencing was F2 located downstream from the limiting primer for asymmetric PCR (F1).

RESULTS AND DISCUSSION

Restriction isotyping of A-IV-1 and A-IV-2 isoforms with a mismatched primer

Fig. 1 shows nucleotide sequences for the common A-IV-1 and A-IV-2 isoforms as recently determined by Lohse and coworkers (10). Although the nucleotide changes responsible for the Gln to His substitution alter cleavage sites for Fnu4HI and BbvI, these restriction enzymes are expensive and cut frequently in apoA-IV sequences (27). We have developed an alternate strategy using restriction isotyping after amplification with a mismatched primer (28). **Fig. 2a** shows results of restriction isotyping of the A-IV-1 and A-IV-2 isoforms. Lane A shows amplification of hepatic DNA using a forward primer (F1, see Fig. 1) and the mismatched reverse primer (R1) that produced a 222 bp fragment. Lane B shows digestion of this PCR product with PvuII that resulted in a 192 bp cleavage product. This individual was classified as an A-IV-1/A-IV-1 homozygote bearing both alleles that are cut with PvuII. Lane C shows results of PvuII digestion of DNA from an A-IV-1/A-IV-2 heterozygote. The AIV-2 allele was distinguished by the 222 bp fragment corresponding to the absence of the PvuII site. These results were confirmed using Fnu4H1 (cuts GCNGC) which is not affected by the mismatch introduced by primer R1. Lane E of Fig. 2a shows amplification with primers F1 and R1 (222 bp fragment), and lane F shows Fnu4HI digestion of amplified DNA from this A-IV-1/A-IV-1 homozygote (cleavage product of 190 bp). Lane G shows Fnu4HI digestion of amplified DNA from

