Characterization of a new apolipoprotein E5 variant detected in two French-Canadian subjects

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Abstract We have found a novel apoE5 mutation, using isoelectric focusing (IEF), in two apparently unrelated French-Canadian subjects. Co-dominant inheritance was demonstrated in the family of the first proband, a healthy male subject. The presence of the apoE5 form was not associated with lipid abnormalities or cardiovascular disease in this family. The second proband was a hyperlipidemic female patient suffering from angina, with no informative relatives available for study. In both individuals, monoclonal antibody studies demonstrated that the mutation was associated with the loss of two overlapping epitopes at the amino terminus of the protein. Cysteamine treatment of the very low density lipoproteins indicated that the mutant apoE contained only one cysteine residue, suggesting that apoE3 was the parental form. Two-dimensional electrophoresis suggested that the mutated protein had a slightly lower molecular weight (by 1-2 kDa). However, DNA sequencing of the third exon of the apoE gene in both probands revealed a single G to A substitution at the 48th nucleotide, changing the amino acid at position 13 from glutamic acid to lysine. These results were confirmed by oligo melting experiments with allele-specific probes in relatives of the probands. The study of this apoE variant should provide additional insight into the structure-function relationship of apoE. —**Mailly, F., C-f. Xu, M. Xhignesse, S. Lussier-Cacan, P. J. Talmud, J. Davignon, S. E. Humphries, and A. C. Nestruck.** Characterization of a new apolipoprotein E5 variant detected in two French-Canadian subjects. *J. Lipid Res.* 1991. **32:** 613-620.

Supplementary key words DNA sequence • oligonucleotide probes • monoclonal antibodies • epitope loss

Human apolipoprotein E (apoE) is a polymorphic glycoprotein involved in the transport and metabolism of both cholesterol and triglycerides (1, 2). In addition to contributing to the structure and stability of lipoproteins, apoE mediates the clearance of these complexes from plasma through specific binding to cell surface receptors. Genetic variation at the apoE gene locus underlies the protein polymorphisms, giving rise to three common isoforms. These differ by single cysteine-arginine interchanges at amino acids 112 and 158 of the polypeptide chain and can be resolved by isoelectric focusing (3-5). More recently, methods have been developed that allow the direct detection of the underlying nucleotide substitutions through the use of the polymerase chain reaction (PCR) in combination with allele-specific oligonucleotides (ASO) or digestion with the restriction enzyme *Hha*I (6-8).

Variation at these amino acid residues affects cholesterol homeostasis (reviewed in reference 9). Normolipidemic subjects bearing the E4 isoform have higher mean plasma cholesterol than individuals with the common E3 isoform, while those with the E2 isoform have lower mean plasma cholesterol. Furthermore, the presence of the apoE2 protein increases the risk of hyperlipidemia (10, 11). The basis for this is thought to be the reduced ability of the apoE2 variant to bind to the LDL receptor, which may delay the clearance of triglyceride-rich lipoproteins (12, 13). In the presence of compounding genetic or environmental factors, apoE2 homozygosity may result in type III hyperlipidemia, characterized by a marked accumulation of abnormal chylomicron remnants and intermediate density lipoproteins (14, 15).

In addition, a number of rare apoE variants have been identified which are associated with hyperlipidemia and heart disease (16-22). Several of these mutations are in or near the putative receptor binding domain (amino acids 140-150) (23, 24) and display reduced binding affinity in vitro (16, 19-22). However, the presence of a positively charged residue at position 158, outside this proposed region, appears to be critical for maintaining the tertiary structure of this region, as shown by the severely defective binding of the apoE2 isoform (25).

Abbreviations: apo, apolipoprotein; HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; ASO, allele-specific oligonucleotides; PCR, polymerase chain reaction; MAbs, monoclonal antibodies; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing; PBS, phosphate-buffered saline.

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It has been suggested recently that apoE may be implicated in processes other than plasma lipid transport (reviewed in reference 26). These include lipid transport and redistribution in the brain and regulation of growth or differentiation in several cell types. These functions are not yet understood completely and the structural relationships involved have not been well defined. However, it is likely that domains other than the receptor binding area may be involved. One approach to study these domains is to identify and characterize naturally occurring variants of apoE and to investigate the biological impact of the mutations.

We have identified an abnormal band by IEF in delipidated very low density lipoproteins (VLDL) from two subjects. We show this protein to be a previously undescribed apoE mutant by characterizing the mutation at the gene level and discuss its implications with regards to apoE function.

MATERIALS AND METHODS

Subjects

The propositus from the first family ("G" family) was identified through a large screening project aimed at examining lipid levels and genetic variation in a highly selected healthy population where relatively little admixture had taken place. The subject was a 41-year-old male white-collar worker with normal lipid levels. There was no family history of heart disease but the occurrence of several cerebrovascular accidents and brain tumors in previous generations was reported. The second proband was the 52-year-old wife of a patient with familial hypercholesterolemia attending the lipid clinic. She had elevated cholesterol and triglyceride levels and suffered from angina (**Table 1**).

