

# Identification of a New Structural Variant of Human Apolipoprotein E, E2(Lys<sub>146</sub>→Gln), in a Type III Hyperlipoproteinemic Subject with the E3/2 Phenotype

STANLEY C. RALL, JR., KARL H. WEISGRABER, THOMAS L. INNERARITY, THOMAS P. BERSOT, and ROBERT W. MAHLEY, *Gladstone Foundation Laboratories for Cardiovascular Disease, Cardiovascular Research Institute, Departments of Pathology and Medicine, University of California, San Francisco, California 94140*

CONRAD B. BLUM, *Department of Medicine and the Arteriosclerosis Research Center, Columbia University, College of Physicians and Surgeons, New York, New York 10032*

**ABSTRACT** A type III hyperlipoproteinemic subject having the apolipoprotein E (apo E) phenotype E3/2 was identified. From isoelectric focusing experiments in conjunction with cysteamine treatment (a method that measures cysteine content in apo E), the E2 isoform of this subject was determined to have only one cysteine residue, in contrast to all previously studied E2 apoproteins, which had two cysteines. This single cysteine was shown to be at residue 112, the same site at which it occurs in apo E3. From amino acid and sequence analyses, it was determined that this apo E2 differed from apo E3 by the occurrence of glutamine rather than lysine at residue 146. When phospholipid-protein recombinants of the subject's isolated E3 and E2 isoforms were tested for their ability to bind to the human fibroblast apo-B<sub>100</sub> receptor, it was found that the E3 bound normally (compared with an apo E3 control) but that the E2 had defective binding (~40% of normal). Although they contained E3 as well as E2, the  $\beta$ -very low density lipoproteins ( $\beta$ -VLDL) from this subject were very similar in character to the  $\beta$ -VLDL from an E2/2 type III hyperlipoproteinemic

subject; similar subfractions could be obtained from each subject and were shown to have a similar ability to stimulate cholesteryl ester accumulation in mouse peritoneal macrophages. The new apo E2 variant has also been detected in a second type III hyperlipoproteinemic subject.

## INTRODUCTION

The genetically determined polymorphism of human apolipoprotein E (apo E),<sup>1</sup> first demonstrated by Utermann et al. (1, 2), was subsequently shown by Zannis et al. (3, 4) to be the result of multiple alleles occurring at a single genetic locus. These alleles are the structural genes for apo E and specify apo E molecules that differ from the most frequently occurring apo E (apo E3, the "wild type" or parent) by a series of single amino acid substitutions (5-7). To date, four such alleles have been identified from studies of their protein products (7). Two apo E variants have been shown to be functionally defective, although to different degrees, in their ability to interact in vitro with specific lipoprotein receptors on cell membranes (7, 8).

The lipid disorder type III hyperlipoproteinemia has usually been associated with the apo E phenotype

Address all correspondence to Dr. R. W. Mahley, The Gladstone Foundation Laboratories, P. O. Box 40608, San Francisco, CA 94140.

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<sup>1</sup> Abbreviations used in this paper: apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine.

E2/2 (9-11). Not only has the E2/2 phenotype proven to be genotypically heterogeneous regarding apo E structure (7), but also functionally heterogeneous regarding apo E binding activity (7, 12). The severity of the apo E binding defect does not necessarily correlate with the severity of the disease in the individual from whom the apo E was derived (7, 12). Furthermore, severely defective apo E has been shown to occur in individuals who have neither the clinical manifestations (xanthomatosis and/or premature vascular disease) nor the hyperlipidemia that is characteristic of the disorder (13).

In this manuscript, a new structural variant of apo E2 and its receptor binding activity are described. The relationship of the structure and binding ability of apo E to the manifestation of type III hyperlipoproteinemia is also discussed.

## METHODS

**Apoprotein preparation and characterization.** Apo E was isolated from the  $d < 1.02$  lipoproteins by chromatography on Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) in the presence of 4 M guanidine containing 0.1 M Tris-HCl and 0.01% EDTA (pH 7.4) as previously described (5). Prior to application to the Sephacryl column, the lipoproteins were delipidated with  $\text{CHCl}_3/\text{MeOH}$  (2:1, vol/vol), also as described (5, 13).

Analytical isoelectric focusing and cysteamine treatment of lipoproteins or apoproteins were carried out as described (5, 13). Preparative isoelectric focusing was performed on an LKB Multiphor flatbed unit (LKB Produkter, Bromma, Sweden) using Sephadex G-200 (Pharmacia Fine Chemicals) as a support medium. Focusing was carried out for 16 h at 4°C in 8 M urea over the pH range 4-6.5 (2% Pharmalyte, Pharmacia Fine Chemicals) at a constant power of 8 W. A paper print was used to locate the isoforms, and the protein was recovered and freed of ampholytes by  $(\text{NH}_4)_2\text{SO}_4$  precipitation as previously described (5).

**Amino acid and sequence analysis.** Samples for amino acid analyses were hydrolyzed for 20 h at 110°C in 6 N HCl in sealed, evacuated tubes. The contents were dried under reduced pressure at 40°C, dissolved in 0.2 N sodium citrate, pH 2.2, and analyzed on a Beckman 121MB analyzer equipped with a model 126 data system (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). No corrections were made for hydrolytic destruction or incomplete release.