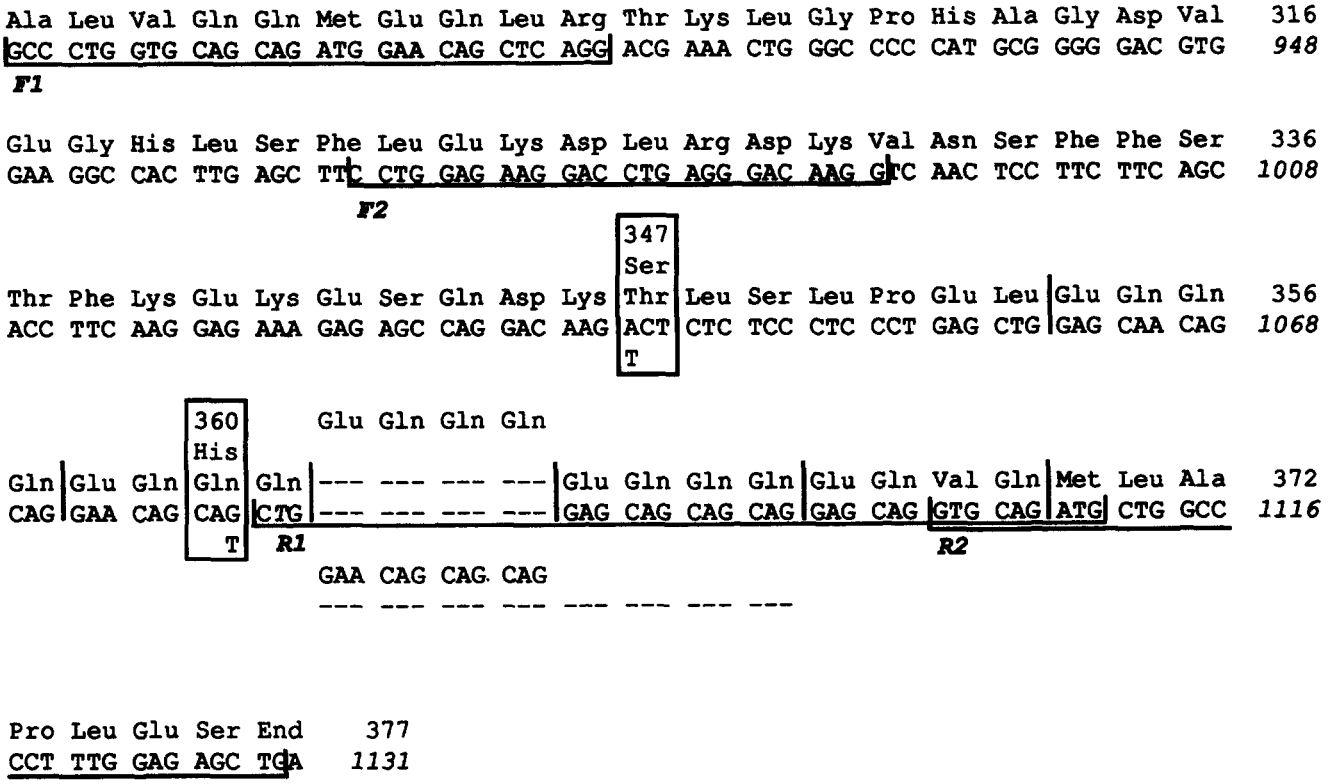

Fig. 1. Sequences of apoA-IV isoforms and PCR primers. Amino acid sequences (numbered from the mature apoA-IV) and nucleotide sequences (numbered according to aa sequence) are presented for the common A-IV-1 isoform (9). The A to T change for the 347Ser allele, and the G to T change in codon 360 for the A-IV-2 isoform are aligned and boxed along the sequence. The inserted sequences for apoA-IV-0 are aligned on the second lines above and below the A-IV-1 sequence. The 12 nucleotide deletion (shown by dashes) for the apoA-IV-2* isoform is aligned on the third line below the sequence. The amplification primers (F1 and F2, reverse complements of R1 and R2) are shown in brackets, and the mismatched T (replaces A in the wild-type apoA-IV sequence) in primer R1 is italicized. Vertical lines show the 4 aa hydrophilic repeats (Glu Gln X Gln) in the carboxy terminus region of apoA-IV.

an A-IV-1/A-IV-2 heterozygote that produced an additional 199 bp fragment reflecting the absence of the polymorphic Fnu4HI site and cleavage at another Fnu4HI site located in the R1 primer (nucleotide positions 1086–1091, Fig. 1).

To examine the nucleotide substitutions that were detected by restriction isotyping, we used asymmetric PCR amplification to produce single-stranded DNA templates for direct nucleotide sequencing (25). Fig. 2b shows sequencing gels for an A-IV-1/A-IV-1 homozygote (gel A) and an A-IV-1/A-IV-2 heterozygote (gel B) that were previously typed by restriction isotyping. We detected a G at position 1080 encoding Gln in the A-IV-1/A-IV-1 homozygote, and both G (Gln) and T (His) in the A-IV-1/A-IV-2 heterozygote.

After development and verification of restriction isotyping, we determined A-IV-1 and A-IV-2 genotypes for 509 PDAY subjects and used allele counting to determine the frequencies of each isoform. The frequency of the A-IV-1 isoform was 0.955 and the frequency of the A-IV-2 allele was 0.034. Relative to results from other studies, these frequencies fall between those for American whites (0.909 for apoA-IV-1, 0.088 for apoA-IV-2) and blacks (0.961 for apoA-IV-1, 0.035 for apoA-IV-2) (29), the racial groups represented in the PDAY study.

Restriction isotyping of 347Ser and 347Thr isoforms for apoA-IV

Using nucleotide sequencing after PCR amplification, Boerwinkle, Visvikis, and Chan (14) detected an A to T polymorphism that results in Thr to Ser substitution at amino acid position 347 (see Fig. 1). This substitution does not alter apoA-IV charge properties and has not been detected by isoelectric focusing methods. Fig. 2c shows the results of restriction isotyping to distinguish 347Thr and 347Ser isoforms by cleavage with HinfI (cuts GANTC). For 347Ser/347Ser homozygote (lane A), the PCR products (222 bp) remained uncut due to the absence of the HinfI site. For the 347Thr/347Thr homozygote (lane B), 150 bp fragments were produced from cleavage by HinfI. Lane C shows a 347Ser/347Thr heterozygote with 222 and 150 bp fragments corresponding to the presence of both alleles. The sequencing gels in Fig. 2b show the A to T nucleotide substitutions that underlie these isoform genotypes. The frequency of the 347Thr allele was 0.856 in 509 PDAY cases, and the fre-

