

## Transgenic Mice Carrying the Apolipoprotein E3-Leiden Gene Exhibit Hyperlipoproteinemia\*

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Arn M. J. M van den Maagdenberg‡, Marten H. Hofker‡, Paul J. A. Krimpenfort§, Inge de Bruijn‡, Bart van Vlijmen¶, Hans van der Boom¶, Louis M. Havekes¶, and Rune R. Frants‡

From the ‡MGC-Department of Human Genetics, Leiden University, Leiden, the §Gene Pharming Europe B.V., Leiden, and ¶IVVO-TNO Gaubius Laboratory, 2300 RA Leiden, The Netherlands

**Apolipoprotein (apo) E3-Leiden, described in a large Dutch family, is associated with a dominantly inherited form of familial dysbetalipoproteinemia. To study the effect of the APOE\*3-Leiden mutation *in vivo*, transgenic mice were generated using a genomic 27-kilobase DNA construct isolated from the APOE\*3-Leiden proband. This construct carried the APOE gene, the APOC1 gene, and all known regulatory elements including an element that mediates liver expression. Three strains were generated that showed human APOE and APOC1 expression. All strains had significantly elevated levels of total plasma cholesterol and triglycerides on a regular diet. When mice of one strain were fed a semisynthetic cholesterol-rich diet, total plasma cholesterol and triglyceride levels increased dramatically. This increase was observed mainly in the very low density lipoprotein (VLDL)- and low density lipoprotein (LDL)-sized fractions. In cholesterol-fed mice, the apoE3-Leiden protein became equally distributed between the VLDL/LDL and HDL-sized fractions, while in mice kept on a regular diet, apoE3-Leiden protein was mainly associated with HDL-sized fractions. The presence of hyperlipoproteinemia in the APOE\*3-Leiden-expressing transgenic mice supports our finding that the apoE3-Leiden variant behaves like a dominant trait in the expression of familial dysbetalipoproteinemia. ApoE3-Leiden transgenic mice may serve as a model to elucidate additional factors involved in the metabolism of apoE containing remnant lipoproteins in general and the etiology of familial dysbetalipoproteinemia in particular.**

Apolipoprotein (apo)<sup>1</sup> E is one of the major structural components of chylomicron and very low density lipoprotein (VLDL) remnants (1). ApoE functions as a ligand in the receptor-mediated uptake of these remnant lipoprotein particles from the blood by the liver. Mutant forms of apoE have been described leading to an impaired clearance of remnant particles by the liver and thus resulting in familial dysbetalipoproteinemia (FD) or type III hyperlipoproteinemia (for a review, see Ref. 2). More than 90% of FD patients are homo-

zygous for the APOE\*2(Arg-158 → Cys) allele. However, only 4% of all homozygous carriers for this allele express hyperlipidemia, suggesting that other genetic and/or environmental factors are required for the expression of the disease (3).

The APOE\*3-Leiden mutation is characterized by a tandem duplication of codons 120-126 and yields a mature protein of 306 amino acid residues (4, 5). Family studies have demonstrated that this mutation is associated with a dominantly inherited form of FD (6). Recently, we have shown that the expression of FD in apoE3-Leiden carriers was influenced by other factors, such as body mass index and the second APOE allele (7). To further investigate the effect of APOE mutants on lipoprotein metabolism, these genes can be introduced in mice, allowing control of both genetic and environmental factors. Recent studies already demonstrated the feasibility of manipulating the mouse lipid metabolism by introducing human genes and studying the effect of overexpression (8-12). Overexpression of rat apoE in mice resulted in a reduction in plasma lipoproteins and resistance against diet-induced hypercholesterolemia (13).

It has previously been shown that liver expression of APOE can be obtained with constructs including 23 kb of 3' sequences, which harbor the APOC1 gene and a DNA element mediating liver expression (14). No abnormalities in lipid phenotype were observed in these mice, which express both APOE and APOC1 at high levels.

In the present study, we have examined the effect of the expression of the APOE\*3-Leiden gene in transgenic mice. We used a 27-kb fragment containing the APOE\*3-Leiden gene in addition to the APOC1 gene and the liver-positive element. ApoE3-Leiden transgenic mice display hyperlipoproteinemia, which becomes more prominent during cholesterol feeding. Since the apoE3-Leiden-expressing transgenic mice have a hyperlipoproteinemic phenotype, we have generated a mouse model to study lipoprotein remnant metabolism.