For CNBr digestion, apo E was dissolved in 70% HCOOH at 5 mg/ml and digested with a 30-fold excess (wt/wt) of CNBr (Pierce Chemical Co., Rockford, IL) for 24 h at room temperature. The resultant peptides were then separated on Sephadex G-50 (Pharmacia Fine Chemicals) as previously described (5, 6, 13).

The peptides that correspond to those previously identified as cysteine containing (5-7, 13) from the G-50 chromatography were lyophilized, redissolved in 0.5 ml 50% acetic acid, and sequenced in the presence of 2 mg of polybrene (Sigma Chemical Co., St. Louis, MO) on a Beckman 890C sequencer (Beckman Instruments, Inc., Fullerton, CA) using a 0.1 M Quadrol program (No. 122974) and standard Beckman reagents. Methods of analysis and criteria for identification of the phenylthiohydantoin amino acids by high performance liquid chromatography have been described (5).

**Fibroblast binding studies of apo E.** Phospholipid complexes of apo E were prepared by incubating apo E (150  $\mu\text{g}$  in 150-300  $\mu\text{l}$  0.1 M  $\text{NH}_4\text{HCO}_3$ , containing 0.1%  $\beta$ -mercaptoethanol) with 56  $\mu\text{l}$  of dimyristoylphosphatidylcholine (DMPC, Sigma Chemical Co.) vesicles (10 mg/ml, prepared by sonication) for 1 h at 25°C. The resultant apo E-DMPC complexes were isolated by density gradient centrifugation (14). The complexes were dialyzed against 0.15 M NaCl, 0.01% EDTA (pH 7.4), and tested for their ability to compete with  $^{125}\text{I}$ -low density lipoproteins (LDL) for binding to the apo-B,E receptors of intact human fibroblasts grown on 35-mm petri dishes. The methodology for the maintenance of the cells and the conditions for the competitive binding assay were as described (14).

**Preparation and characterization of  $\beta$ -very low density lipoproteins (VLDL) and  $\beta$ -VLDL subfractions.** Lipoproteins of the  $d < 1.006$  fraction were prepared from plasma by ultracentrifugation and then subjected to Geon-Pevikon block electrophoresis to separate the pre- $\beta$ - and  $\beta$ -migrating lipoproteins (15). The  $\beta$ -VLDL were then subfractionated by chromatography on a Biogel A-15m (Bio-Rad Laboratories, Richmond, CA) column (2.5  $\times$  95 cm, Kontes Glass Co., Vineland, NJ) as previously described by Fainaru et al. (16). Two subfractions, Fraction I and Fraction II, were tested for their ability to stimulate [ $^{14}\text{C}$ ]oleate incorporation into cholesteryl esters in mouse peritoneal macrophages (16, 17).

**Chemical determinations.** Protein levels were determined by the method of Lowry et al. (18) using bovine serum albumin (BSA) as the standard. Total cholesterol and triglyceride levels were determined enzymatically (Bio-Dynamics, Boehringer-Mannheim Corp., Indianapolis, IN), and phospholipid levels were determined from the phosphorus content (19).

## RESULTS

**Subject description.** The subject (D.F., 51 yr old in 1982) had a 15-yr history of known hyperlipidemia (Table I). In 1967, both tuberoeruptive xanthomas on the elbows and bilateral palmar xanthomas appeared. Plasma cholesterol and triglyceride levels were 555 mg/dl and 1,015 mg/dl, respectively. In 1969, his VLDL cholesterol and VLDL triglyceride levels were 344 mg/dl and 686 mg/dl, respectively;  $\beta$ -VLDL were also present in the patient's plasma. Therapy, which included clofibrate treatment, weight reduction, and dietary restrictions, led to a reduction in plasma cholesterol and triglyceride levels to 185 mg/dl and 255 mg/dl, respectively. The palmar xanthomas resolved in less than a year, and by 1972 the tuberoeruptive xanthomas had also resolved. The marked hyperlipidemia reappeared in subsequent years; it was possibly associated with the subject's weight gain (Table I). In 1982,  $\beta$ -VLDL were again identified, and the subject's plasma apo E level was measured at 27.0 mg/dl by radioimmunoassay (20). The range for normal plasma apo E levels is 3-7 mg/dl (see, for example, reference 20).

The subject has never had symptoms of coronary or peripheral vascular disease, and there is no known

TABLE I  
Lipid Values of Subject D.F.

Age	Weight	TC	TG	VLDL-C/TG	Comment
yr	kg	mg/dl			
36	82	555	1015	ND	
38	80	484	657	0.50	
39	73	185	255	ND	Clofibrate
42	81	354	368	0.47	Clofibrate
43	82	405	563	0.40	Clofibrate
47	82	408	808	0.33	
47	82	390	1056	ND	Clofibrate
48	83	394	516	0.47	Clofibrate
49	91	385	924	ND	Clofibrate
49	90	209	283	ND	Clofibrate/nicotinic acid
50	89	224	301	ND	Clofibrate/nicotinic acid
51	91	281	682	0.24	Clofibrate/nicotinic acid

Abbreviations used in this table: TC, total cholesterol; TG, triglycerides; VLDL-C, VLDL cholesterol; ND, no determination.

family history of premature coronary heart disease. One of the proband's grandparents died in his tenth decade, two in their ninth, and one in her eighth. The mother of the proband died in her fourth decade of pneumonia. The proband's father is still living.