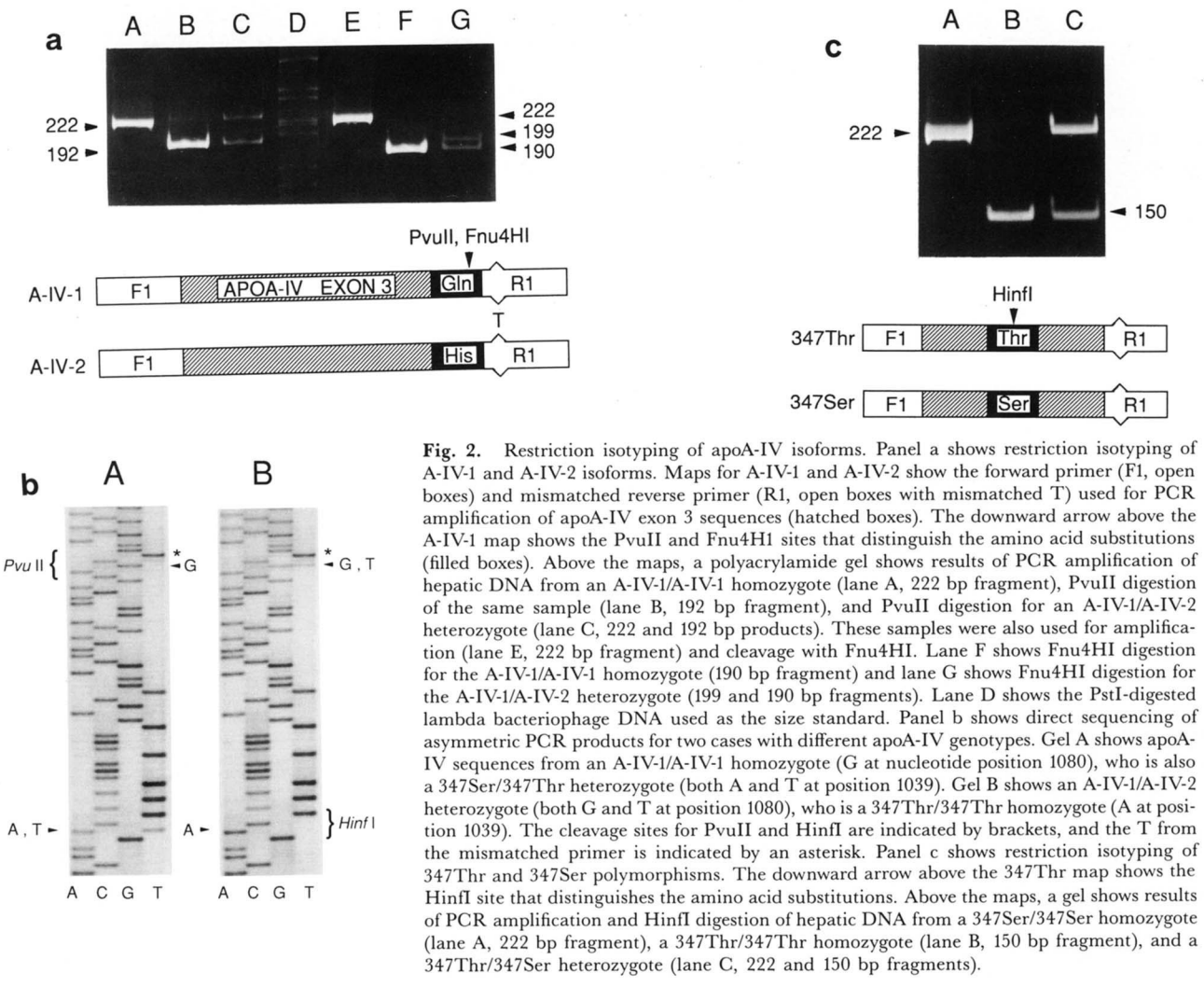

Fig. 2. Restriction isotyping of apoA-IV isoforms. Panel a shows restriction isotyping of A-IV-1 and A-IV-2 isoforms. Maps for A-IV-1 and A-IV-2 show the forward primer (F1, open boxes) and mismatched reverse primer (R1, open boxes with mismatched T) used for PCR amplification of apoA-IV exon 3 sequences (hatched boxes). The downward arrow above the A-IV-1 map shows the PvuII and Fnu4H1 sites that distinguish the amino acid substitutions (filled boxes). Above the maps, a polyacrylamide gel shows results of PCR amplification of hepatic DNA from an A-IV-1/A-IV-1 homozygote (lane A, 222 bp fragment), PvuII digestion of the same sample (lane B, 192 bp fragment), and PvuII digestion for an A-IV-1/A-IV-2 heterozygote (lane C, 222 and 192 bp products). These samples were also used for amplification (lane E, 222 bp fragment) and cleavage with Fnu4HI. Lane F shows Fnu4HI digestion for the A-IV-1/A-IV-1 homozygote (190 bp fragment) and lane G shows Fnu4HI digestion for the A-IV-1/A-IV-2 heterozygote (199 and 190 bp fragments). Lane D shows the PstI-digested lambda bacteriophage DNA used as the size standard. Panel b shows direct sequencing of asymmetric PCR products for two cases with different apoA-IV genotypes. Gel A shows apoA-IV sequences from an A-IV-1/A-IV-1 homozygote (G at nucleotide position 1080), who is also a 347Ser/347Thr heterozygote (both A and T at position 1039). Gel B shows an A-IV-1/A-IV-2 heterozygote (both G and T at position 1080), who is a 347Thr/347Thr homozygote (A at position 1039). The cleavage sites for PvuII and HinfI are indicated by brackets, and the T from the mismatched primer is indicated by an asterisk. Panel c shows restriction isotyping of 347Thr and 347Ser polymorphisms. The downward arrow above the 347Thr map shows the HinfI site that distinguishes the amino acid substitutions. Above the maps, a gel shows results of PCR amplification and HinfI digestion of hepatic DNA from a 347Ser/347Ser homozygote (lane A, 222 bp fragment), a 347Thr/347Thr homozygote (lane B, 150 bp fragment), and a 347Thr/347Ser heterozygote (lane C, 222 and 150 bp fragments).

