

## Transgenic Mice Expressing High Plasma Concentrations of Human Apolipoprotein B100 and Lipoprotein(a)

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### Abstract

The B apolipoproteins, apo-B48 and apo-B100, are key structural proteins in those classes of lipoproteins considered to be atherogenic [e.g., chylomicron remnants,  $\beta$ -VLDL, LDL, oxidized LDL, and Lp(a)]. Here we describe the development of transgenic mice expressing high levels of human apo-B48 and apo-B100. A 79.5-kb human genomic DNA fragment containing the entire human apo-B gene was isolated from a P1 bacteriophage library and microinjected into fertilized mouse eggs. 16 transgenic founders expressing human apo-B were generated, and the animals with the highest expression had plasma apo-B100 levels nearly as high as those of normolipidemic humans (~50 mg/dl). The human apo-B100 in transgenic mouse plasma was present largely in lipoproteins of the LDL class as shown by agarose gel electrophoresis, chromatography on a Superose 6 column, and density gradient ultracentrifugation. When the human apo-B transgenic founders were crossed with transgenic mice expressing human apo(a), the offspring that expressed both transgenes had high plasma levels of human Lp(a). Both the human apo-B and Lp(a) transgenic mice will be valuable resources for studying apo-B metabolism and the role of apo-B and Lp(a) in atherosclerosis. (*J. Clin. Invest.* 1993. 92:3029–3037.) Key words: P1 bacteriophage • low density lipoproteins • cholesterol

### Introduction

Both B apolipoproteins (apo-B48 and apo-B100),<sup>1</sup> play central roles in mammalian lipoprotein metabolism (1, 2). apo-B100 is the key structural protein in the formation of triglyceride-rich VLDL and is virtually the only protein in the cholesteryl ester-enriched LDL. Epidemiologic (3) and pathologic studies (4) repeatedly have demonstrated that high plasma levels of apo-B100 and LDL cholesterol are risk factors for premature atherosclerosis. Moreover, individuals with genetic disorders such as familial hypercholesterolemia (5, 6) and familial defec-

tive apo-B100 (7–9), which are characterized by high plasma concentrations of LDL cholesterol, have an increased incidence of premature atherosclerotic disease. apo-B48, the amino-terminal 48% of the full-length apo-B100, is an obligate structural protein in chylomicron formation in the small intestine. Its synthesis results from the enzymatic editing of a single nucleotide of the apo-B mRNA (apo-B100 cDNA nucleotide 6666), which converts glutamine-2153 into a stop codon (10–12). In humans, virtually no apo-B48 is synthesized in the liver (13). In rats and mice, however, the liver expresses the apo-B mRNA editing activity (14) and therefore synthesizes both apo-B48 and apo-B100. Greeve et al. (15) found that ~70% of mouse hepatic apo-B mRNA is edited. In many mouse strains, the concentration of apo-B48 in mouse plasma exceeds that of apo-B100 (16).

apo-B100 is an important component of another atherogenic lipoprotein in human plasma, lipoprotein(a) [Lp(a)]. Lp(a) is formed by the high affinity association of apo-B100 LDL with apo(a) (17, 18). The interaction between apo-B100 and apo(a) is assumed to involve an intermolecular disulfide bond between apo(a) and one of the carboxyterminal cysteines of human apo-B100, although the existence of the disulfide bond and its location within the apo-B100 sequence have yet to be definitively established (19–22). In transgenic mice expressing human apo(a) (21), the apo(a) circulates free in the plasma rather than associating with murine lipoproteins. Infusion of human LDL into the apo(a) transgenic mice resulted in the rapid association of the apo(a) with the human lipoproteins. Based on these studies, it is presumed that human apo(a) fails to associate with mouse apo-B100 due to primary structural differences between mouse and human apo-B. It would be predicted that transgenic mice expressing both apo-B100 and apo(a) would form human Lp(a).

To study the role of apo-B100 in lipoprotein metabolism and the role of apo-B100 and Lp(a) in atherogenesis, we have developed transgenic mice expressing human apo-B100 and Lp(a).

### Methods

*Generation of human apo-B transgenic mice.* Oligonucleotides B1 (5'-GAA GAA CTT CCG GAG AGT TGC AAT-3') and B2 (5'-CTC TTA GCC CCA TTC AGC TCT GAC-3') were used to amplify a 300-bp fragment located 4543–4244 bp 5' of the apo-B transcriptional start site; oligonucleotides B3 (5'-CGG AAG GTC TCT GAA CTC AGA AG-3') and B4 (5'-CCC TCC ATA ATT TCT CCG TTT CCA-3') were used to amplify a 261-bp fragment located 179 bp downstream from the TAA stop codon in exon 29 of the apo-B gene. After optimization of the enzymatic amplification conditions, the oligonucleotides were sent to Genome Systems, Inc. (St. Louis, MO) for PCR screening

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1. Abbreviations used in this paper: apo, apolipoproteins; Lp(a), lipoprotein(a).

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of a human genomic DNA library constructed in the bacteriophage P1 (23). A single P1 clone (Dupont Merck Pharmaceutical Company-Human Foresk in Fibroblast [DMPC-HFF] no. 1-261G, here designated p158) yielded positive PCRs with both sets of primers. The identity of clone p158 was confirmed by PCRs using apo-B-specific oligonucleotides from various regions of the apo-B gene (24, 25). Automated DNA sequencing was used to verify the nucleotide sequence of 5,424 bp of clone p158, including a large portion of apo-B exons 26 and 29. In addition, p158 and pSV2neo were cotransfected into a rat hepatoma cell line, and human apo-B100-secreting stable transformants were obtained (M. Linton and S. Young, unpublished observations).