Protein analysis

Plasma was obtained from venous blood after a 12-h fast. VLDL isolated by ultracentrifugation were delipidated sequentially with acetone–ethanol 1:1 (twice) and diethyl ether. VLDL apoproteins were resuspended in 0.01 M Tris-HCl, pH 8.6, 8.0 M urea, and 0.001 M dithiothreitol. Isoelectric focusing was performed according to the method of Bouthillier, Sing, and Davignon (4). Detection of the separated apoE isoforms was achieved with protein staining (Coomassie Blue G-250) or with a two-step immunoreaction. After IEF, the gel was cut longitudinally and the proteins from one half of the gel were transferred electrophoretically to a nylon membrane (Hybond-N, Amersham) in a Bio-Rad Trans-Blot cell for 2 h in 0.025 M Tris-HCl and 0.15 M glycine, pH 8.3. After a 1-h blocking step with 10% BSA (Fraction V, Sigma) in PBS buffer, the membrane was incubated for 2 h with one of three mouse anti-apoE monoclonal antibodies (kind gift from Drs. Ross W. Milne and Yves L. Marcel), diluted 1:10,000 in PBS, 3% BSA. The epitopes for antibodies 6C5 and 7C9 mapped to the first 13 amino acids of the polypeptide chain and the epitope for antibody 1D7 to the receptor-binding domain (amino acids 140–160) (27). The membrane was then washed and further incubated (in the same buffer) for 1 h with goat anti-mouse antibodies labeled with ^{125}I, as described previously (28). All incubations were carried out at 37°C. Autoradiography was for 24–48 h.

Cysteamine modification of apoE isoforms was carried out as described previously (3) as were neuraminidase treatments (4).

Two-dimensional analysis of apoE isoforms was achieved on Pharmacia 4–30% acrylamide gradient gels in a Pharmacia GE-2/4 apparatus. The first dimension gel rod was laid on top of the vertical acrylamide gel, sealed with 1% agarose, and electrophoresed in Tris-borate, SDS buffer at 150 V for 3 h as described in Pharmacia Polyacrylamide Gel Electrophoresis handbook, 1984.

DNA preparation

DNA was obtained from frozen whole blood by the Triton X-100 lysis method (29).

Oligonucleotide synthesis

Oligonucleotides were synthesized on a Pharmacia Gene assembler (Pharmacia, Sweden). Purification was performed using a NAP-10 column (Pharmacia) and NENSORB PREP column (Du Pont). The sequences of all oligonucleotides used are presented in **Table 2**. The amplification primers were designed to flank the third exon of the apoE gene and synthetic linkers containing ScaI and EcoRI restriction sites, respectively, were added

TABLE 1. Lipid levels of the two probands

Proband	Sex	Age	TCHOL	LDL-C	LDL-B	HDL-C	TG
		yr			*mg/dl*		
Proband 1	M	41	220	154	127	42	76
Proband 2	F	52	331	235	187	44	164

TCHOL, total cholesterol; LDL-C, LDL cholesterol; LDL-B, LDL apoB; HDL-C, HDL cholesterol; TG, total triglycerides.

TABLE 2. Sequence of primers and ASOs and their relative position in the apoE gene

Oligo	Sequence	Nucleotide Position
Primer 1	5' GGAGTACTAAAGAAGCATTTGTGGAGCACCTTCTGTGT 3'	2721-2750
Primer 2	5' GGGAATTCTAAAGCCAGGAGTCAGAAATGGGAAGAGGA 3'	3133-3162
ASO1	5' AAGGTGGAGCAAGCG 3'	2830-2844
ASO2	5' AAGGTGAAGCAAGCG 3'	2830-2844
ASO3	5' GCGCAGCTCGGGCTC 3'	2860-2874
ASO4	3' CGCGTCGAACCCGAG 5'	2860-2874

The linkers added to the primers for cloning purposes are underlined. The position of all the oligonucleotides is specified from the corresponding sequence in the coding strand of the apoE gene, according to the numbering system of Paik et al. (31).

directly to the 5' end of each primer. One allele-specific oligonucleotide pair (ASO1 and 2) represent, respectively, the wild-type allele and the reported Japanese ε5 allele with the G to A change at the 18th nucleotide of the third exon (30). The other ASO pair (ASO3 and ASO4) recognize the wild-type and French-Canadian ε5 alleles with a G to A substitution at the 48th nucleotide of the third exon, respectively.

DNA amplification

The polymerase chain reaction (PCR) (32) was carried out in a final volume of 100 μl containing 1.0 μg of genomic DNA, 2.5 μM of each primer, one unit of Taq polymerase enzyme per sample, and the reaction buffer recommended by the manufacturer (Cetus Perkin-Elmer). Amplification was performed in a Cambio Intelligent Heating Block (Cambio, Cambridge, UK). Samples were heated to 95°C for 5 min to denature DNA, and cooled to 65°C for 2 min for annealing and extension of the primers. Forty-nine subsequent cycles were as follows: 95°C for 1 min and 65°C for 2 min. Successful amplification of the correct fragment was assessed by electrophoresis in 1% agarose gels and visualization with ethidium bromide (5 μg/ml) on a Chromato-vue TM-20 transilluminator (UVP, San Gabriel, CA).

Oligonucleotide melting

ASOs were phosphorylated at their 5' ends with [γ^{32}P]ATP (Amersham International, Amersham, UK) and T4 nucleotide kinase (BRL, Uxbridge, UK) at 37°C for 1 h (6). The labeled probes were separated from unincorporated ATP by elution with 3× SSC (1× SSC: 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.6) on a G-25 Sephadex column. The specific activity of the probes was approximately 0.1 μCi/pmol.