quency of the 347Ser allele was 0.144. In 51 unrelated individuals, Boerwinkle et al. (14) found frequencies of 0.784 and 0.216 for 347Thr and 347Ser, respectively.

Restriction isotyping of A-IV-0 isoforms in PDAY cases

In addition to the common apoA-IV alleles, we also detected heterozygotes for a rare allele with a larger PCR product. **Fig. 3a** (lane A) shows results of PCR amplification (primers F2 and R2, Fig. 1) from a heterozygote that possessed the larger allele (177 bp), as well as the normal allele (165 bp). For comparison, Fig. 3a (lane B) also shows PCR ampification of an A-IV-1/A-IV-1 homozygote. To determine the basis for allele size differences, we gel-purified both sizes of PCR products from a heterozygote for asymmetric amplification and nucleotide sequencing (Fig. 3b). The larger allele contained an in-frame 12 bp insertion encoding Glu Gln Gln Gln between aa positions 361 and 362 in the normal allele. Sequence comparisons from this and other heterozygotes in the PDAY survey showed that this allele corresponded to the A-IV-0 isoform that contains one additional acidic charge (Glu) relative to the A-IV-1 allele (11). The frequency of the A-IV-0 isoform as determined by restriction isotyping was 0.010 in 509 PDAY subjects (total of 10 A-IV-0/A-IV-1 heterozygotes) compared to a frequency of 0.002 for a large Dutch population (30).

Restriction isotyping detects a new allele that deletes 4 aa of apoA-IV

In our survey, we also detected a heterozygote for a rare allele with a smaller PCR product. Fig. 3a (lane C) shows the PCR products of this heterozygote that carried the smaller allele (153 bp) and the normal allele (165 bp). To determine the basis for this size difference, we gel-purified both sizes of PCR products from the heterozygote for asymmetric amplification and nucleotide sequencing. Fig. 3c shows the sequence of both the normal and smaller alleles. The smaller allele contained a 12 bp deletion that does not alter the reading frame, but removes Glu Gln