To prepare p158 DNA for microinjection, p158 DNA was isolated from the NS3529 strain of *Escherichia coli* by alkaline lysis, purified by CsCl<sub>2</sub> ultracentrifugation (26), and then cleaved with NruI. The cut DNA was subjected to electrophoresis on a 0.8% agarose gel, and the 79.5-kb band containing the apo-B gene was electroeluted into 0.5% TBE; the fragment was then repurified on a CsCl<sub>2</sub> gradient (27). The CsCl<sub>2</sub> fractions containing the 79.5-kb NruI fragment were pooled, dialyzed against microinjection buffer (27), adjusted to a concentration of 3 ng/ $\mu$ l, and then microinjected into 120 fertilized ICR eggs at the Gladstone Institutes (San Francisco, CA). The same DNA preparation also was microinjected into 185 and 59 (C57BL/6J  $\times$  SJL) F<sub>1</sub> fertilized eggs at DNX Biotherapeutics, Inc. (Princeton, NJ) and the University of Texas Southwestern Medical Center at Dallas, respectively.

Transgenic animals were identified by Southern blot or dot blot analysis of tail DNA using a <sup>32</sup>P-labeled human apo-B probe. Founders that expressed human apo-B were mated with ICR, C57BL/6J, or (C57BL/6J  $\times$  SJL) F<sub>1</sub> mice, and the transgenic offspring were identified by a specific human apo-B radioimmunoassay (see below). All mice were fed a normal chow diet.

**RNA slot blot studies.** Total cellular RNA was isolated as previously described (28) from tissues of a 5-wk-old transgenic offspring of founder M11 and one nontransgenic littermate. The slot blot studies of apo-B mRNA expression were performed as previously described (26). Three amounts of total cellular RNA (4, 1, and 0.25  $\mu$ g) were loaded onto a sheet of nitrocellulose membrane using a slot blot apparatus and then probed with a <sup>32</sup>P-labeled human apo-B-specific cDNA probe (apo-B cDNA nucleotides 7335–10068) as well as a 3.7-kb EcoRI fragment of the apo-B gene extending from intron 24 to cDNA nucleotide 6507 within exon 26. The membrane was washed at high stringency (26) and autoradiography was performed.

**Determination of transgene copy number.** For founders 1–11, tail DNA was prepared as described (29), digested with HindIII, and subjected to electrophoresis on 0.8% agarose gels. After transfer to nylon membranes, the blots were hybridized simultaneously with <sup>32</sup>P-labeled probes from exon 26 of the human apo-B gene and exon 25–intron 25 of the mouse apo-B gene. Copy number was quantitated using a Fujix phosphorimager (BAS1000; Fuji, Stamford, CT). For founders 12–16, copy number was quantitated using dot blot analysis and a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

**Human apo-B100 RIAs.** To assess the amount of human apo-B in mouse plasma samples, two different mAb-based RIAs for human apo-B100, similar to those described previously (30–33), were used. The first RIA, a very sensitive, direct-binding “sandwich RIA” using two different human apo-B-specific mAbs, was used to identify transgenic founders and their transgenic offspring, and to assess the relative amounts of human apo-B100 in fractions from density gradients or Superose 6 chromatography. For this assay, flexible polyvinyl chloride 96-well plates were coated for 4 h at 4°C with 50  $\mu$ l of PBS containing 2  $\mu$ g/ml of immunopurified mAb MB47 (30). (Antibody MB47 binds near human apo-B100 residue 3500 [34] and therefore does not bind to apo-B48.) The plates were washed four times with PBS containing 0.1% RIA-grade bovine serum albumin, 0.05% Tween 20, and 0.04% sodium azide (SPRIA) and incubated with SPRIA containing 2% BSA (SPRIA-BSA) for 1 h at 20°C to block nonspecific binding sites. To test for the presence of human apo-B100, samples (aliquots of mouse plasma, Superose 6 chromatography fractions, or density gradient fractions) were diluted in SPRIA-BSA, added in triplicate to the 96-well

plate, and incubated overnight at 4°C. The plates were washed six times with SPRIA. Then, 50  $\mu$ l of SPRIA-BSA-containing <sup>125</sup>I-labeled human apo-B-specific mAb C1.4 (35) (8,000 cpm/ $\mu$ l), which binds near apo-B100 amino acid 500, was added to each well and incubated at 4°C for 4 h. (Antibody C1.4, which was generously provided by E. Krul of Washington University [St. Louis, MO] was radiolabeled using the lactoperoxidase method [BioRad, Richmond, CA] to a specific activity of  $\sim$  10,000 cpm/ng). The plates were washed again with SPRIA and individual wells counted. Because the RIA was quite sensitive, detecting as little as 0.3 ng of human apo-B100 per well, it was capable of measuring relative amounts of apo-B100 in density fractions and Superose 6 chromatography fractions. It also was useful for determining whether a mouse expressed human apo-B since 1  $\mu$ l of transgenic mouse plasma (even from mice with very low apo-B expression levels) typically yielded > 20,000–30,000 cpm in this assay, whereas 1  $\mu$ l of nontransgenic plasma yielded a background of only 200–300 cpm. This RIA was not useful, however, for measuring the human apo-B100 content of transgenic mouse plasma because the binding curves for dilutions of human plasma and mouse plasma were not parallel.