There is some evidence of hyperlipidemia in the proband's family; it possibly involves his father and at least three of the proband's four children. Family studies are incomplete, primarily because of poor cooperation on the part of the subjects. In 1972, when it was possible to obtain samples from all family members, the proband's daughters, M.F. (13 yr old at that time) and Do.F. (9 yr old), had plasma cholesterol levels of 215 mg/dl and 275 mg/dl, respectively, and plasma triglyceride levels of 108 mg/dl and 132 mg/dl, respectively. The son, G.F. (12 yr old), had a plasma cholesterol level of 250 mg/dl, and his triglyceride level was 216 mg/dl. The other son (T.F., 7 yr old) had cholesterol and triglyceride levels of 175 mg/dl and 196 mg/dl, respectively. The proband's father (71 yr old in 1972) had cholesterol and triglyceride levels of 281 mg/dl and 270 mg/dl, respectively.

The only family members from which it has been possible to obtain a recent sample are the daughter Do.F. (now 20 yr old) and the proband's spouse (I.F., 45 yr old). The daughter had plasma cholesterol and triglyceride levels of 266 mg/dl and 160 mg/dl, respectively; also, she had a plasma apo E level of 4.3 mg/dl and was an E4/3 phenotype. The proband's spouse had plasma cholesterol and triglyceride levels of 205 mg/dl and 214 mg/dl, respectively; also, she had a plasma apo E level of 3.4 mg/dl and was an E4/4 phenotype.

Description of a second type III hyperlipoproteinemic subject (P.H.) will be presented in a later section of Results.

**Characterization of D.F.'s apo E.** Analytical isoelectric focusing of VLDL apoproteins was carried out to determine the apo E phenotype of subject D.F. As shown in Fig. 1, subject D.F. had the E3/2 heterozygous phenotype. Therefore, this subject was a rare case of an individual with type III hyperlipoproteinemia who did not have the E2/2 homozygous phenotype. Cysteamine treatment, a method used previously to determine cysteine content in apo E (5), caused a one-charge shift in the entire apo E pattern for subject D.F. (Fig. 1). This was in contrast to the pattern usually seen for individuals with the E3/2 heterozygous phenotype, where both the E3 and E2 isoforms shifted to the E4 position (Fig. 1). In the latter case, this has been interpreted to mean that the E3 contained one cysteine residue and the E2 contained two cysteine residues, which is consistent with our previous observations (5-7, 13). The cysteamine-treated pattern of D.F.'s apo E was interpreted to mean that either (a) the E2 contained two cysteines and therefore shifted to the E4 position, while the E3 contained no cysteine and did not shift or (b) the E3 contained one cysteine and shifted to the E4 position, while the E2 also contained one cysteine and shifted to the E3 position.

To distinguish between these two possibilities, column-isolated apo E from D.F. was subjected to preparative isoelectric focusing; the individual isoforms were isolated and then separately focused with or with-

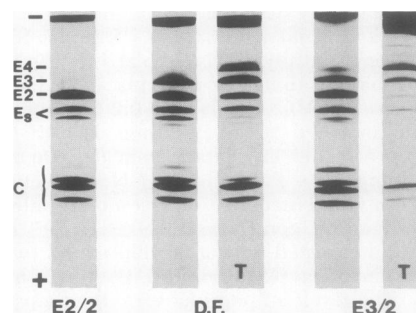


FIGURE 1 Isoelectric focusing gels of VLDL apoproteins. *Left*, control gel from a type III hyperlipoproteinemic subject with the E2/2 phenotype; *center*, control and cysteamine-treated (T) gels from subject D.F.; *right*, control and cysteamine-treated (T) gels from a subject with the E3/2 phenotype. The anode is at the bottom and the cathode is at the top. The focusing positions of E4, E3, and E2 are indicated. E<sub>s</sub> denotes sialylated forms of E3 and E2. Note that monosialylated E3 focuses in the E2 position and may contribute to the intensity of the E2 isoform when E3 is present. The C denotes positions of the apo C-II and apo C-III isoforms.

out cysteamine treatment on analytical isoelectric focusing gels. As shown in Fig. 2, both the E3 and E2 isoforms moved one charge unit after cysteamine treatment and therefore contained one cysteine residue each. These results suggested that the E2 from subject D.F. was different from all other previously described E2 apoproteins, which contained two cysteines (5-7, 13).

**Structural analysis of D.F.'s apo E.** The E3 and E2 isoforms isolated by flatbed preparative isoelectric focusing were subjected to amino acid analysis (Table II). The compositions of the E3 and E2 isoforms were very similar to each other as well as to the composition of the apo E3 previously derived from sequence analysis (6). A comparison of the amino acid analyses of the E3 and E2 isoforms did suggest, however, the possibility that D.F.'s E2 might contain one less lysine residue than D.F.'s E3 (Table II). The substitution of a neutral amino acid for lysine could explain the charge difference between E3 and E2 from D.F.