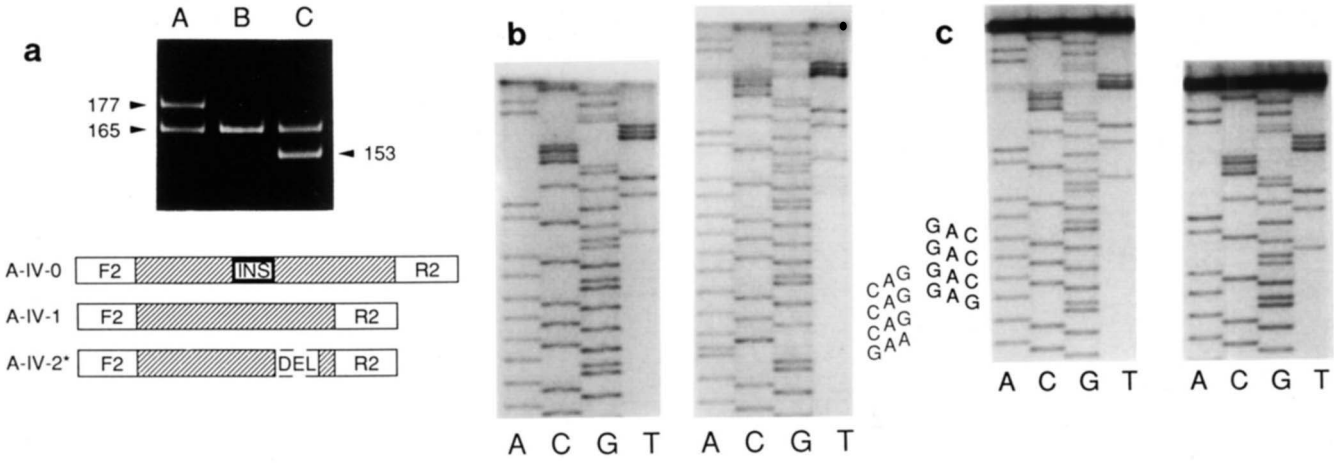

Fig. 3. PCR amplification and direct sequencing of A-IV-0 and A-IV-2* insertion/deletion isoforms. Panel a shows amplification products for A-IV-0, A-IV-1, and A-IV-2* isoforms. Maps for each isoform show the primers (F2 and R2, open boxes) used for PCR amplification of apoA-IV sequences (hatched boxes). The insertion in A-IV-0 (filled box labeled INS) and the deletion in A-IV-2* (open box with dashed line labeled DEL) are shown relative to A-IV-1. Above the maps, an ethidium bromide-stained gel shows results of PCR amplification of an A-IV-0/A-IV-1 heterozygote (lane A, 177 and 165 bp fragments), an A-IV-1/A-IV-1 homozygote (lane B, 165 bp fragment), and an A-IV-2*/A-IV-1 heterozygote (lane C, 165 and 153 bp fragments). Panel b shows direct sequencing of gel-separated A-IV-1 and A-IV-0 alleles from a single individual. The gel on the left shows the sequence of the gel-purified 165 bp PCR product (A-IV-1 isoform). The gel on the right shows the sequence for the 177 bp PCR product (A-IV-0) from the same individual. The 12 bp that are inserted in the A-IV-0 isoform are shown adjacent to the gel on the right. Panel c shows direct sequencing of gel-separated A-IV-1 and A-IV-2* alleles from a single individual. The gel on the left shows the sequence for the gel purified 165 bp PCR product (A-IV-1). The gel on the right shows the sequence for the 153 bp PCR product (A-IV-2*) from the same individual. The 12 bp that are deleted from the A-IV-2* isoform are shown adjacent to the gel on the left (A-IV-1).

Gln Gln relative to A-IV-1 (positions 362–365, Fig. 1). This deletion removes one acidic charge (Glu) from the A-IV-1 isoform, resulting in a more basic protein. Because this charge difference corresponds to the more basic apoA-IV-2 isoform, we have called this deletion allele apoA-IV-2*. Thus far in our PDAY survey of 509 cases, we have detected one heterozygote giving an allele frequency of 0.001 for this new apoA-IV-2* allele. Because this and other isoforms (e.g., 347 Ser) may not possess distinct charge properties, apoA-IV may be more polymorphic than previously believed based on results of isoelectric focusing surveys.