A second competitive RIA was used to measure the human apo-B100 concentration in transgenic mice. Antibody MB47-coated 96-well plates were prepared as described above. Serial dilutions of human and transgenic mouse plasma were prepared in 25  $\mu$ l of SPRIA and pipetted into the MB47-coated wells. Then, a total of 25  $\mu$ l of a fixed concentration of <sup>125</sup>I-human LDL (6,000 cpm/ $\mu$ l) was added to each well. The human LDL sample was prepared by sequential ultracentrifugation and radioiodinated using the lactoperoxidase method to a specific activity of 5,000–10,000 cpm/ng. The 96-well plates were incubated overnight at 4°C, washed six times with SPRIA, and individual wells counted. Competition curves were plotted on semilog graph paper as  $B/B_0$  vs. the log of the  $\mu$ l of plasma added to each well, where  $B$  and  $B_0$  represent the cpm bound in the presence and absence of competitor, respectively. The competition curves for human apo-B100 in human and transgenic mouse plasma were invariably parallel. The amount of human apo-B in mouse plasma samples was determined using a human plasma secondary standard and a linear regression of logit  $B/B_0$  vs. the log of the standard concentration. The apo-B100 concentration of the secondary standard was 55 mg/dl, as judged by competitive RIA (32). The standard was stored at –70°F and used within 6 wks of the phlebotomy.

**Lipoprotein separation and analysis.** Blood was taken from the tail or retroorbital plexus of mice during the light cycle, and the plasma was isolated after centrifugation at 14,000 rpm in a microcentrifuge for 10 min at 4°C. In some experiments, phenylmethylsulfonyl fluoride (final concentration, 1 mM) was added to the plasma samples. Plasma samples (50–100  $\mu$ l) from individual mice were chromatographed on a Superose 6 10/50 column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS, and the column was eluted at a flow rate of 0.5 ml/min. 55 fractions (0.5 ml each) were collected. Cholesterol and triglycerides were measured by a colorimetric method on a 100- $\mu$ l sample of each 500- $\mu$ l Superose 6 fraction (36).

Sequential ultracentrifugation of human and mouse plasma samples (100–150  $\mu$ l) was used to isolate the VLDL ( $d < 1.006$  g/ml), intermediate density lipoproteins ( $d = 1.006$ – $1.019$  g/ml), LDL ( $d = 1.019$ – $1.063$  g/ml), and HDL ( $d = 1.063$ – $1.21$  g/ml) in a tabletop centrifuge (TL-100; Beckman Instrs., Inc., Fullerton, CA) for 2.5 h at 100,000 rpm, 10°C. After dialysis against a 0.9% NaCl, 1 mM EDTA solution (pH 7.4), each fraction was assayed to determine its cholesterol, triglycerides, and phospholipid content. The cholesterol ester content of each fraction was determined by subtracting the free cholesterol, measured with a kit (Free Cholesterol C; Wako, Osaka, Japan), from the total cholesterol, determined by an enzymatic method (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Electrophoresis of plasma samples and lipoprotein fractions was performed using 1% agarose gels as previously described (31). The gels either were dried and stained for lipid with Fat red 7B, or the proteins were transferred to a nitrocellulose membrane and immunoblotted (31) with <sup>125</sup>I-labeled antibody C1.4 or a rabbit antiserum to rat apo-B

(generously provided by R. Davis, San Diego State University, San Diego, CA). To insure that the rat antiserum would not detect human apo-B, 2 ml of the antiserum was passed over a 4-ml human LDL-Sepharose 4B column (prepared with cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions; Pharmacia, Uppsala, Sweden). The IgG was purified from the antiserum on an ImmunoPure (G) IgG Purification Kit (Pierce, Rockford, IL) according to the manufacturer's instructions and then radioiodinated using the lactoperoxidase method.

To assess the relative amounts of human apo-B48 and apo-B100 in the transgenic mouse plasma, 1–2  $\mu$ l of mouse plasma was size fractionated on 3–12% SDS-polyacrylamide slab gels as previously described (37). The separated proteins were then electrophoretically transferred to polyvinylidene difluoride membrane (Imobilon-P; Millipore, Bedford, MA) in (3-[cyclohexylamino]-1-propanesulfonic acid) (CAPS) buffer (10 mM CAPS, 10% methanol, pH 11) at 150 mA for 16 h or 300 mA for 4 h. Immunoblots were performed using  $^{125}$ I-labeled antibodies MB47 or C1.4 as previously described (30, 37). To quantitate the relative amounts of apo-B48 and apo-B100 in mouse plasma, the antibody C1.4 immunoblots were then scanned using a gel scanner (AMBIS, Inc., San Diego, CA).

**Analysis of apo(a) distribution in transgenic mouse plasma.** The human apo-B100 transgene founder 620-1 (see Table I) was bred with a male mouse hemizygous for the human apo(a) transgene (21). The resulting litter of six mice was screened for human apo-B expression in the plasma by Western blotting using mAb MB3 (31, 38). To evaluate the presence of human apo(a) in the mouse plasma and to determine if the apo(a) circulated freely or bound to lipoproteins, 1  $\mu$ l of mouse plasma was loaded onto a 4% nondenaturing polyacrylamide gel and immunoblotted as described previously (21) using a mAb specific for human apo(a), IgG-1A<sup>2</sup>, conjugated to horseradish peroxidase.