### MATERIALS AND METHODS

**Cosmid Library**—Genomic DNA used for the construction of a cosmid library was prepared from proband C.V. of the apoE3-Leiden pedigree (7). Proband C.V. is a heterozygous carrier for both the mutant APOE\*3-Leiden allele and the common APOE\*2(Arg-158 → Cys) allele. For the construction of the library, DNA was partially digested with *Mbo*I and cloned into the Supercos1 vector (Stratagene). After transformation into DH5 $\alpha$ -MCR cells (Bethesda Research Laboratories), approximately 8 × 10<sup>6</sup> colonies were obtained and screened using a human APOC1 cDNA probe (15). Recombinant clones were characterized using standard techniques (16).

**Generation and Analysis of Transgenic Mice**—Preparation of the DNA fragment for microinjection and the production of transgenic mice was performed according to standard procedures (17). In brief, a DNA solution (2.5 μg/ml) was microinjected into male pronuclei of fertilized mouse eggs taken from superovulated (C57Bl/6J × CBA/J)F1 females. Thirty-six pups were born. Genomic DNA was isolated

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<sup>1</sup> The abbreviations used are: apo, apolipoprotein(s); VLDL, very low density lipoprotein(s); LDL, low density lipoprotein(s); HDL, high density lipoprotein(s); FD, familial dysbetalipoproteinemia; kb, kilobase(s).

from 1-cm portions of their tails by standard procedures (17). Polymerase chain reaction analysis was performed on genomic tail DNA using a primer set, spanning the APOE\*3-Leiden mutation (4). Transgenic founder mice were bred with C57Bl/6J mice to establish transgenic strains. Transgenic mice of strains 2, 181, and 195 and nontransgenic littermates of generations F1 and F2 (between 6 and 10 weeks of age) were used for experiments. For Southern blot analysis, 7  $\mu$ g of genomic DNA was digested with the appropriate restriction enzyme, fractionated on 0.7% agarose gels, and transferred to Hybond N<sup>+</sup> nylon membrane filters (Amersham). Filters were hybridized with a <sup>32</sup>P-labeled human APOC1 cDNA probe, according to Church and Gilbert (18). Hybridization signals were scanned with a PhosphorImager using the software program Imagequant (Molecular Dynamics) and compared to that of a human control sample.

**Isolation of Total RNA and Northern Blot Analysis**—Total RNA was isolated from kidney and liver tissue, according to Cromczynski and Sacchi (19). Some 10  $\mu$ g of total RNA was separated through formaldehyde-containing agarose gels and blotted to Hybond N<sup>+</sup> nylon membranes, according to the manufacturer's recommendations. Blots were hybridized using <sup>32</sup>P-labeled probes of human APOC1 cDNA, human APOE cDNA (20), mouse *ApoE* cDNA (21), or 28 S rRNA (human genomic 7.3-kb *Eco*RI-fragment cloned in pAT153). Hybridizations were performed at 55 °C in a solution containing 50% formamide according to Krumlauf (22). Hybridization signals were scanned using the PhosphorImager as above.

**Analysis of Human ApoE and ApoC1 Proteins**—Lipoproteins were isolated by centrifugation of sera for 1.5 h (at 28 p.s.i.g.) in an Airfuge, using an A-100 fixed-angle rotor (Beckman), followed by dialysis overnight at 4 °C against phosphate-buffered saline. Samples were subjected to SDS-polyacrylamide gel electrophoresis, using 5–20% gradient gels. Proteins were transferred to nitrocellulose membranes, which were incubated with either polyclonal goat anti-human apoE antiserum or rabbit antiserum recognizing human apoC1, apoC2, and apoC3. As a second antibody, rabbit anti-goat IgG or goat anti-rabbit IgG antibodies, conjugated to horseradish peroxidase, were used. Human apoE concentrations in serum were measured by enzyme-linked immunosorbent assay as described earlier by Bury *et al.* (23).

**Measurement of Lipids and Lipoproteins**—After an overnight fasting period, approximately 300  $\mu$ l of blood was obtained through tail bleeding. Cholesterol and triglyceride (without free glycerol) were measured enzymatically, using commercially available kits, CHOD-PAP (No. 236691) and No. 877557, respectively (Boehringer Mannheim).