Size variation in human apoA-IV coding sequences

In addition to the amphipathic 22 aa repeats that span apoA-IV, comparisons of sequences from human (8), rat (31), and mouse (32) revealed a small region of highly conserved hydrophilic repeats (Glu Gln X Gln) at the carboxy terminus of apoA-IV. This sequence is repeated four times in human and mouse apoA-IV, and three times in the rat. Nucleotide sequencing of apoA-IV isoforms has shown that differences in repeat numbers also occur within human populations. The A-IV-0 allele adds 12 nucleotides that encode an additional hydrophilic repeat (Figs. 3a and 3b). The insertion is a duplication of repeat number 2 (Fig. 1, aa positions 358–361), resulting in a total of five repeats in the A-IV-0 allele (11). The A-IV-2* allele deletes 12 nucleotides (Figs. 3a and 3c) that encode the third hydrophilic repeat (Fig. 1, aa positions 362–365) in the A-IV-1 sequence, resulting in a total of three repeats. Although strong conservation between species indicates a functional role for these repeats, we do not yet know the physiological effects of these insertion/deletion alleles. However, detection of these alleles demonstrates how restriction isotyping can facilitate genetic analysis by identification of "natural" mutations for future studies of apoA-IV structure and function

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REFERENCES

1. Green, P. H. R., R. M. Glickman, J. W. Riley, and E. Quinet. 1980. Human apolipoprotein A-IV. Intestinal origin and distribution in plasma. *J. Clin. Invest.* **65:** 911–919.
2. Steinmetz, A., and G. Utermann. 1985. Activation of lecithin:cholesterol acyltransferase by human apolipoprotein A-IV. *J. Biol. Chem.* **260:** 2258–2264.
3. Scheraldi, C. A., C. L. Bisgaier, H. N. Ginsberg, and I. J. Goldberg. 1988. Modulator role of apolipoprotein A-IV in the activation of lipoprotein lipase. *Circulation (Suppl. II)* **78:** 199.
4. Goldberg, I. J., C. A. Scheraldi, L. K. Yakoub, U. Saxena, and C. L. Bisgaier. 1990. Lipoprotein apoC-II activation of lipoprotein lipase. Modulation of apolipoprotein A-IV. *J. Biol. Chem.* **265:** 4266–4272.
5. Stein, O., Y. Stein, M. Lefevre, and P. S. Roheim. 1986. The role of apolipoprotein A-IV in reverse cholesterol transport studied with cultured cells and liposomes derived from an ether analog of phosphatidylcholine. *Biochim. Biophys. Acta.* **878:** 7–13.
6. Steinmetz, A., R. Barbaras, N. Ghalim, V. Clavey, J-C. Fruchart, and G. Ailhaud. 1990. Human apolipoprotein A-IV binds to apolipoprotein A-I/A-II receptor sites and promotes cholesterol efflux from adipose cells. *J. Biol. Chem.* **265:** 7859–7863.
7. Dvorin, E., N. L. Gorder, D. M. Benson, and A. M. Gotto, Jr. 1986. Apolipoprotein A-IV. A determinant for binding and uptake of high density lipoproteins by rat hepatocytes. *J. Biol. Chem.* **261:** 15714–15718.
8. Elshourbagy, N. A., D. W. Walker, M. S. Boguski, J. I. Gordon, and J. M. Taylor. 1986. The nucleotide and derived amino acid sequence of human apolipoprotein A-IV mRNA and the close linkage of its gene to the genes of apolipoprotein A-I and C-III. *J. Biol. Chem.* **261:** 1998–2002.
9. Karathanasis, S. K., I. Yunis, and V. I. Zannis. 1986. Structure, evolution and tissue-specific synthesis of human apolipoprotein A-IV. *Biochemistry.* **25:** 3962–3970.
10. Lohse, P., M. R. Kindt, D. J. Rader, and H. B. Brewer, Jr. 1990. Genetic polymorphism of human plasma apolipoprotein A-IV is due to nucleotide substitutions in the apolipoprotein A-IV gene. *J. Biol. Chem.* **265:** 10061–10064.
11. Lohse, P., M. R. Kindt, D. J. Rader, and H. B. Brewer, Jr. 1990. Human plasma apolipoproteins A-IV-O and A-IV-3. Molecular basis for two rare variants of apolipoprotein A-IV-1. *J. Biol. Chem.* **265:** 12734–12739.
12. Menzel, H. J., P. M. Kovary, and G. Assman. 1982. Apolipoprotein A-IV polymorphism in man. *Hum. Genet.* **62:** 349–352.