## Results

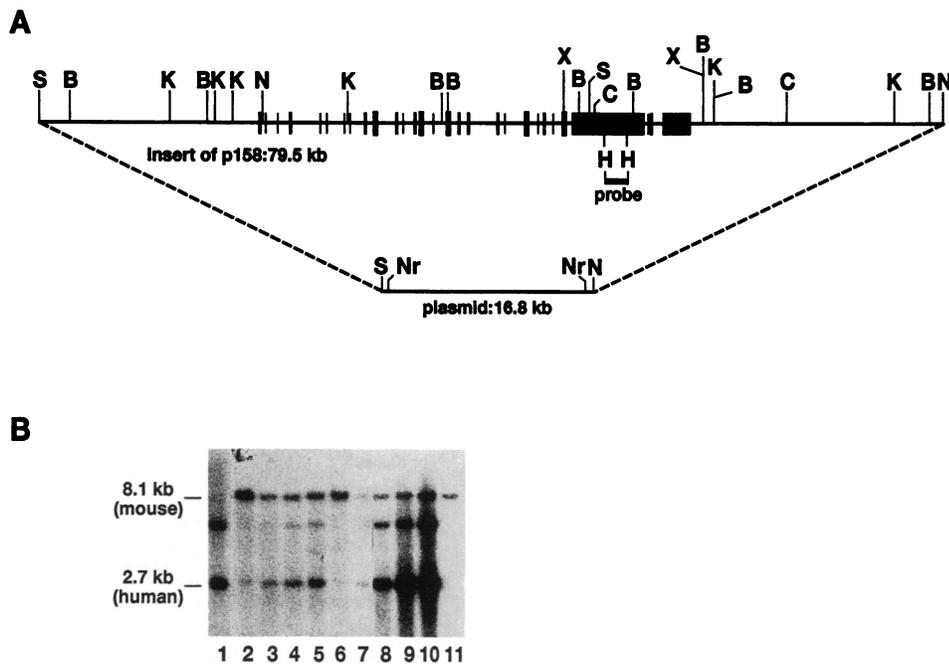
A map of the P1 bacteriophage clone containing the entire apo-B gene is shown in Fig. 1A. The P1 DNA was cleaved with

NruI, yielding a 79.5-kb insert containing < 550 bp of vector sequences. The fragment contained the entire coding region of the apo-B gene as well as 19 kb of 5' flanking sequences and 17.5 kb of sequences 3' to the gene. The purified 79.5-kb DNA fragment was microinjected into fertilized murine eggs in three laboratories, and 16 transgenic founders expressing human apo-B were obtained (Table I). The DNA of each of the founders was analyzed to determine the approximate transgene copy number by Southern blot or dot blot analysis (Fig. 1B and Table I). The human apo-B100 plasma levels of these founders, as measured by competitive RIA, ranged from 0.8 to 50.7 mg/dl (Table I). In addition to the 16 founders expressing human apo-B in their plasma, 4 transgenic animals were identified that did not express apo-B in the plasma.

A competitive RIA demonstrating the ability of several transgenic plasma samples to compete with  $^{125}$ I-human LDL for binding to immobilized antibody MB47 is shown in Fig. 2. The plasma from one of the transgenic founders (founder 1102) was equally effective in competing with  $^{125}$ I-LDL for binding to antibody MB47 as the plasma from a normolipidemic human subject. The plasma from the other two transgenic mice (621-1 and 1101) were slightly less effective competitors. Of note, the displacement curves for human and transgenic mouse plasma were parallel, indicating that the epitope for antibody MB47 is expressed similarly in the lipoproteins of human and transgenic mouse plasma.

To determine the tissue distribution of human apo-B expression, an RNA slot blot assay using tissue from one transgenic and one nontransgenic offspring of founder M11 was performed. The human apo-B mRNA was present largely in liver (Fig. 3). On a much longer exposure, very small amounts of expression could be detected in small intestine and heart.

To quantify the relative amounts of human apo-B100 and



**Figure 1.** Map of the P1 bacteriophage clone containing the human apo-B gene (A) and a Southern blot analysis of human apo-B transgenic mice (B). The P1 clone, p158, which was mapped by restriction endonuclease digestion of plasmid DNA, extends 19 kb 5' to the transcriptional start site and 17.5 kb 3' of the polyadenylation site. S, SalI; B, BamHI; K, KpnI; X, XhoI; C, ClaI; N, NotI; Nr, NruI. Approximately three BamHI and three KpnI sites, located in the far 5' and 3' regions of the insert, are not indicated because they remain unmapped. NruI cleaves the plasmid 370 and 175 bp, respectively, from the polylinker SalI and NotI sites. Genomic DNA was extracted from the tail of the founder mice and digested with HindIII; Southern blots were probed simultaneously with a 2,733-bp HindIII fragment from human exon 26 and a 700-bp exon 25–intron 25 fragment from the mouse apo-B

gene (B). The mouse probe detects an 8.1-kb HindIII fragment that includes almost all of mouse exon 26, and the human probe detects a 2,733 bp fragment. Lane 1 shows DNA from the human hepatoblastoma cell line HepG2; lanes 2–9 include founders that express apo-B in plasma (M3, M6, M11, M15, 1095, 1097, 1101, and 1102, respectively). Lane 10 contains a sample from a founder that expressed no human apo-B100 in the plasma; lane 11 shows DNA from a nontransgenic mouse. The band between the mouse apo-B band and the human apo-B band is most likely a human apo-B gene fragment resulting from a partial HindIII digestion of the genomic DNA.