For each of the amplified DNA samples, two 2-μl aliquots were diluted in 200 μl 15× SSC, denatured by heating at 95°C for 7 min, and applied to Hybond-N filters (Amersham) using a slot-blot apparatus (Schleicher and Schuell, FRG). DNA was bonded to the filter by ultraviolet irradiation for 3 min (Chromato-vue TM-20, UVP transilluminator). Filters were prehybridized in 5 ml 5× SSPE (SSPE: 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 5× Denhardt's solution, 0.5% SDS for 30 min. Each probe (5 pmol) was then added and hybridized at 39°C for ASO1 and ASO2 or at 49°C for ASO3 and ASO4 for 1 h. Filters were rinsed briefly in 2× SSPE, 0.1% SDS at room temperature, followed by a high stringency wash in 5× SSPE, 0.1% SDS for 10 min at 41°C or 51°C depending on the probe pair used. Autoradiography was performed for 16 h at −70°C using a single intensifying screen (Du Pont) with preflashed Konica film.

Subcloning and sequencing

PCR-amplified DNA was purified with Geneclean (Bio101, La Jolla, CA), digested with *EcoRI*/*Sca*I, making use of the restriction sites introduced into the 5' end of the oligonucleotide primers, and further purified with Geneclean. The amplified fragment was next cloned into the *EcoRI*/*Sca*I-digested M13 mp11 vector by standard techniques (M13 cloning and sequencing handbook, Amersham). The recombinant M13 RF molecule was introduced into the JM101 *E. coli* strain. Selection and purification were performed according to the Amersham International plc recommended method. Fourteen (subject 1) and nine (subject 2) clones, respectively, were sequenced using the Sequenase kit (United State Biochemical Corp., Cleveland, Ohio), and analyzed on 6% denaturing polyacrylamide gels.

RESULTS

Using isoelectric focusing, we detected a protein band with a more cathodic migration than apoE4 in the VLDL fraction of subject 1. This band was also found to be present in the VLDL of the proband's two sisters, his son, and one uncle on the paternal side. In addition, all subjects showed the presence of the common E3 form in their plasma. In contrast, the band was absent from the plasma of the proband's mother, his daughter, his wife, and one of his father's sisters. The pedigree is presented in **Fig. 1**,

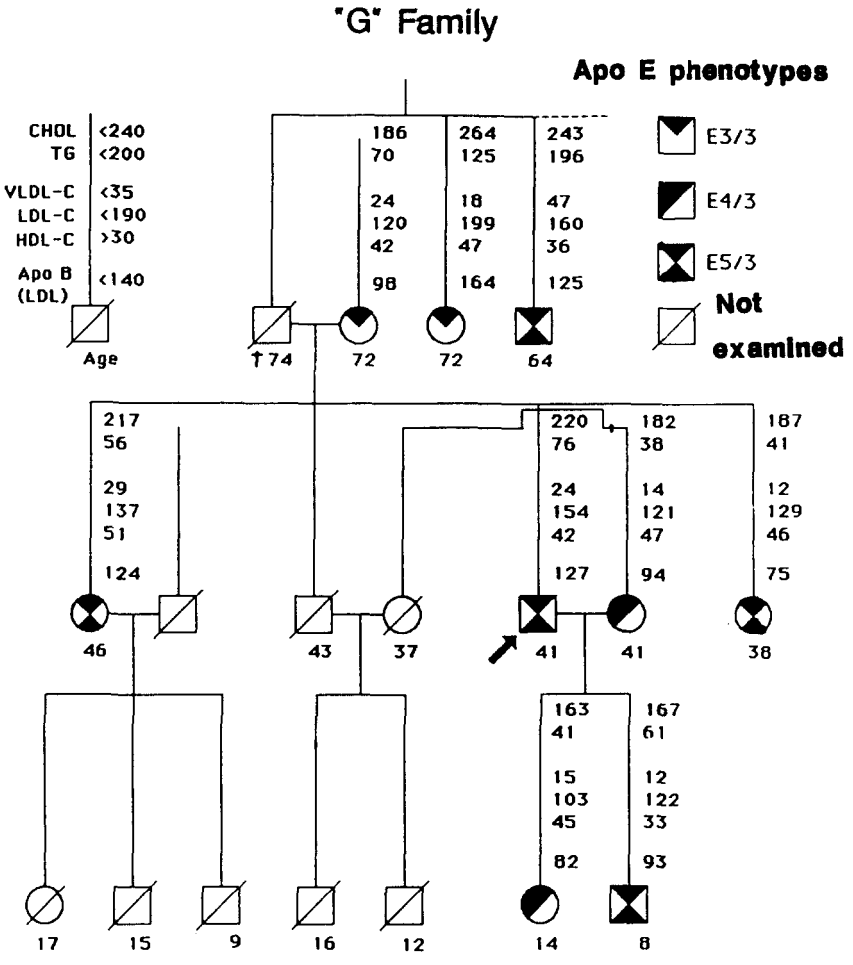

Fig. 1. Pedigree of the G family showing co-dominant transmission of apoE5 over three generations. The propositus is indicated by an arrow. All lipid values are in mg/dl, with normal upper-limit for each parameter indicated at the top left of the figure. Abbreviations as in Table 1, except for LDL apoB, apoB (LDL).

along with lipid values, which were all within the normal range. A band showing very similar migration was found in the VLDL of a second unrelated subject, but not in her husband and one daughter available for study.