13. Utermann, G., G. Feussner, G. Franceschini, and H. J. Steinmetz. 1982. Genetic variants of the group A apolipoproteins. Rapid methods for screening and characterization without ultracentrifugation. *J. Biol. Chem.* **257:** 501–507.
14. Boerwinkle, E., S. Visvikis, and L. Chan. 1990. Two polymorphisms for amino acid substitutions in the APOA4 gene. *Nucleic Acids Res.* **18:** 4966.
15. Menzel, H. J., E. Boerwinkle, J. Schrangl-Will, and G. Utermann. 1988. Human apolipoprotein A-IV polymorphism: frequency and effect on lipid and lipoprotein levels. *Hum. Genet.* **79:** 368–372.
16. Menzel, H. J., G. Sigurdsson, E. Boerwinkle, S. Schrangl-Will, H. Dieplinger, and G. Utermann. 1990. Frequency and effect of human apolipoprotein A-IV polymorphism on lipid and lipoprotein levels in an Icelandic population. *Hum. Genet.* **84:** 344–346.
17. Weinberg, R. B., M. K. Jordan, and A. Steinmetz. 1990. Distinctive structure and function of human apolipoprotein variant apoA-IV-2. *J. Biol. Chem.* **265:** 18372–18378.
18. Wojciechowski, A. P. P., M. Farrall., P. Cullen, T. M. E. Wilson, J. D. Bayliss, B. Farren, B. A. Griffin, M. J. Caslake, C. J. Packard, J. Shepherd, R. Thakker, and J. Scott. 1990. Familial combined hyperlipidaemia linked to the apolipoprotein A-I–C-III–A-IV gene cluster on chromosome 11q23–q24. *Nature.* **349:** 161–164.
19. Hixson, J. E., and D. T. Vernier. 1990. Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with *Hha*I. *J. Lipid Res.* **31:** 545–548.
20. Pathobiological Determinants of Atherosclerosis in Youth Research Group. 1990. Relationship of atherosclerosis in young men to serum lipoprotein cholesterol concentrations and smoking. A preliminary report from the PDAY Research group. *JAMA.* **264:** 3018–3024.
21. Miller, S. A., D. D. Dykes, and H. F. Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* **16:** 1215.
22. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science.* **230:** 1350–1354.
23. Karathanasis, S. K., P. Oettgen, I. A. Haddad, and S. E. Antonarakis, 1986. Structure, evolution, and polymorphisms of the human apolipoprotein A4 gene (*APOA4*). *Proc. Natl. Acad. Sci. USA.* **83:** 8457–8461.
24. Elshourbagy, N. A., D. W. Walker, Y-K. Paik, M. S. Boguski, M. Freeman, J. I. Gordon, and J. M. Taylor. 1987. Structure and expression of the human apolipoprotein A-IV gene. *J. Biol. Chem.* **262:** 7973–7981.
25. Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo, F. X. Villablanca, and A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA.* **86:** 6196–6200.
26. Biggin, M. D., J. Gibson, and G. F. Hong. 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA.* **80:** 963–3965.
27. Tenkanen, H. 1991. Genotyping of apolipoprotein A-IV by digestion of amplified DNA with restriction endonuclease Fnu4HI: use of a tailored primer to abolish addition recognition sites during the gene amplification. *J. Lipid Res.* **32:** 545–549.
28. Haliassos, A., J. C. Chomel, L. Tesson, M. Baudis, J. Kruh, J. C. Kaplan, and A. Kitzis. 1989. Modification of enzymatically amplified DNA for the detection of point mutations. *Nucleic Acids Res.* **17:** 3606.
29. Kamboh, M. I., and R. E. Ferrell. 1987. Genetic studies of human apolipoproteins. I. Polymorphism of apolipoprotein A-IV. *Am. J. Hum. Genet.* **41:** 119–127.
30. de Knijff, P., M. Rosseneu, U. Beisiegel, W. de Keersgieter, R. R. Frants, and L. M. Havekes. 1988. Apolipoprotein A-IV polymorphism and its effect on plasma lipid and apoli-

poprotein concentrations. *J. Lipid Res.* **29:** 1621–1627.
31. Boguski, M. S., N. Elshourbagy, J. M. Taylor, and J. I. Gordon. 1984. Rat apolipoprotein A-IV contains 13 tandem repetitions of a 22-amino acid segment with amphipathic helical potential. *Proc. Natl. Acad. Sci. USA.* **81:** 5021–5025.
32. Williams, S. C., S. M. Bruckheimer, A. J. Lusis, R. C. LeBouef, and A. J. Kinniburgh. 1986. Mouse apolipoprotein A-IV gene: nucleotide sequence and induction by a high-lipid diet. *Mol. Cell. Biol.* **6:** 3807–3814.