Therefore, partial sequence analysis of D.F.'s apo E was undertaken in an attempt to locate the site(s) that could account for the charge difference. Column-isolated apo E (containing a mixture of E3 and E2) was used for this analysis. A CNBr digest was performed on D.F.'s apo E (9.1 mg) and the peptides were separated on Sephadex G-50; this yielded an elution profile indistinguishable from those previously reported (5, 6, 13).

The peptide designated CB4 was investigated first; it is a 17-residue CNBr peptide (residues 109-125) that previously was shown to contain the single cysteine residue of apo E3 and one of the two cysteine residues of apo E2 (5-7, 13). As shown in Tables III and IV, this peptide from D.F.'s apo E also contained one cysteine residue and had the same sequence as the com-

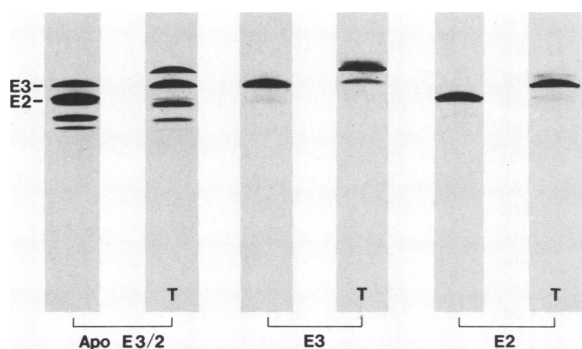


FIGURE 2 Isoelectric focusing gels of isolated D.F. apo-E and the individual isoforms. *Left*, control and cysteamine-treated (*T*) gels of D.F.'s isolated apo E3/2; *center*, control and cysteamine-treated (*T*) gels of the E3 isoform; *right*, control and cysteamine-treated (*T*) gels of the E2 isoform. The anode is at the bottom and the cathode is at the top.

TABLE II  
Amino Acid Compositions of Isolated Apo E Isoforms  
from Subject D.F.\*

Amino acid	D.F.'s E3†	D.F.'s E2‡	Sequence, E3 <sup>¶</sup>
Aspartic acid	12.5±0.2	12.5±0.2	11
Asparagine	—	—	1
Threonine	10.5±0.1	10.4±0	11
Serine	12.8±0	12.8±0.3	14
Glutamic acid	72.4±0.2	72.7±0.2	40
Glutamine	—	—	31
Proline	8.3±0.3	8.3±0.1	8
Glycine	17.6±0.2	17.6±0.6	17
Alanine	34.3±0	34.0±0.2	35
Cysteine	ND	ND	1
Valine	21.5±0.2	22.2±0.6	22
Methionine	6.7±0.2	6.6±0.1	7
Isoleucine	1.8±0	1.8±0.1	2
Leucine	37.5±0.1	37.2±0.1	37
Tyrosine	3.8±0.1	3.9±0.1	4
Phenylalanine	3.1±0	3.2±0.1	3
Tryptophan	ND	ND	7
Lysine	11.6±0.3	10.7±0.1	12
Histidine	2.0±0.1	2.0±0.1	2
Arginine	34.6±0.1	35.0±0.4	34

ND, no determination.

\* Numbers are given in residues/mole±SD.

† Three determinations.

‡ Six determinations.

¶ From reference 6.

parable peptide from previous studies on other subjects (5-7, 13). No heterogeneity was observed at the position where cysteine occurred in the sequence analysis, and the cysteine value from amino acid analysis was 0.9; this suggested that both D.F.'s E3 and E2 contained cysteine at this site, which corresponds to residue 112 in the apo E sequence (6).

The peptide designated CB5, a 93-residue CNBr peptide (residues 126-218) that previously was shown to contain the second cysteine residue of apo E2 (5-7, 13), was subjected to sequence analysis. The peptide CB5 from the apo E of subject D.F. did not contain cysteine, as demonstrated by amino acid analysis (Table III) and sequence analysis (Table V). Previously, apo E2 has been shown to have cysteine at either residue 158 (cycle 33 of peptide CB5) or residue 145 (cycle 20 of peptide CB5) (7). Cysteine's absence in peptide CB5 reinforced the observation made from the CB4 analysis that both D.F.'s E3 and E2 contained a single cysteine residue at a common site, which was residue 112.

The sequence analysis of D.F.'s apo E CB5 differed from all others previously reported (5-7, 13) in that glutamine was found at cycle 21, which corresponds

TABLE III  
Amino Acid Composition of Peptides CB4 and CB5  
from Apo E of Subjects D.F. and P.H.

Amino acid	CB4		CB5	
	D.F.	P.H.	D.F.	P.H.
Aspartic acid	1.0	1.1	3.2	3.6
Threonine	0.2	0.3	2.6	2.3
Serine	0.2	0.3	4.0	3.8
Glutamic acid	4.2	4.6	18.2	18.3
Proline	0	0	2.2	2.2
Glycine	2.2	1.9	8.5	8.1
Alanine	1.0	1.1	11.5	12.0
Cysteine*	0.9	0.9	0.1	0.6
Valine	2.9	2.5	5.1	6.2
Methionine	+†	+	+	+
Isoleucine	0	0	0.9	0.8
Leucine	1.1	1.2	14.6	13.9
Tyrosine	0.8	0.8	1.2	1.3
Phenylalanine	0	0	0.2	0.4
Lysine	0.1	0.1	2.5	2.6
Histidine	0	0	0.9	0.9
Arginine	1.9	1.9	15.8	14.3
Yield (%)	41	18	37	50

\* Determined as cysteic acid.