We have used anti-apoE monoclonal antibodies (MAbs) to establish the identity of the bands found in the VLDL of these two unrelated individuals and family members of proband one. The focused apoE isoforms of the first individual and of three control subjects were transferred to a nylon membrane and immunoreacted with MAbs specific for the N-terminal and receptor binding region. MAb 6C5 (N-terminal epitope) bound all apoE isoforms but failed to bind the more cathodic band. In contrast, MAb 1D7 (epitope located between amino acids 140 and 160) showed a positive reaction with the unknown band as well as with the common isoforms (**Fig. 2A and 2B**). Additional experiments with MAb 7C9, with N-terminal specificity, confirmed the lack of immunoreactivity of this segment of the cathodic protein (result not shown). These results confirmed the identity of the cathodic band as apoE, as well as the loss of N-terminal epitopes. Similar results were obtained for the second proband (Fig. 2A and 2B).

Further characterization of the mutant apoE protein, hereafter designated apoE5, was obtained through cysteamine modification of VLDL apolipoproteins, which adds one positive charge to each cysteine residue present on a polypeptide chain. ApoE5 was shown to be shifted upward by one charge on IEF gels as was apoE3, indicative of the presence of one cysteine residue. The cysteamine-modified monosialo form of apoE5 was aligned with the untreated apoE5 protein. In contrast, glutathione treatment of the proteins, known to modify cysteine residues by the addition of a negative charge, shifted the whole band pattern downward, with the E5 protein co-migrating with apoE4 (results not shown). Neuraminidase treatment of the proteins removed all apoE sialoforms, leaving bands in the E3 and E5 positions only.

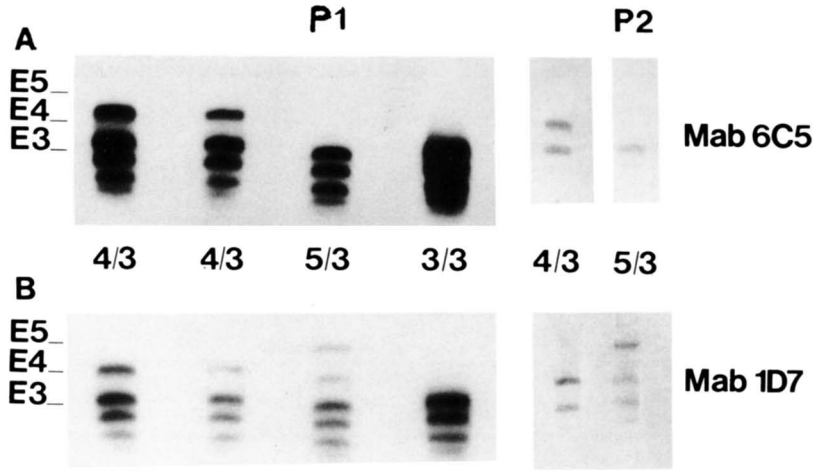

Fig. 2. Immunoblot of IEF-separated VLDL apolipoproteins from the two probands (P1, proband 1; and P2, proband 2) and control subjects. After transfer to Hybond-N filter, apolipoproteins were incubated sequentially with mouse anti-apoE monoclonal antobodies, and ^{125}I-labeled goat anti-mouse antibodies. Panel A: MAb 6C5 (amino terminal specific) as first antibody. Panel B: MAb 1D7 (amino acid 140–160 specific) as first antibody.

In an attempt to determine whether the loss of epitopes affected the size of the apoE5 protein, second-dimension analysis was performed on SDS gel. The apoE5 of subject 1 migrated slightly further than the apoE3 protein, suggesting a smaller molecular mass (by 1–2 kDa) (**Fig. 3**).

We next sought to determine the underlying defect at the DNA level. The portion of the gene coding for the amino terminal end of apoE was amplified by PCR with primers flanking the third exon. One tenth of the amplified DNA was run on a 1% agarose gel. The PCR product was of the expected size (442 bp, result not shown). Oligomelting experiments demonstrated that the DNA of the probands did not hybridize with the Japanese ϵ5 specific probe (ASO2) but did hybridize to the wild-type allele (ASO1) (results not shown). We therefore cloned the amplified fragments from the two probands into the M13 vector and sequenced several subclones from each. All subclones showed an inversion of the GC dinucleotide at the 50th base from the 5' end of the third exon, compared to the published apoE sequence (31). As the sequence in this region was compressed for all subclones, we concluded that the inversion was an artefact. Five of the fourteen subclones for proband 1, and four of the nine for proband 2 were shown to have a G to A substitution in the 48th base from the 5' end of the third exon, changing codon 13 from GAG, coding for glutamic acid, to AAG, coding for lysine (**Fig. 4**). Sequence analysis revealed no other base changes within exon 3. Oligomelting experiments with ASO3 and ASO4 confirmed these results, as both the G and A alleles were found to be present in the probands and members of family 1 with apoE5, while only the wild-type G allele could be detected in family members without apoE5 (**Fig. 5**).