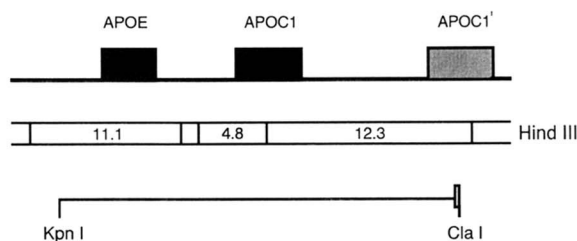
Lipoprotein patterns were determined by gel filtration chromatography through a Superose 6B column (25 ml) (Pharmacia, Uppsala, Sweden), using fast protein liquid chromatography equipment. Fasted sera of three mice of the F2 generation were pooled. Some 200  $\mu$ l of pooled serum was injected into the column and eluted with phosphate-buffered saline at a rate of 0.5 ml/min. The effluent was collected in 0.5-ml fractions.

**Diets**—Mice were housed under standard conditions in conventional cages and given free access to water and food. Mice were given regular mouse diet (SRM-A, Hope Farms, Woerden, The Netherlands). In one experiment, mice of 6 weeks of age were given a semisynthetic cholesterol-rich diet, containing 40.5% sucrose (w/w), 15% cacao butter (w/w), 1% cholesterol (w/w), and 0.5% sodium cholate (w/w) (Hope Farms, Woerden, The Netherlands), essentially according to Nishina *et al.* (24), for a period of 9 weeks.

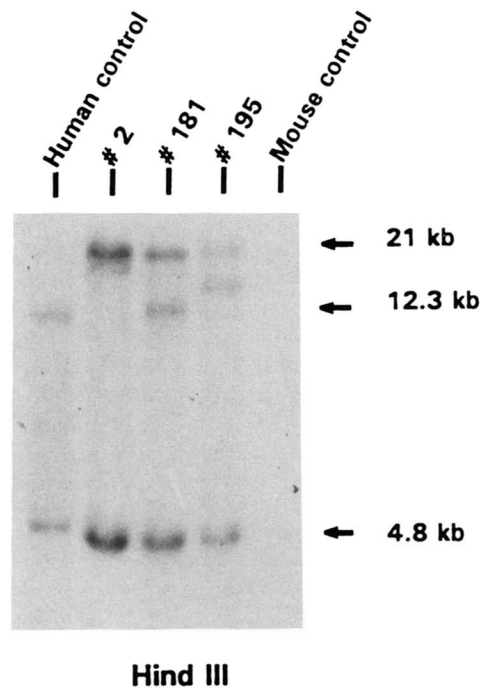
## RESULTS

**Generation of Transgenic Mice Carrying the Human APOE\*3-Leiden Gene**—A cosmid library prepared from genomic DNA of an APOE\*3-Leiden carrier was screened with a human APOC1 cDNA probe. Some 20 positive clones were identified. Three cosmids were found to carry both the APOE\*3-Leiden gene and the APOC1 gene. A 27-kb gene fragment was obtained from one of these cosmids by digestion at the *Kpn*I site located 4 kb 5' of the APOE gene and the *Cl*aI site located in the cosmid vector adjacent to the cloning site (Fig. 1). This fragment contains the APOE\*3-Leiden gene, the APOC1 gene, and the complete intergenic region between APOC1 and the APOC1 pseudogene, harboring the postulated liver regulatory element (25).

The gene fragment was injected into fertilized eggs of (C57Bl/6J  $\times$  CBA/J)F1 mice. Six out of thirty-six newborn



**FIG. 1. Schematic representation of a part of the human APOE-C1-C2 gene cluster and of the DNA construct used for microinjection.** APOE and APOC1 sequences are indicated by solid black boxes on the upper line. The APOC1 pseudogene is indicated by a gray box. A HindIII restriction map is given. Sizes of relevant restriction fragments are given in kilobases. The bottom line shows the 27-kb DNA construct, which contains at the 5'-end an endogenous *Kpn*I site and at the 3'-end a *Cl*aI site, which is present in the vector (open box). As a consequence, about 30 base pairs of vector sequences are present in the construct.