Table I. Human Apo-B Transgenic Mouse Founders

Transgenic founders*	Sex	Transgene copy number <sup>‡</sup>	Human Apo-B100 <sup>§</sup>	mg/dl	
				Chol	TG
1. M3	F	1	1.9	117	80
2. M6 <sup>  </sup>	F	3	1.5	68	174
3. M11 <sup>  </sup>	M	3	5.9	126	140
4. M15 <sup>  </sup>	F	4	6.8	136	202
5. M44	F	6	12.1	146	114
6. M45	F	3	3.8	199	85
7. 620-1 <sup>  </sup>	F	42	28.3	143	224
8. 620-2 <sup>†</sup>	F	17	4.4	97	172
9. 621-1 <sup>  </sup>	F	25	41.1	175	207
10. 621-2	F	7	10.2	109	196
11. 621-6 <sup>  </sup>	M	6	11.2	130	182
12. 1095 <sup>  </sup>	F	1	0.8	99	133
13. 1097 <sup>  </sup>	M	3	2.8	133	360
14. 1099	M	1	0.9	113	229
15. 1101	F	9	31.8	155	195
16. 1102 <sup>  </sup>	F	10	50.8	164	116

Chol, total plasma cholesterol; TG, triglycerides. \* Founders 1–6 are ICR mice generated in San Francisco, CA; founders 7–11 are C57 × SJL mice generated in Dallas, TX; founders 12–16 are C57 × SJL mice generated in Princeton, NJ. <sup>‡</sup> Transgene copy numbers for animals 1–6 and 12–16 were determined from Southern blots; for animals 7–11, copy number was determined from a slot blot. <sup>§</sup> Competitive RIA for human apo-B measures only human apo-B100, not human apo-B48. <sup>||</sup> Transmission of transgene to offspring documented. <sup>†</sup> Animal died.

apo-B48 in transgenic mouse plasma, plasma samples were subjected to size fractionation on 3–12% SDS-polyacrylamide gels, and Western blots were performed using the human apo-B-specific mAb C1.4. The Western blots revealed that apo-B100 was the predominant species of human apo-B in the plasma of two founders expressing large amounts of human apo-B, 1101 and 1102 (Fig. 4 A, lanes 4 and 5); gel scanning revealed that human apo-B100/apo-B48 ratios in these mice were 3.7 and 5.4, respectively. Plasma from mice expressing lower plasma levels of apo-B100 had lower apo-B100/apo-B48 ratios: 0.09 in M11 and 1.0 in 1097 (Fig. 4 A, lanes 2 and 3).

The plasma cholesterol levels in the transgenic mice expressing high levels of human apo-B were higher than those of nontransgenic mice. For transgenic founders 7–11 and 12–16 (Table I), plasma lipid data were compared with data from nontransgenic littermates. The mean plasma cholesterol level in founders 7–11 was 130 ± 31 mg/dl vs. 97 ± 12 mg/dl in six nontransgenic littermates ( $P = 0.033$ ). The mean plasma triglyceride level in founders 7–11 was 197 ± 20 vs. 128 ± 20 in the seven nontransgenic controls ( $P < 0.001$ ). The mean plasma cholesterol level in founders 12–16 was 132 ± 27 mg/dl vs. 71 ± 15 mg/dl in five nontransgenic littermates ( $P = 0.002$ ). For the ICR founders 1–6, the plasma apo-B levels did not correlate well with the total plasma cholesterol levels. This is probably due to the diverse genetic background and cholesterol levels in this outbred strain, and due to the relatively low plasma human apo-B levels in these founders.

Founder 1102 was crossed with a C57BL/6J male, and founders 620-1 and 621-1 were crossed with (C57BL/6J

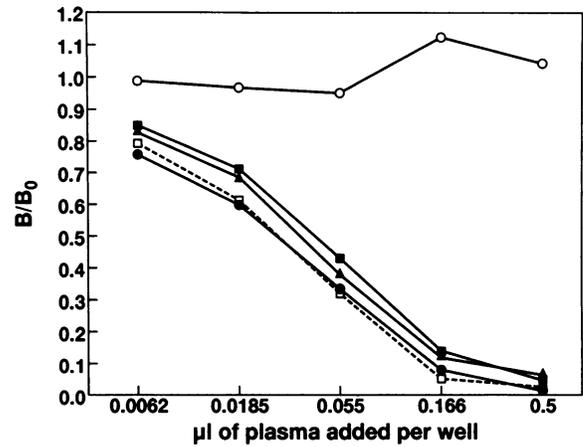


Figure 2. Competitive RIA of human and transgenic mouse plasma. The RIA was performed as described in Methods. Briefly, human or transgenic mouse plasma (from 0.0061 to 0.5  $\mu$ l) were diluted in 25  $\mu$ l of SPRIA-BSA and added to the 96-well plates. Then, 25  $\mu$ l of a fixed concentration of <sup>125</sup>I-human LDL (diluted in SPRIA-BSA) was added to each well, and the plates were incubated overnight at 4°C. The plates then were washed and individual wells counted. The human plasma sample used in this experiment had a total cholesterol level of 165 mg/dl, an LDL cholesterol level of 100 mg/dl, and an apo-B100 level of 55 mg/dl. (□---□) Human plasma; (■—■) founder 1101; (●—●) founder 1102; (▲—▲) founder 621-1; (○—○) a nontransgenic offspring of founder 1102.