DISCUSSION

We have been able to show Mendelian transmission over three generations of a rare apoE isoform in a French-Canadian pedigree. The isoprotein migrated at a position one charge more cathodic than apoE4, hence its designation as apoE5. This French-Canadian apoE5 was also present in a second unrelated proband. The specific defect at the gene level in both subjects is a G to A substitution, at the 48th position from the 5' end of the third exon. This is therefore a novel apoE mutation, differing from the previously described Japanese apoE5, where the 18th base of the third exon is changed also from G to A, substituting lysine for glutamic acid in the protein's 3rd amino acid (30).

The French-Canadian mutation changes the first position of the codon for amino acid 13 of the mature polypeptide chain. This is expected to result in the substitution of the normal glutamic acid residue with a lysine residue, adding two positive charges to the protein. This single mutation is therefore sufficient to explain the IEF pattern. As the cysteamine treatment shifts the position of the E5 protein by one charge, it can be inferred that it is derived

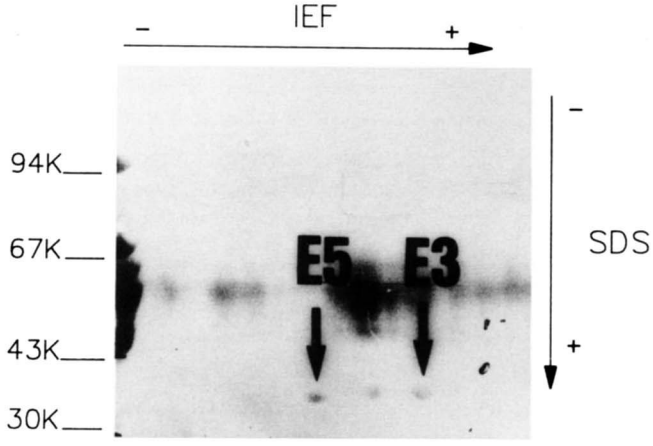

Fig. 3. Two-dimensional analysis of VLDL apolipoproteins from proband 1. Delipidated VLDL were separated in the first dimension by IEF and in the second by acrylamide gradient gel electrophoresis in the presence of SDS, then stained with 0.2% Coomassie Blue R-250 in 95% ethanol.

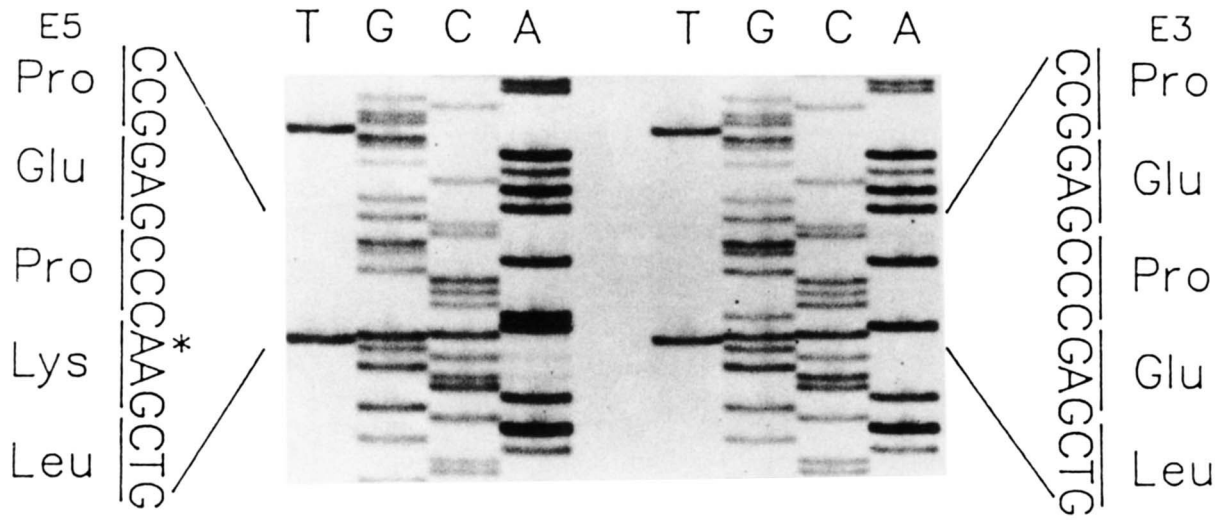

Fig. 4. Nucleotide sequence of representative M13 subclones containing, respectively, the wild-type G (right) and mutant A (left) residues at the 48th nucleotide of exon 3 of the apoE gene. The artefactual inversion of the GC dinucleotide at position 50 and 51 is due to a compression in this region of the gel.

from an apoE3 parental form. Thus, it seems highly unlikely that this E5 isoform is identical to the one reported by Ordovas et al. (33), as the latter appears to have originated from an apoE4 parental form.

The altered migration of the E5 protein observed with SDS electrophoresis is somewhat unexpected in view of the nature of the mutation, but this phenomenon has been described previously for several apoE variants (18, 34), including apoE5 $glu_3 \rightarrow lys$, which shows an altered migration similar to our mutant. The glu_{13} to lys substitution in apoE5 markedly alters the conformation of two overlapping, but distinct epitopes (27) at the amino terminus of the delipidated protein, completely preventing their recognition by monoclonal antibodies specific for this region. Undoubtedly, the replacement of a negatively charged amino acid by a positively charged one at amino acid 13 affects the interactions between other residues. It is possible that the ordered structure of this region is preserved to a sufficient degree under these conditions to allow the effect of this mutation to be reflected in the migration pattern. Alternatively, the mutation may modify the interaction between SDS and the positively charged amino acids.