**FIG. 2. Southern blot of HindIII digests of genomic DNA isolated from tails of transgenic mice strains 2, 181, 195, and control mice.** A human DNA sample was loaded as control. The 21-kb band junction fragment and the intragenic 4.8-kb fragment were obtained in every transgenic line. Note that the variable 3'-border fragments in the transgenic lines are larger than the endogenous human 12.3-kb band.

mice were found to carry the APOE\*3-Leiden transgene, as could be detected by polymerase chain reaction analysis, using primers for the APOE gene (not shown). Three transgenic strains 2, 181, and 195 were established by breeding the founder mice with C57Bl/6J mice and used for further study.

The mode of integration of the APOE\*3-Leiden/APOC1 construct in the three strains was examined more closely by Southern blot analysis using a human APOC1 cDNA probe. Correct bands were observed in HindIII-digested DNA for every strain (Fig. 2). Digestion with HindIII yielded a 4.8-kb internal fragment and a junction fragment of approximately 21 kb, which will arise when multiple copies have integrated in a head-to-tail manner. In all strains, single unique 3' border fragments were observed, suggesting that the gene construct is integrated at a single chromosomal site in all cases. The inheritance patterns of the transgenes were in agreement with

this observation. Scanning of the hybridization signals indicated that strains 2 and 181 contained 4 and 3 copies, respectively, and strain 195 contained 2 copies (data not shown).

**Expression of the Transgenes**—Total RNA of both kidney and liver tissue was isolated to study the expression of the transgenes. Northern blot analysis showed that all three transgenic strains express human APOE and APOC1 mRNA in the liver (Fig. 3). The expression level in liver of human APOE mRNA was lower in strain 195 than in strains 2 and 181. Also for APOC1, mRNA expression levels were lower in strain 195 than in strains 2 and 181. Expression of the transgenes in strains 2 and 195 was found in the liver and not in the kidney. However, in strain 181, considerable expression of human APOE and APOC1 mRNA in kidney was observed as well. Northern blot analysis of total RNA isolated from nontransgenic littermates showed that mouse *ApoE* mRNA was expressed at high levels in liver and only in very low amounts in kidney (Fig. 3). Expression of the endogenous mouse *ApoE* mRNA in the transgenic strains was comparable with that of nontransgenic littermates.

**Apolipoprotein Analysis**—The size of human apoE3-Leiden produced in the transgenic strains was compared to mouse and human apoE by SDS-polyacrylamide gel electrophoresis, using 5–20% gradient gels, and subsequent Western blotting (Fig. 4). For immunostaining of the blot, a polyclonal antibody was used that showed partial cross-reaction with mouse apoE. Human apoE3-Leiden produced by the transgenic strains was indistinguishable in size from a human apoE3-Leiden control sample, but clearly somewhat larger than mouse apoE. With the same method of SDS-polyacrylamide gel electrophoresis and Western blotting using antibodies recognizing human apoC1, apoC2, and apoC3, significant amounts of human apoC1 were found in the transgenic mice (Fig. 4). In the human control lanes, all three apoC proteins are visible, as expected. The results show that in strains 2 and 181 the levels of human apoE and apoC1 are much higher than in strain 195. These findings agree with the plasma level of human apoE of strains 181 and 195, which is  $71 \pm 29$  mg/dl and  $3 \pm 2$  mg/dl, respectively (Table I).

**Lipoprotein Analysis**—On a regular diet, total plasma cholesterol and triglycerides were elevated about 2 and 4 times, respectively, in transgenic animals of strains 2 and 181, when compared to nontransgenic littermates (Table I). In strain

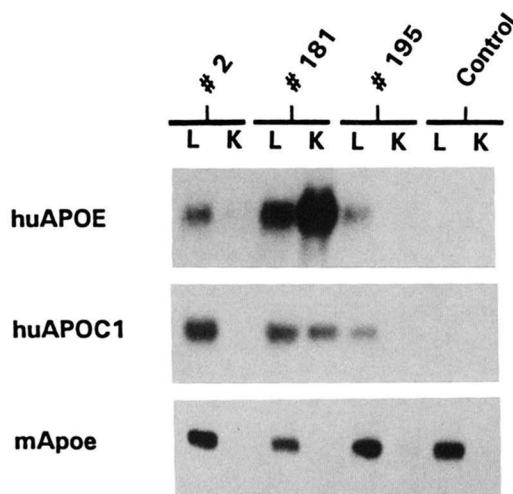


FIG. 3. Northern blotting analysis of 10  $\mu$ g of total RNA isolated from liver (L) or kidney (K) tissue of transgenic mice strains 2, 181, 195, and of a nontransgenic mouse. Blots were hybridized with human APOE probe (top), human APOC1 probe (middle), or mouse *ApoE* (bottom) probe.