× SJL) F<sub>1</sub> males. The plasma lipids were analyzed in their transgenic and nontransgenic offspring. Founder 1102, the highest expresser, had nine offspring, four of which were transgenic and had human apo-B100 levels equivalent to those in the founder (Fig. 5 C). The mean cholesterol level in the transgenic offspring was 110 ± 8 vs. 66 ± 9 mg/dl in the five nontransgenic littermates ( $P < 0.001$ ) (Table II). The mean triglyceride level in the four transgenic mice was 152 ± 10 vs. 102 ± 11 mg/dl in the nontransgenic littermates ( $P < 0.001$ ). A statistically significant difference in the plasma cholesterol level was also observed in the transgenic and nontransgenic offspring of founders 620-1 and 621-1 (Table II). The triglyceride level was higher in the transgenic offspring of 620-1 and 621-1, but the

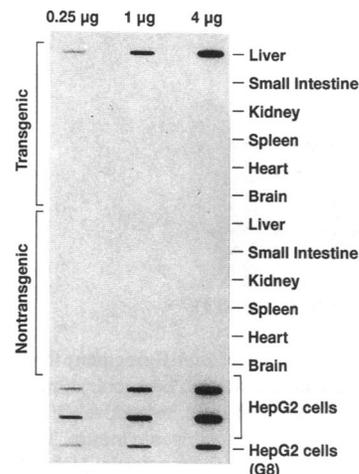
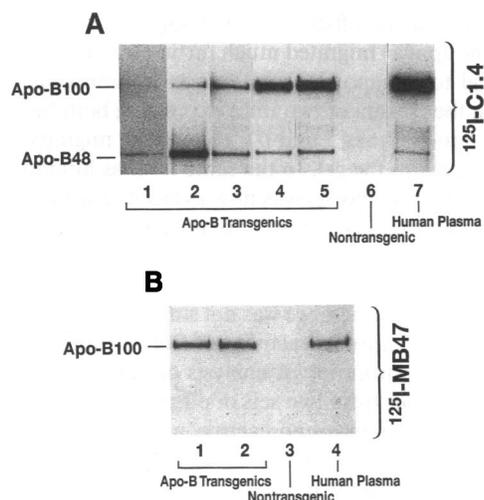


Figure 3. Slot blot analysis of the tissues sites of human apo-B mRNA expression in a 5-wk-old transgenic mouse and a nontransgenic littermate. Positive controls include two different RNA preparations from HepG2 cells and one RNA preparation from a HepG2 cell clone (G8) in which one of the apo-B alleles had been inactivated by gene targeting techniques (53).



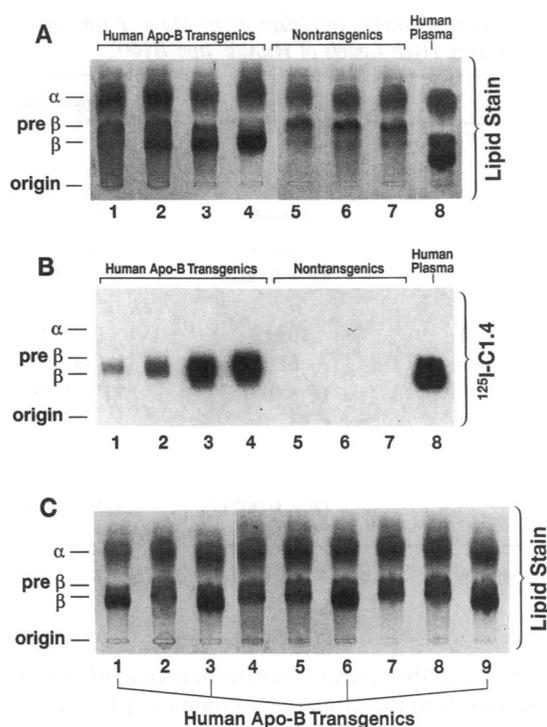
**Figure 4.** Immunoblot analysis of plasma from apo-B transgenic mice. A total of 2  $\mu$ l of mouse plasma was size fractionated on 3–12% SDS-polyacrylamide gels, and the separated proteins were then electrophoretically transferred to a sheet of Immobilon-P membrane for Western blots. (A) Antibody C1.4 immunoblot. Antibody C1.4 binds near apo-B100 amino acid 500 (35). Lane 1, plasma from founder 1095; lane 2, founder M11; lane 3, founder 1097; lane 4, founder 1101; lane 5, founder 1102; lane 6, nontransgenic mouse plasma; lane 7, plasma taken from a human subject after a fat-rich meal. The exposure for lane 1 was 4 d; for all other lanes, it was 16 h. (B) Blot using an  $^{125}$ I-labeled human apo-B100-specific mAb, MB47. Lane 1, plasma from founder 1101; lane 2, from founder 1102; lane 3, from a nontransgenic mouse; lane 4, from a human subject.

difference was not statistically significant (Table II). When all of the offspring of 620-1 and 621-1 were analyzed as a single group, however, the higher level of triglycerides in the transgenics achieved statistical significance ( $P = 0.045$ ).

To determine the lipoprotein distribution of the human apo-B100 in the plasma of the transgenic mice, plasma samples from selected founder mice and their offspring were analyzed by agarose gel electrophoresis (Fig. 5). In the plasma of the high expressing transgenic mice, the amount of  $\beta$ -migrating lipoproteins increased dramatically (Fig. 5 A, lanes 3 and 4). By Western blot analysis, the vast majority of the human apo-B was  $\beta$  migrating (Fig. 5 B). The four transgenic offspring of founder 1102 also had intense  $\beta$  bands upon lipoprotein electrophoresis (Fig. 5 C).

The analysis of mouse apo-B expression in the human apo-B transgenic mice was performed with Western blots of agarose gels using a rabbit antiserum to rat apo-B that had been passed over a human LDL-Sepharose 4B column. An  $^{125}$ I-labeled IgG fraction did not bind to human apo-B in human plasma, but detected mouse apo-B in all of the mouse samples (Fig. 6). In human apo-B transgenic mice, the mouse apo-B levels, as judged from the Western blot, were not decreased, and possibly were slightly increased (see Fig. 6, lanes 3 and 4) when compared with levels in nontransgenic littermates.