The occurrence of this particular G to A substitution in two apparently unrelated subjects warrants further investigation. The mutation seems to have arisen recently in genetic terms since it has not been reported in other Caucasian populations. As French-Canadians are descendants of a relatively small number of founders (35), the probability of this mutation arising independently more than once is low. However, examination of the DNA sequence at the site of the mutation reveals the presence of a highly mutable CpG dinucleotide on the non-coding strand (36). Recurrent mutations involving this dinucleotide are known to occur in several inherited diseases (36, 37). It is also present at the two common polymorphic sites for apoE. By contrast, both the E5 and E7 Japanese mutations replace glutamic acid codons with lysine ones, but do not involve CpG sequences (30, 38).

If these two probands are in fact distantly related, it is expected that they will share a common chromosome 19 haplotype. To investigate this, we have used DNA poly-

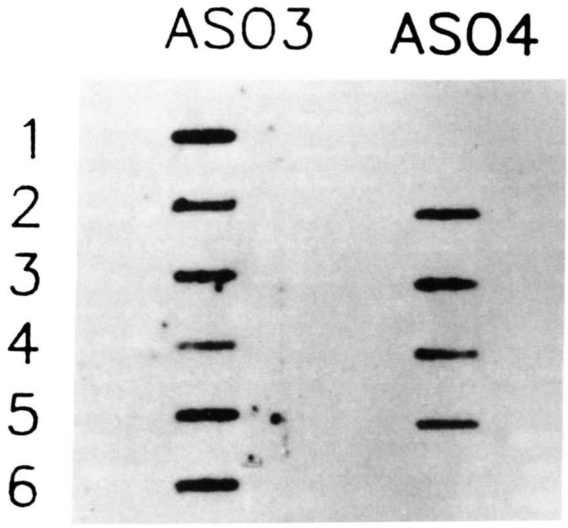

Fig. 5. Allele-specific hybridization of amplified DNA with ASO3 (wild-type) and ASO4 (mutant) probes confirming the co-segregation of the G to A substitution with the presence of apoE5 protein in both probands and members of proband 1's family. 1, mother of proband 1; 2, proband 1; 3, sister of proband 1; 4, sister of proband 1; 5, proband 2; 6, husband of proband 2.

morphisms of the apoE-C-I-C-II gene cluster to establish the haplotype carrying the E5 mutation. Preliminary studies have shown that both probands share a common allele for the CA dinucleotide repeat polymorphism in the apoC-II gene (39). However, this marker is not informative in the "G" family to determine which of the chromosomes bears the mutation. Both probands are also homozygous for the *Hpa*I polymorphism of the CI gene (40) (C-f. Xu and F. Mailly, unpublished results). These results increase the likelihood of the two subjects being related.

The biological significance of the apoE5 mutation is not clear. It is not associated with type III dyslipoproteinemia in either of the subjects, and it does not seem to be associated with hyperlipidemia, as the members of the G family are normolipidemic. This is not surprising as the mutation is far removed from the receptor-binding domain of apoE. However, this region has been shown to contain a heparin-binding site, which could be blocked by Mab 7C9 (27). Thus, this raises the question whether this mutation could alter the ability of apoE to bind heparin and thereby interfere with other postulated functions of apoE. In particular, apoE is synthesized and secreted by astrocytic glia but not neurons in the brain and expressed in a stage-specific manner in human glial neoplasms (41, 42). It is also known that cell growth and division are accelerated by interaction with heparin-like structures on the extracellular matrix (43). Accordingly, it has been suggested that, through its ability to bind heparin, apoE may modulate cell growth either by interacting with heparin-like structures on the extracellular matrix, or by displacing other growth factors (26). Family history for proband 1 revealed the existence of several cases of cerebrovascular incidents and brain tumours on the paternal side, from which apoE5 is inherited. Whether the presence of the apoE5 mutant can be related in any way to these events is not known at present. Patients with complete apoE deficiency only present with type III dyslipoproteinemia (44), and no impairment of neurological function is known. This suggests that any physiological role played by apoE in these processes can be compensated by other proteins. However, if a functional mutation increased a growth-related activity of apoE, it might have a significant effect, perhaps detrimental, even in heterozygous subjects.

It would therefore be interesting to test the effect of the mutation reported here on the heparin-binding activity of apoE. Any detected variation in this activity could then be used to test models for proposed apoE functions unrelated to lipid metabolism.