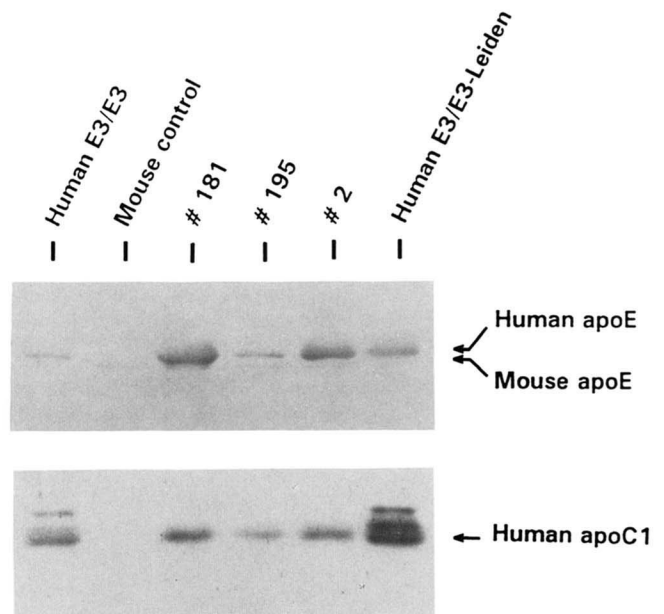


FIG. 4. SDS-polyacrylamide gel electrophoresis followed by Western blotting of isolated lipoproteins. Lipoproteins of sera of transgenic mice, control mice, and humans were isolated as described under "Materials and Methods" were used. In the human control lanes of the top panel, 12  $\mu$ l was loaded. In all other lanes, either 6  $\mu$ l (top) or 9  $\mu$ l (bottom) was loaded. Top panel, immunostaining with anti-human apoE. Note that the antibody cross-reacts with mouse apoE. Bottom panel, immunostaining with antiserum recognizing human apoC1, apoC2, and apoC3. The positions of human and mouse apoE, as well as human apoC1, are indicated. Extra bands in the human lanes correspond to human apoC2 and apoC3.

TABLE I  
Effect of human APOE\*3-Leiden expression on serum cholesterol and triglyceride levels in transgenic mice

Diet	Mice	Plasma levels		Human apoE <sup>b</sup>
		Cholesterol <sup>a</sup>	Triglycerides <sup>a</sup>	
		mmol/liter		mg/dl
Regular (SRM-A)	Control	2.46 $\pm$ 0.43	0.59 $\pm$ 0.29	
	Strain 195	3.42 $\pm$ 0.27 <sup>a</sup>	1.01 $\pm$ 0.45 <sup>c</sup>	3 $\pm$ 2
	Strain 181	4.27 $\pm$ 1.16 <sup>d</sup>	2.06 $\pm$ 0.90 <sup>d</sup>	71 $\pm$ 29
	Strain 2	4.65 $\pm$ 2.16 <sup>c</sup>	2.76 $\pm$ 1.40 <sup>a</sup>	15 $\pm$ 3
Cholesterol-rich	Control	4.37 $\pm$ 0.56	0.06 $\pm$ 0.13	
	Strain 181	67.1 $\pm$ 12.3 <sup>d</sup>	3.50 $\pm$ 2.00 <sup>d</sup>	198 $\pm$ 38

<sup>a</sup> Values are the mean  $\pm$  S.D. of six mice per group.

<sup>b</sup> Values are the mean  $\pm$  S.D. of four mice per group.

<sup>c</sup>  $p < 0.05$ , indicating the difference between transgenic and nontransgenic groups of mice on the same diet, using nonparametric Mann-Whitney test.

<sup>d</sup>  $p < 0.01$ .

195, total plasma cholesterol and triglycerides were elevated about 1.5 to 2 times. After separation of lipoprotein fractions by gel filtration chromatography of serum using Superose 6B, it is obvious that the increase of cholesterol and triglycerides in transgenic mice of strains 2 and 181 occurred mainly in the VLDL/LDL-sized fractions (Fig. 5, A and B). The amount of cholesterol in HDL-sized fractions was comparable to that observed in the nontransgenic littermates (Fig. 5D). In the less hyperlipidemic strain 195, the distribution of cholesterol and triglycerides over the lipoprotein fractions was comparable to that of nontransgenic control mice (Fig. 5, C and D).