† Present as homoserine lactone.

to residue 146 (Table V). Lysine, which usually occupies this position (5-7, 13), was also present but in a lower yield than would be expected if the peptide was an equal mixture of sequences containing either lysine or glutamine at this position. This may have resulted from differential recovery of the two isoproteins (or peptides) during the preparative procedures before sequence analysis. Despite this apparent discrepancy, the sequence analysis and the other findings indicated that D.F.'s E3 contained lysine at residue 146, while D.F.'s E2 contained glutamine at this site. Therefore, this E2 represents a new variant form of apo E (specified by a heretofore unrecognized fifth apo E allele) that differs from normal apo E3 by the occurrence of glutamine rather than lysine at residue 146.

*Receptor binding studies of D.F.'s apo E.* The ability of apo E · DMPC complexes to compete with <sup>125</sup>I-LDL for binding to the human fibroblast apo-B,E receptor was tested. A summary of the binding activities of DMPC complexes of the total apo E of subject D.F. and the individual E3 and E2 isoforms (Fig. 2) is given in Table VI. The total apo E of subject D.F. was only slightly defective in its ability to displace <sup>125</sup>I-LDL from fibroblast receptors as compared with control apo E prepared from an individual with the E3/3 phe-

TABLE IV  
Sequence of Peptide CB4

Residue no.	Cycle no.	D.F.		Amino acid identified	P.H.	
		nmol	(n + 1)/n*		nmol	(n + 1)/n*
	0	102			29	
109	1	30.2	0.03	Glu	11.0	0.14
110	2	12.8	0.12	Asp	10.3	0.03
111	3	41.6	0.08	Val	13.0	0.05
112	4	6.4	0	Cys	1.3	0
113	5	36.6	0.05	Gly	10.1	0.12
114	6†	14.7	ND	Arg	4.5	ND
115	7	32.0	0.11	Leu	9.5	0.14
116	8	36.0	0.14	Val	10.6	0.13
117	9	13.3	0.26	Gln	4.4	0.18
118	10	21.9	0.24	Tyr	6.8	0.22
119	11†	8.3	ND	Arg	3.3	ND
120	12	27.4	0.43	Gly	7.1	0.31
121	13	16.9	0.51	Glu	7.1	0.38
122	14	13.7	0.52	Val	4.0	0.30
123	15	3.0	0.53	Gln	0.9	0.39
124	16	0.5	ND	(Ala)	trace	ND
125	17	ND	ND	(Met)	ND	ND

ND, no determination.

\* The ratio of the nanomoles at cycle n + 1 to cycle n of the amino acid identified at cycle n.

† From aqueous phase analysis.

TABLE V  
Partial Sequence of Peptide CB5

Residue no.	Cycle no.	D.F.		Amino acid identified	P.H.	
		nmol	(n + 1)/n*		nmol	(n + 1)/n*
	0	96			90	
126	1	36.8	0.04	Leu	23.8	0.09
127	2	43.1	0.05	Gly	27.3	0.09
128	3	15.1	0.29	Gln	11.5	0.24
129	4	7.8	0.23	Ser	9.6	0.26
130	5	20.5	0.08	Thr	14.9	0.04
131	6	32.2	1.18	Glu	25.8	1.12
132	7	37.9	0.25	Glu	28.8	0.22
133	8	37.5	0.17	Leu	28.9	0.21
134	9†	5.5	ND	Arg	6.8	ND
135	10	39.6	0.20	Val	27.7	0.22
136	11†	7.9	ND	Arg	8.1	ND
137	12	37.7	0.26	Leu	26.2	0.24
138	13	41.7	0.24	Ala	24.6	0.28
139	14	11.2	0.21	Ser	6.3	0.11
140	15†	4.2	ND	His	2.1	ND
141	16	31.8	0.25	Leu	22.1	0.31
142	17†	4.7	ND	Arg	4.7	ND
143	18	10.1	0.13	Lys	9.7	0.22
144	19	29.2	0.29	Leu	20.6	0.35
145	20†	5.7	ND	Arg	4.2	ND
146	21	10.0	0.37	Gln	7.6	0.34
		1.9	0.29	Lys	1.3	0.65
147	22†	5.9	ND	Arg	6.1	ND
148	23	23.9	1.17	Leu	18.6	1.06
149	24	27.9	0.35	Leu	19.7	0.58
150	25†	5.6	ND	Arg	5.9	ND
151	26	7.8	0.31	Asp	4.4	0.41
152	27	22.4	0.50	Ala	17.8	0.48
153	28	5.5	1.20	Asp	4.6	1.30
154	29	6.6	0.39	Asp	6.0	0.32
155	30	18.0	0.59	Leu	14.5	0.46
156	31	6.4	0.56	Gln	4.8	0.54
157	32	3.8	0.39	Lys	4.0	0.28
158	33†	3.9	ND	Arg	1.7	ND
159	34	16.1	0.58	Leu	12.4	0.50
160	35	18.2	0.49	Ala	13.2	0.56
161	36	12.8	0.49	Val	9.4	0.44
162	37	8.5	0.48	Tyr	6.5	0.51
163	38	5.2	0.65	Gln	4.7	0.47
164	39	13.5	0.64	Ala	10.5	0.64
165	40	8.8	0.59	Gly	6.3	0.73
166	41	13.6	0.65	Ala	10.9	0.59
167	42†	3.2	ND	Arg	3.0	ND
168	43	6.4	0.56	Glu	7.1	0.65
169	44	10.5	0.61	Gly	7.8	0.65
170	45	10.8	0.71	Ala	8.8	0.66
171	46	6.5	0.74	Glu	5.8	0.55
172	47†	1.7	ND	Arg	2.5	ND

ND, no determination.