To determine the cholesterol, triglyceride, and human apo-B100 distributions among the various lipoprotein fractions, the plasma of transgenic and nontransgenic mice was analyzed by chromatography on Superose 6 columns (Fig. 7). Two of the high expresser transgenic mice (founders 1101 and 1102) had large LDL cholesterol peaks (chromatography fractions 22–



**Figure 5.** Agarose gel electrophoresis of plasma lipoproteins from a human subject and transgenic and nontransgenic mice. (A) Agarose gel stained for neutral lipids with Fat red 7B. Lane 1, the plasma of founder 1095; lane 2, founder 1097; lane 3, founder 1101; lane 4, founder 1102; lanes 5–7, nontransgenic littermates; lane 8, normolipidemic human subject. (B)  $^{125}$ I-labeled antibody C1.4 Western blot of an agarose gel. Lanes 1–8 are identical to those shown in A. For lane 1, a 22-h exposure was used; for the other lanes, a 5-h exposure. (C) Lipid-stained agarose gel of plasma samples from the nine offspring of founder 1102. Lanes 1, 3, 6, and 9 show plasma samples of offspring that, by RIA, had human apo-B100 levels identical to founder 1102. Lanes 2, 4, 5, 7, and 8 show plasma samples from nontransgenic littermates.

27) that were nearly as tall and somewhat wider than their respective HDL cholesterol peaks (Fig. 7 A). A triglyceride peak was present in the LDL fractions (Fig. 7 B) of these mice, but appeared somewhat diminished in the VLDL-sized fractions compared with nontransgenic mice. By solid phase RIA, the vast majority of the human apo-B100 resided within the LDL fractions (Fig. 7 C). Discontinuous salt density gradient ultracentrifugation confirmed that most of the human apo-B100 within the transgenic plasma of both high and low expressers was contained in the LDL density range, very similar to the distribution of LDL in the plasma of a human subject (Fig. 8).

The VLDL, IDL, LDL, and HDL fractions of founders 12–16 and five nontransgenic littermates were isolated by sequential ultracentrifugation. On lipid-stained agarose gels, the LDL fraction contained largely  $\beta$ -migrating lipoproteins, with a small amount of  $\alpha$ -migrating lipoproteins; the HDL fraction contained both  $\alpha$ - and  $\beta$ -migrating lipoproteins (data not shown). In two high-expresser founders, 1101 and 1102, the amount of cholesterol in the LDL fraction was 18-fold greater than in the nontransgenic mice. In founders 1101 and 1102, the cholesterol/triglyceride ratios in the LDL fraction were 0.75 and 2.04, respectively.

Table II. Lipid Measurements in Offspring of Three Transgenic Founders Expressing High Levels of Human apo-B100

Offspring	Cholesterol	Triglycerides
	mg/dl	
1102		
Transgenic (n = 4)	110±8	152±10
Nontransgenic (n = 5)	66±9	102±11
	(P < 0.001)	(P < 0.001)
620-1		
Transgenic (n = 2)	109±2	177±18
Nontransgenic (n = 4)	82±9	146±27
	(P = 0.015)	(P = 0.21)
621-1		
Transgenic (n = 3)	146±8	195±69
Nontransgenic (n = 3)	82±13	135±8
	(P = 0.002)	(P = 0.20)

Mean±SD are shown.

To determine whether coexpression of human apo(a) and human apo-B results in the formation of human Lp(a) in the plasma, a female human apo-B transgenic mouse (620-1) was crossed with a male mouse that was hemizygous for the human apo(a) transgene (line 275-4). Plasma from the six offspring were screened for the presence of both human apo-B and apo(a) by Western blot analysis. In addition, aliquots of plasma from each of the offspring were loaded onto a 4% non-denaturing polyacrylamide gel and immunoblotted using the human apo(a)-specific mAb, 1A<sup>2</sup> (Fig. 9). This method was shown previously to separate free apo(a) from lipoprotein-

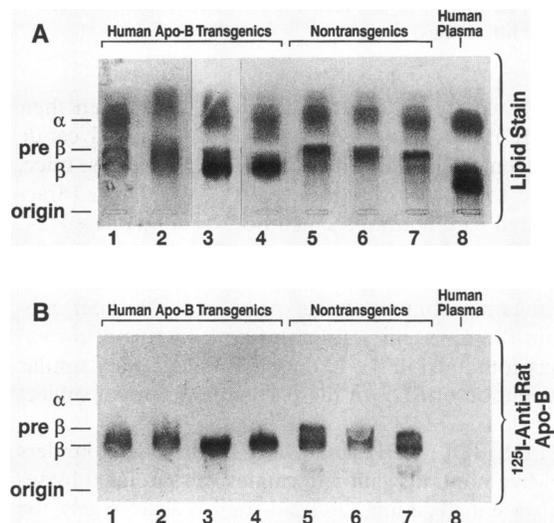


Figure 6. Agarose gel electrophoresis of the plasma lipoproteins of a human subject and transgenic and nontransgenic mice. (A) Lipid-stained agarose gel, and (B) Western blot using an <sup>125</sup>I-labeled IgG specific for rat apo-B. For both panels, lane 1, founder 1095; lane 2, founder 1097; lane 3, founder 1101; lane 4, founder 1102; lanes 5-7, nontransgenic littermates; lane 8, plasma from a normolipidemic human subject. In the high expressers (1101 and 1102), the mouse apo-B was found largely in the  $\beta$  position.