Mice of strain 181 and nontransgenic control mice were fed a cholesterol-rich diet for 9 weeks. This diet led to an approximately 15 times increase of total plasma cholesterol in trans-

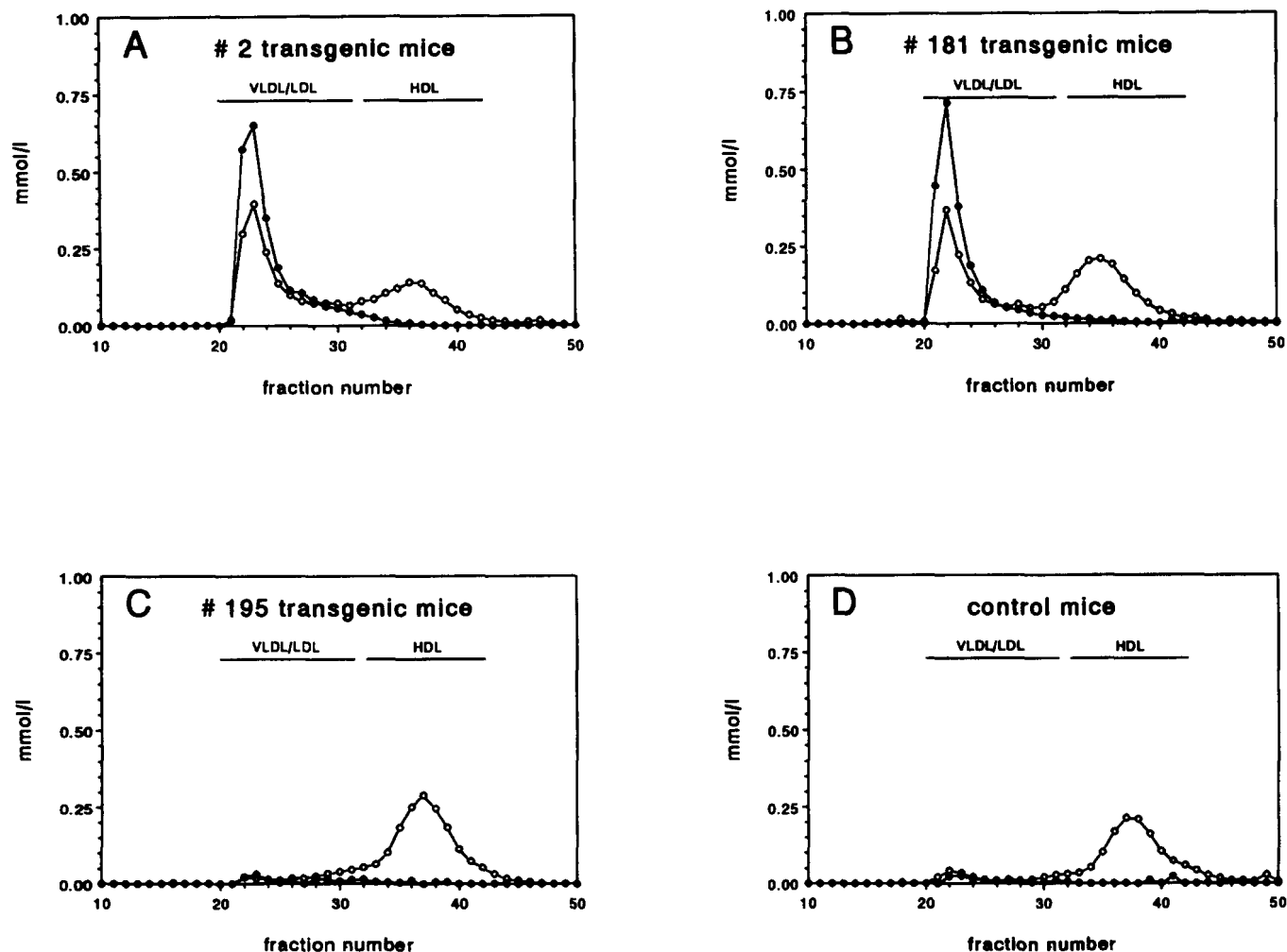


FIG. 5. Distribution of cholesterol and triglycerides over lipoprotein fractions. Lipoprotein fractions were separated by gel filtration chromatography using Superose 6B. Sera were used of mice which were kept on a regular diet. A, serum of strain 2; B, serum of strain 181; C, serum of strain 195; D, serum of control mice. Cholesterol (○) and triglyceride (●) levels (mmol/liter) are shown. The separation of VLDL/LDL- and HDL-sized fractions as observed in control mice on chow diet is indicated by horizontal bars in the figure.