\* The ratio of the nanomoles at cycle  $n + 1$  to cycle  $n$  of the amino acid identified at cycle  $n$ .

† From aqueous phase analysis.

TABLE VI  
Receptor Binding Activity of Apo E · DMPC

Apo E	Concentration of apo E · DMPC at which 50% of <sup>125</sup> I-LDL was displaced from fibroblasts (ng protein/ml)*
Control E3/3	44
D.F.'s E3/2	67
D.F.'s E3 isoform	49
D.F.'s E2 isoform	129

\* Cultured human fibroblasts in which the apo-B,E receptors had been induced by preincubations with Dulbecco's modified Eagle's medium containing 10% lipoprotein-deficient serum were incubated at 4°C for 2 h with 1 ml of medium containing 2 μg of <sup>125</sup>I-LDL, various concentrations of apo E · DMPC, and 10% lipoprotein-deficient serum. Logit-log plots of the percent displacement of <sup>125</sup>I-LDL from the apo-B,E receptors at various concentrations of apo E · DMPC yielded straight and parallel lines. From these plots, the concentration of apo E · DMPC needed to displace 50% of the <sup>125</sup>I-LDL from the monolayers of fibroblasts was determined. The data are the average of separate experiments on two different preparations. The 100% control value was 97 and 88 ng of <sup>125</sup>I-LDL bound/mg of cellular protein, respectively, for the two experiments.

In an additional third experiment, D.F.'s E3 and E2 isoforms were tested at one concentration (50 ng protein/ml). The percent displacement of <sup>125</sup>I-LDL was 20% with D.F.'s E2 · DMPC and 53% with D.F.'s E3 · DMPC. There were no differences among the isoforms in their ability to recombine with DMPC. In each case, ~75% of the added protein was recovered in the DMPC complex.

notype. Furthermore, D.F.'s E3 isoform appeared to have normal binding activity, whereas D.F.'s E2 isoform had 35–40% of the activity of either control E3 or D.F.'s E3. Therefore, the new variant of apo E2 that has glutamine at residue 146 was functionally defective in its ability to bind to the apo-B,E receptor. The binding activity of D.F.'s apo E2 was similar to that reported for the apo E2 variant that has cysteine at residue 145 (7). A summary of apo E alleles, sites of substitution, and receptor binding activity is presented in Table VII. In this table, the variants are listed according to the site(s) and amino acid(s) that differ from apo E3. The nomenclature system used in this table is our proposed system for the designation of apo E variants.

**Characteristics of D.F.'s β-VLDL.** One of the major criteria for identification of type III hyperlipoproteinemia is the presence of β-VLDL. Almost all E2/2 individuals, whether they have hyperlipidemia (i.e., type III hyperlipoproteinemia) or not, demonstrate β-VLDL (i.e., dysbetalipoproteinemia) in their plasma; however, only a small number of E3/2 individuals have detectable β-VLDL (21). Therefore, it was of interest to examine the β-VLDL from subject

TABLE VII  
Summary of Apo E Alleles, Substitution Sites, and Receptor Binding Activity

Allele	Apo E	Receptor binding activity
ε3	E3 (parent)	100% (reference 8)
ε4	E4(Cys <sub>112</sub> →Arg)	100% (reference 8)
ε2	E2(Arg <sub>158</sub> →Cys)	<2% (references 7, 8)
ε2°	E2(Arg <sub>145</sub> →Cys)	45% (reference 7)
ε2**	E2(Lys <sub>146</sub> →Gln)	35–40%

Each variant form of apo E is designated by its isoelectric focusing position and by the amino acid difference compared with apo E3, which contains Cys at residue 112, Arg at 145, Lys at 146, and Arg at 158.

D.F. to determine whether its characteristics were similar to those reported for E2/2 type III hyperlipoproteinemic subjects (16).