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REFERENCES

1. Mahley, R.W., T. L. Innerarity, S. C. Rall, Jr., and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.* **25:** 1277–1294.
2. Mahley, R. W., and T. L. Innerarity. 1983. Lipoprotein receptor and cholesterol haemostasis. *Biochim. Biophys. Acta.* **737:** 197–222.
3. Weisgraber, K. H., S. C. Rall, Jr., and R. W. Mahley. 1981. Human E apoprotein heterogeneity: cysteine-arginine interchanges in the amino acid sequence of the apoE isoforms. *J. Biol. Chem.* **256:** 9077–9083.
4. Bouthillier, D., C. F. Sing, and J. Davignon. 1983. Apolipoprotein E phenotyping with a single gel method: application to the study of informative matings. *J. Lipid Res.* **24:** 1060–1069.
5. Havekes, L. M., P. de Kniff, U. Beisiegel, J. Havinga, M. Smit, and E. Klasen. 1987. A rapid micro-method for apolipoprotein E phenotyping directly in serum. *J. Lipid Res.* **28:** 455–463.
6. Houlston, R. S., C. Snowden, F. Green, K. G. M. M. Alberti, and S. E. Humphries. 1989. Apolipoprotein E genotyping by polymerase chain reaction and allele-specific probes: no detectable linkage disequilibrium between apoE and apoC-II. *Hum. Genet.* **83:** 364–368.
7. Smeets, H. J. M., J. Poddighe, P. M. J. Stuyt, A. F. H. Stalenhoef, H. H. Rogers, and B. Wieringa. 1988. Identification of apolipoprotein E polymorphism by using synthetic oligonucleotides. *J. Lipid Res.* **29:** 1231–1237.
8. Hixson, J. M., and D. T. Vernier. 1990. Restriction isotyping of human apolipoprotein E polymorphism by gene amplification and cleavage with *Hha*I. *J. Lipid Res.* **31:** 545–548.
9. Davignon, J., R. E. Gregg, and C. F. Sing. 1988. Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis.* **8:** 1–21.
10. Utermann, G., N. Pruin, and A. Steinmetz. 1979. Polymorphism of apolipoprotein E. III. Effect of a single polymorphic gene locus on plasma lipid levels in man. *Clin. Genet.* **15:** 63–72.
11. Utermann, G., I. Kindermann, H. Kaffernik, and A. Steinmetz. 1984. Apolipoprotein E phenotypes and hyperlipoproteinemia. *Hum. Genet.* **65:** 232–236.
12. Weisgraber, K. H., T. L. Innerarity, and R. W. Mahley. 1982. Abnormal lipoprotein receptor-binding activity of the human apoE apoprotein due to cysteine-arginine interchanges at a single site. *J. Biol. Chem.* **257:** 2518–2521.
13. Schneider, W. J., P. T. Kovanen, M. S. Brown, J. L. Goldstein, G. Utermann, W. Weber, R. J. Havel, L. Kotite, J. P. Kane, T. L. Innerarity, and R. W. Mahley. 1981. Familial