genic mice, whereas in nontransgenic mice only a 2 times increase was observed (Table I). Total plasma triglycerides in the transgenic mice were almost 2 times increased, whereas in nontransgenic mice triglycerides were decreased to barely detectable levels (<0.1 mmol/liter). The elution pattern, obtained after Superose 6B gel filtration chromatography of serum, showed that the increase in cholesterol upon cholesterol feeding was mainly confined to the VLDL/LDL-sized fractions (Fig. 6). In addition, the HDL fraction has moved to a larger size range, in particular in the transgenic mice. After cholesterol feeding, triglycerides were present only in VLDL/LDL-sized fractions (not shown).

On a regular diet, in strain 181, the majority of human apoE3-Leiden protein was associated with HDL-sized particles (Fig. 7). However, upon cholesterol feeding, about one-half of the human apoE3-Leiden was associated with lipoproteins in the VLDL/LDL-sized range. Human apoC1 co-eluted with human apoE3-Leiden on both diets.

#### DISCUSSION

FD is a genetically heterogeneous disorder of lipoprotein metabolism predisposing to premature coronary and/or peripheral vascular disease (for a review, see Ref. 26). Homozygosity for the APOE\*2(Arg-158 → Cys) allele has been recognized as the primary cause in more than 90% of FD

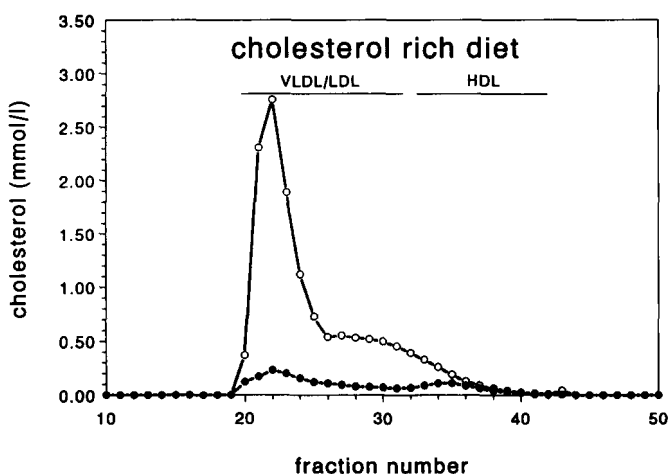


FIG. 6. Distribution of cholesterol over lipoprotein fractions. Lipoprotein fractions were separated after gel filtration chromatography using Superose 6B. Sera were used of transgenic mice of strain 181 (○) and control mice (●), which were both kept on a cholesterol-rich diet for 9 weeks. The separation of VLDL/LDL- and HDL-sized fractions as observed in control mice on chow diet is indicated by horizontal bars in the figure.



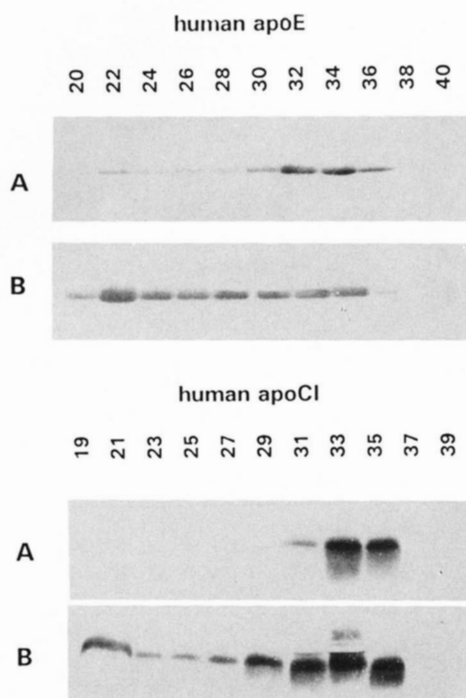


FIG. 7. Distribution of human apoE3-Leiden and human apoC1 over lipoprotein fractions. Lipoprotein fractions were separated by gel filtration chromatography. Sera were used of transgenic mice of strain 181 kept on a regular diet (A) or on a cholesterol-rich diet (B). Fraction numbers correspond to those in Figs. 5 and 6. Samples (50  $\mu$ l) were loaded on 5–20% SDS-polyacrylamide gels. After Western blotting, immunostaining was performed using either goat anti-human apoE antiserum (top panel) or rabbit antiserum against human apoC1 (bottom panel).