Fainaru et al. (16) demonstrated that the *d* < 1.006 lipoproteins from type III hyperlipoproteinemic subjects and from cholesterol-fed dogs can be subfractionated into several distinct components, some of which have the property of stimulating cholesteryl ester synthesis in macrophages. The techniques of Fainaru et al. (16) were applied to the *d* < 1.006 lipoproteins of subject D.F. (Fig. 3 shows the agarose

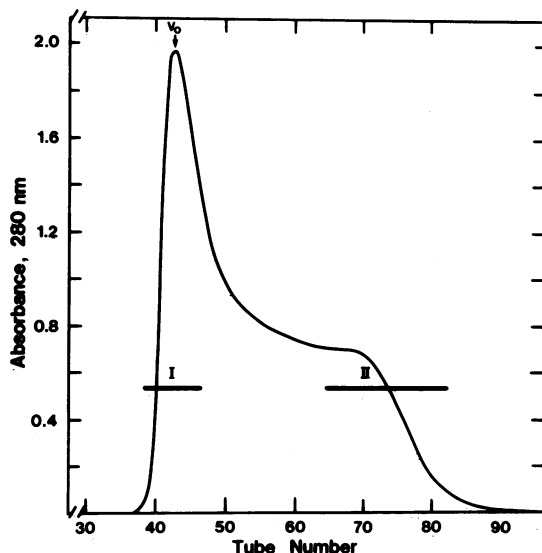


FIGURE 3 Biogel A-15m chromatography of the β-VLDL from subject D.F. The column (2.5 × 95 cm) was loaded with β-VLDL (35 mg protein) and eluted with 0.15 M NaCl and 10 mM phosphate (pH 7.4) at a flow rate of 15 ml/h at 4°C. Each tube contained 3.7 ml. The indicated fractions (I and II) were dialyzed against 0.15 M NaCl and 0.01% EDTA (pH 7.4) before use. The void volume (V<sub>0</sub>) is indicated by an arrow.

chromatography of the Geon-Pevikon block-isolated  $\beta$ -VLDL.) As shown in Table VIII, the  $\beta$ -VLDL fraction from Pevikon block electrophoresis caused significant [ $^{14}$ C]oleate incorporation into cholesteryl esters, whereas the pre- $\beta$ -migrating VLDL fraction did not. Furthermore, the Fraction I  $\beta$ -VLDL obtained from agarose column chromatography was characterized by much greater [ $^{14}$ C]oleate incorporation than Fraction II. (Fainaru et al. [16] have shown that Fraction I is probably of intestinal origin, whereas Fraction II is apparently of hepatic origin.) These results were very similar to those for  $\beta$ -VLDL subfractions from E2/2 type III hyperlipoproteinemic subjects (16); at least by this criterion, D.F.'s  $\beta$ -VLDL showed no unexpected behavior.

**Detection of E2(Lys<sub>146</sub>→Gln) in a second type III hyperlipoproteinemic subject.** The new apo E variant has also been detected in a second subject (P.H., deceased in 1979 at the age of 69). Apo E had been isolated several times from this subject in the mid-1970s and stored lyophilized since that time. The subject's lipid levels at the time of apo E isolation are reported in Table IX. The subject had a history of peripheral vascular disease and palmar xanthomas (which were resolved by therapy), and he always displayed  $\beta$ -VLDL in his plasma.

Observations of isoelectric focusing gels of P.H.'s apo E suggested that the protein had deteriorated upon storage; this was judged by increased amounts of the more acidic isoforms (E1 and below), which could possibly be due to specific deamidation, carbamyla-

TABLE IX  
Lipid Values of Subject P.H.\*

	TC	TG	VLDL-C	LDL-C	HDL-C
March 1975	453	412	273	159	21
August 1975	211	219	84	99	28
December 1976	228	145	100	92	36
January 1977	245	189	ND	ND	ND

*Abbreviations used in this table:* TC, total cholesterol; TG, triglycerides; VLDL-C, LDL-C; VLDL, and LDL cholesterol; HDL-C, high density lipoprotein cholesterol; ND, no determination.

\* Reported in milligrams per deciliter.

tion, or oxidation. However, the subject was tentatively assigned the E2/2 homozygous phenotype. Cysteine treatment caused at least some of the apo E to shift to the E4 position (not shown). In spite of these difficulties, the apo E was deemed suitable for sequence studies. Digestion of 6.6 mg of P.H.'s apo E with CNBr was carried out and the CB4 and CB5 peptides were investigated in the same manner as D.F.'s apo E. The compositions and sequences of these two peptides are reported in Tables III, IV, and V. The results indicated that cysteine occurred at residue 112 (Table IV) and that glutamine occurred at residue 146 in P.H.'s apo E (Table V). Some lysine was also detected at residue 146. In addition, the arginine value at cycle 33 of peptide CB5 was less than that expected for a full residue. Although no cysteine was detected at cycle 33 (possibly because of oxidation), it appeared that subject P.H. had either the  $\epsilon 2/\epsilon 2^*$  genotype, or, less likely, the  $\epsilon 2^*/\epsilon 2^*$  genotype. In either case, it was apparent that the glutamine for lysine substitution at residue 146 also occurred in the apo E of this subject.

TABLE VIII  
Stimulation of Cholesteryl Ester Formation in Mouse Peritoneal Macrophages by  $\beta$ -VLDL

Lipoprotein fraction added to medium*	Cholesteryl [ $^{14}$ C]oleate synthesis†	
	Subject D.F.	Subject D.R.‡
None	0.7	1.2
$d < 1.006$	7.1	2.0
Pevikon fractions		
$\beta$ -VLDL	8.1	5.0
pre- $\beta$ -VLDL	1.3	2.7
Agarose fractions		
I	18.1	13.3
II	1.9	2.7

\* The concentration of each lipoprotein in the medium was 100  $\mu$ g cholesterol/ml.

† Nanomoles per milligram cellular protein.