dysbetalipoproteinemia: abnormal binding of a mutant apoprotein E to low density lipoprotein receptors of human fibroblasts and membranes from liver and adrenal of rats, rabbits and cows. *J. Clin. Invest.* **68:** 1075–1085.
14. Stalenhoef, A. F. H., M. J. Malloy, J. P. Kane, and R. J. Havel. 1986. Metabolism of apolipoprotein B-48 and B-100 of triglyceride-rich lipoproteins in patients with familial dysbetalipoproteinemia. *J. Clin. Invest.* **78:** 722–728.
15. Brewer, H. B., Jr., L. A. Zech, R. E. Gregg, D. Schwartz, and E. J. Schaefer. 1983. Type III hyperlipoproteinemia: diagnosis, molecular defects, pathology and treatment. *Ann. Intern. Med.* **98:** 623–640.
16. Rall, S. C., Jr., K. H. Weisgraber, T. L. Innerarity, and R. W. Mahley. 1982. Structural basis for receptor-binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects. *Proc. Natl. Acad. Sci. USA.* **79:** 4696–4700.
17. Gregg, R. E., G. Ghiselli, and H. B. Brewer, Jr. 1983. Apolipoprotein E Bethesda: a new variant of apolipoprotein E associated with type III hyperlipoproteinemia. *J. Clin. Endocrinol. Metab.* **57:** 969–974.
18. Yamamura, T., A. Yamamoto, T. Sumiyoshi, K. Hiramori, Y. Nishioeda, and S. Nambu. 1984. New mutants of apolipoprotein E associated with atherosclerotic diseases but not type III hyperlipoproteinemia. *J. Clin Invest.* **74:** 1229–1237.
19. Rall, S. C., Jr., Y. M. Newhouse, H. R. Clarke, K. H. Weisgraber, B. J. McCarthy, R. W. Mahley, and T. P. Bersot. 1989. Type III hyperlipoproteinemia associated with apoE phenotype E3/3: structure and genetics of an apolipoprotein E3 variant. *J. Clin. Invest.* **83:** 1095–1101.
20. Mann, W. A., R. E. Gregg, D. L. Sprecher, and H. B. Brewer, Jr. 1989. ApoE-1 Harrisburgh: a new variant of apolipoprotein E dominantly inherited with type III hyperlipoproteinaemia. *Biochim. Biophys. Acta.* **1005:** 239–244.
21. Wardell, M. R., K. H. Weisgraber, L. M. Havekes, and S. C. Rall, Jr. 1989. ApoE-3 Leiden contains a seven amino acid insertion that is a tandem repeat of residues 121–127. *J. Biol. Chem.* **264:** 21205–21210.
22. Wardell, M. R., S. O. Brennan, E. D. Janus, R. Fraser, and R. W. Carrell. 1987. Apolipoprotein E-2 Christchurch (136 Arg---Ser). New variant of human apolipoprotein E in a patient with type III hyperlipoproteinemia. *J. Clin Invest.* **80:** 483–490.
23. Innerarity, T. L., E. J. Friedlander, S. C. Rall, Jr., K. H. Weisgraber, and R. W. Mahley. 1983. The receptor binding domain of human apolipoprotein E: binding of apolipoprotein fragments. *J. Biol. Chem.* **258:** 12341–12347.
24. Weisgraber, K. H., T. L. Innerarity, K. J. Harder, R. W. Mahley, R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1983. The receptor binding domain of human apolipoprotein E: monoclonal antibody inhibition of binding. *J. Biol. Chem.* **258:** 12348–12354.
25. Innerarity, T. L., K. S. Arnold, S. C. Rall, Jr., and R. W. Mahley. 1984. Normalization of receptor binding of apolipoprotein E2: evidence for modulation of the binding site conformation. *J. Biol. Chem.* **259:** 7261–7267.
26. Mahley, R. W. 1988. Apolipoprotein E: cholesterol transport protein with an expanding role in cell biology. *Science.* **240:** 622–630.
27. Weisgraber, K. H., S. C. Rall, Jr., R. W. Milne, Y. L. Marcel, and J. T. Sparrow. 1986. Human apolipoprotein E: determination of the heparin-binding sites of apolipoprotein E3. *J. Biol. Chem.* **261:** 2068–2076.
28. Mellman, I. S., and J. Unkeless. 1980. Purification of a functional mouse Fc receptor through the use of a monoclonal antibody. *J. Exp. Med.* **152:** 1048–1069.
29. Kunkel, L. M., D. K. Smith, S. H. Boyer, P. S. Borgoankor, S. S. Wachtel, D. J. Miller, H. W. Jones, and J. M. Rary. 1977. Analysis of human Y-chromosome-specific reiterated DNA in chromogene variants. *Proc. Natl. Acad. Sci. USA.* **74:** 1245–1249.
30. Tajima, S., T. Yamamura, and A. Yamamoto. 1988. Analysis of apolipoprotein E5 gene from a patient with hyperlipoproteinemia. *J. Biochem.* **104:** 730–734.
31. Paik, Y-K., D. J. Chang, C. A. Reardon, G. E. Davies, R. W. Mahley, and J. M. Taylor. 1985. Nucleotide sequence and structure of the human apolipoprotein E gene. *Proc. Natl. Acad. Sci. USA.* **82:** 3445–3449.
32. Saiki, R. K., T. L. Bugawan, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1986. Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele-specific-oligonucleotide probes. *Nature.* **324:** 163–166.
33. Ordovas, J. M., L. Litwack-Klein, P. W. F. Wilson, M. M. Schaefer, and E. J. Schaefer. 1987. Apolipoprotein E isoform phenotyping methodology and population frequency with identification of apoE1 and apoE5 isoforms. *J. Lipid Res.* **28:** 371–380.
34. Utermann, G., K. H. Weisgraber, W. Weber, and R. W. Mahley. 1984. Genetic polymorphism of apolipoprotein E: a variant form of apolipoprotein E2 distinguished by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. *J. Lipid Res.* **25:** 378–382.
35. Gagne, C., L-D. Brun, P. Julien, S. Moorjani, and P-J. Lupien. 1989. Primary lipoprotein-lipase-activity deficiency: clinical investigation of a French Canadian population. *Can. Med. Assoc. J.* **140:** 405–411.
36. Cooper, D. N., and H. Youssoufian. 1988. The CpG dinucleotide and human genetic disease. *Hum. Genet.* **78:** 151–155.
37. Youssoufian, H., S. E. Antonarakis, W. Bell, A. M. Griffin, and H. H. Kazakian, Jr. 1988. Nonsense and missense mutations in hemophilia A: estimate of the relative mutation rate at CG dinucleotides. *Am. J. Hum. Genet.* **42:** 718–725.
38. Tajima, S., T. Yamamura, M. Menju, and A. Yamamoto. 1989. Analysis of apolipoprotein E7 gene from a patient with hyperlipoproteinemia. *J. Biochem.* **65:** 249–253.
39. Weber, J. L., and P. E. May. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* **44:** 388–396.
40. Smit, M., E. Van de Kooij-Meijs, R. R. Frants, L. M. Havekes, and E. C. Klasen. 1988. Apolipoprotein gene cluster on chromosome 19: definitive localization of the apoC-II gene and the polymorphic *Hpa*I site associated with type III hyperlipoproteinemia. *Hum. Gent.* **78:** 90–93.
41. Boyles, J. K., R. E. Pitas, E. Wilson, R. W. Mahley, and J. M. Taylor. 1985. Apolipoprotein E is associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J. Clin. Invest.* **76:** 1501–1513.
42. Murakami, M., Y. Ushio, Y. Morino, O. Takao, and Y. Matsukado. 1988. Immunochemical localization of apolipoprotein E in human glial neoplasms. *J. Clin. Invest.* **82:** 177–182.
43. O'Neill, C., P. Jordan, and G. Ireland. 1986. Evidence for two distinct mechanisms of anchorage stimulation in freshly explanted and 3T3 Swiss mouse fibroblasts. *Cell.* **44:** 489–496.
44. Schaefer, E. J., R. E. Gregg, G. Ghiselli, T. M. Forte, J. M. Ordovas, L. A. Zech, and H. B. Brewer, Jr. 1986. Familial apolipoprotein E deficiency. *J. Clin. Invest.* **78:** 1206–1219.