patients. Interestingly, heterozygosity for some rare apoE mutants, such as APOE\*3-Leiden (7), APOE\*2(Lys-146  $\rightarrow$  Gln) (27), and APOE\*3(Cys-112  $\rightarrow$  Arg; Arg-142  $\rightarrow$  Cys) (28) is associated with dominantly inherited forms of FD. In addition to this genetic heterogeneity, FD is characterized by a highly variable expression of the hyperlipidemic phenotype, which has been shown to be influenced by environmental and genetic factors (3, 7). In the present study, we describe a transgenic mouse model carrying APOE\*3-Leiden that may serve to elucidate some of these additional factors involved in lipoprotein remnant metabolism.

Three independent transgenic mouse strains were generated carrying two to four copies of a 27-kb APOE\*3-Leiden/APOC1 gene construct. Two strains (2 and 195) show liver-specific expression of both APOE and APOC1, which confirms earlier results with a similar gene construct (14). In one strain (181), the transgene is expressed also in the kidney, which might be due to a position effect. However, it has been shown that kidney-produced apoE associates normally with lipoproteins (14, 29).

The total human APOE mRNA level is lower in strain 195 than in strains 2 and 181, while the endogenous *ApoE* mRNA level is comparable to that in control mice. In all transgenic strains, both plasma cholesterol and plasma triglycerides are significantly elevated, which is mainly due to increased levels of VLDL/LDL-sized particles. This increase is most prominent in the strains with the highest apoE3-Leiden plasma levels. During cholesterol feeding, plasma cholesterol increases to extreme values (>60 mmol/liter). Also, human subjects with FD respond strongly to dietary changes. In addition, the observation that the majority of apoE3-Leiden was associated with lipoproteins in the size range of VLDL

indicates that in these transgenic mice the expression of the APOE\*3-Leiden gene causes, like in humans, an impaired clearance of apoE3-Leiden-containing lipoproteins. Interestingly, the difference in protein levels between strains 2 and 195 is much larger than the difference in mRNA levels. Apparently, a small increase of APOE\*3-Leiden mRNA level leads to a progressive plasma accumulation of apoE3-Leiden containing VLDL/LDL-sized lipoproteins, which suggests that a certain threshold level is required to cause hyperlipidemia. Despite the presence of the endogenous mouse *ApoE* gene, the transgenic mice show a hyperlipidemic phenotype, which implies that the effect of the APOE\*3-Leiden mutation is dominant in mice as well.

Previous reports demonstrated that transgenic mice overexpressing rat apoE had decreased plasma cholesterol and triglyceride levels in a dose-dependent manner (13). This study confirmed an earlier finding, that cholesterol levels could be lowered in rabbits by intravenous injection of purified apoE (30). Our transgenic mice also express the human APOC1 gene. High level expression of APOC1 in transgenic mice was shown to lead to elevated cholesterol and triglyceride levels (25) suggesting that the hyperlipidemia in the APOE\*3-Leiden mice is caused by APOC1. Although apoE and apoC1 influence lipoprotein levels, mice that express high levels of both APOE and APOC1 do not show hyperlipidemia (25). The gene construct of the present study closely resembles the construct used in the former study. Therefore, it is likely that APOE\*3-Leiden is the major cause of hyperlipidemia in these mice.

In man, mutations in the APOE gene mainly disturb chylomicron remnant VLDL and metabolism, leading to an accumulation of these particles in plasma (FD patients). This study suggests that the severity of the hyperlipidemia depends on the level of expression of APOE\*3-Leiden. This transgenic mouse model carrying APOE\*3-Leiden may help to study additional factors modulating the metabolism of apoE-containing remnant lipoproteins in general and the etiology of FD in particular.

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