‡ Data from reference 16. The type III hyperlipoproteinemic subject D.R. had the E2/2 phenotype and was homozygous for E2(Arg<sub>158</sub>→Cys) (7).

## DISCUSSION

A new variant of human apo E has been described in which glutamine appears at residue 146 instead of the lysine that occurs in the parent apo E3. This substitution, which can result from a single nucleotide change in the gene for apo E3, causes the variant apo E to migrate in the E2 position on isoelectric focusing gels. The variant, apo E2(Lys<sub>146</sub>→Gln), appears to have a functional defect, as judged from its in vitro binding to human fibroblast receptors. It represents the third structurally different apo E2 to be reported and the fifth apo E structure (Table VII). Unlike the other apo E variants reported, this one does not involve a cysteine/arginine substitution. Furthermore, apo E2(Lys<sub>146</sub>→Gln) is unique among the E2 variants in that it contains only one cysteine residue, whereas the others contain two.



Homozygosity for E2 (the E2/2 phenotype) has been used as a predictor of risk for type III hyperlipoproteinemia (22), but this association is not invariant. One of the subjects in this report had the E3/2 heterozygous phenotype (D.F.,  $\epsilon 3/\epsilon 2^{**}$  genotype), and others have reported phenotypes other than E2/2 for type III individuals (22, 23). Furthermore, in light of the heterogeneity of apo E structures possible within a single phenotype, correlations will become even more difficult. For example, the three variant structures for apo E2 (Table VII) can give rise to six different genotypes for the E2/2 phenotype alone. Four of these six genotypes have been identified from this study and previous studies (7).

It is likely that other variants of human apo E exist, and there is no reason to believe that they are limited to E2 isoproteins. Furthermore, although the amino acid interchanges identified so far all involve alterations in charge, there is a strong possibility that "electrophoretically silent" mutations exist as well. The extensiveness of these phenomena remains to be determined. To date, however, all data on apo E variants are consistent with the hypothesis that the structural gene for apo E occupies a single locus (3, 4).

Although identified in two type III hyperlipoproteinemic individuals, apo E2(Lys<sub>146</sub>→Gln) displays only a slight in vitro binding defect, which is similar to that of the variant apo E2(Arg<sub>145</sub>→Cys) described previously (7). This finding reinforces the conclusion that there is an apparent discrepancy between the severity of the binding defect and its effect on risk for type III hyperlipoproteinemia (7, 12, 13).

It is of interest that certain individuals with the E3/2 phenotype have  $\beta$ -VLDL in their plasma, whereas many others do not (21). It might be speculated that the apo E3 component as well as the apo E2 component are receptor-defective and that this is what accounts for the occurrence of  $\beta$ -VLDL in those E3/2 individuals. However, as demonstrated in this study with subject D.F., receptor-defective apo E2 was present in the  $\beta$ -VLDL in association with receptor-normal apo E3. In spite of the presence of normal apo E3, the  $\beta$ -VLDL accumulated in the plasma of this patient. These findings demonstrate that the severity of the apo E in vitro binding defect is not necessarily a sufficient predictor for  $\beta$ -VLDL accumulation.

Furthermore, D.F.'s  $\beta$ -VLDL displayed the abnormal activity of delivering cholesterol to macrophages, a receptor-mediated process that may not involve apo E as the ligand. Significantly, in the macrophage study, the  $\beta$ -VLDL of D.F. were essentially identical to the  $\beta$ -VLDL from an E2/2 type III hyperlipoproteinemic subject who had the severely defective apo E2(Arg<sub>158</sub>→Cys). This suggests that neither the type of apo E nor its receptor binding activity may be crit-

ical in determining the ability of  $\beta$ -VLDL to deliver cholesterol to macrophages.

There have been previous suggestions that type III hyperlipoproteinemia may be a complex, multifactorial disorder (13, 22, 24, 25); abnormalities in addition to the presence of receptor-defective apo E2 may be required for its expression. Of the possible additional contributing factors (13, 22, 24, 25), one that is likely to be operative in the case of subject D.F. is a separate heritable hyperlipidemia, which is perhaps similar to that suggested in the studies of Utermann et al. (26) and Hazzard et al. (27). Several of the subject's offspring showed an early tendency toward hyperlipidemia. In at least one case, this cannot be due to the presence of the newly described mutant apo E, E2(Lys<sub>146</sub>→Gln), because the daughter Do.F. has the apo E phenotype E4/3. Also, this daughter does not demonstrate elevated plasma levels of apo E. (Elevated plasma apo E, as observed in the proband, is another characteristic of the type III disease and is associated with the presence of  $\beta$ -VLDL in these subjects.)

Because the total apo E of subject D.F. is relatively receptor-active, it seems less likely that accumulation of  $\beta$ -VLDL in this subject is due solely to retarded uptake (catabolism) of  $\beta$ -VLDL caused by the defective apo E. Instead,  $\beta$ -VLDL accumulation may occur in this subject because of the contribution of a separate genetic abnormality, as suggested by the family analysis. It is possible that while the newly described mutant apo E may be the underlying basis for the disease in D.F., this additional abnormality is required to trigger the expression of the gross hyperlipidemia. The nature of the supposed second defect in lipid metabolism in this family is not known at present.

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