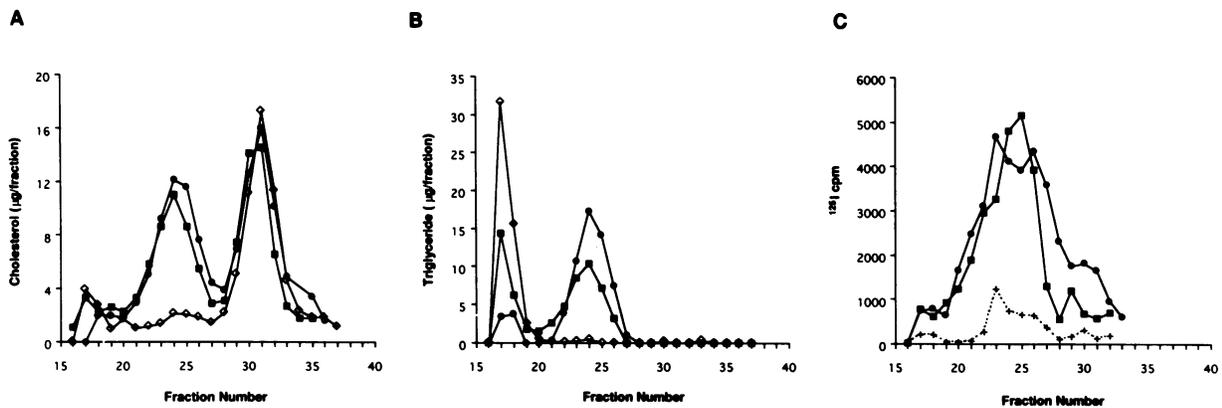
bound apo(a) (21). In the offspring expressing only apo(a) (Fig. 9, lane 4), the apo(a) migrated much further into the gel, to a level identical to the apo(a) from an apo(a)-transgenic mouse control. In the plasma of two mice expressing both human apo(a) and apo-B (lanes 5 and 6), the apo(a) migrated only a short distance into the gel, to the same level as human Lp(a), indicating that the apo(a) was now associated with lipoproteins. In the offspring, the intensity of the apo(a) immunoreactive material was greater in the plasma of the apo(a)  $\times$  apo-B mice (lanes 5 and 6) than in the mouse expressing only apo(a) (lane 4). This difference was not simply an artifact due to variation in the immunoreactivity of free vs. lipoprotein-associated apo(a). Immunoblot analysis of reduced and denatured plasma samples from two sets of offspring disclosed significantly more apo(a) immunoreactive material in the apo(a)  $\times$  apo-B mice than the mice expressing only apo(a) (H. Hobbs, unpublished observations).

## Discussion

In this study, we used DNA from a P1 bacteriophage clone that contained the entire human apo-B gene to generate transgenic mice expressing human apo-B48 and apo-B100. 16 founders were obtained that expressed a wide range of plasma concentrations of apo-B100, from 0.8 to 50.7 mg/dl. In many of the founders, the level of human apo-B100 greatly exceeded the normal apo-B levels in mice, which are  $\sim$ 3.5 mg/dl in C57BL/6J and SJL mice (16). The human apo-B mRNA was expressed in high levels in the liver. High levels of expression of the human apo-B transgene completely transformed the lipoprotein pattern of the mouse from one having extremely low levels of LDL cholesterol to one having a large amount of LDL cholesterol. The animals expressing large amounts of apo-B had a "human-like" pattern upon agarose gel electrophoresis, with an intense  $\beta$  band, corresponding to  $\beta$ -migrating LDL, and they had large human apo-B100 and LDL cholesterol peaks by chromatographic analysis of the plasma. These transgenic mice had significantly higher total plasma cholesterol levels than nontransgenic littermates, and the increase in cholesterol levels was due to increased cholesterol in the LDL fraction. In addition to markedly increased levels of LDL cholesterol, there was also a statistically significant increase in the plasma triglycerides in the transgenic mice, which was associated with the presence of triglyceride-rich LDL particles.

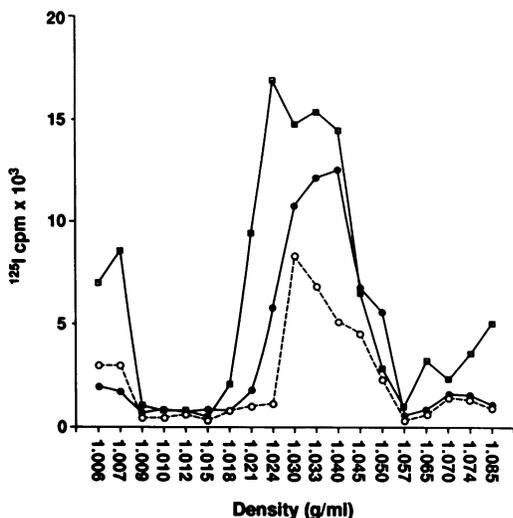
The high levels of human apo-B100 in transgenic mouse plasma are most likely the consequence of both a high rate of human apo-B100 synthesis and a slow catabolism of human apo-B100-containing particles. High levels of human apo-B mRNA in transgenic liver undoubtedly result in high levels of human apo-B synthesis and secretion. In addition to increased synthesis, the human apo-B100-containing LDL in the transgenic mice may not be cleared rapidly from the circulation; in vitro binding studies using mouse fibroblasts have demonstrated that human apo-B100 binds poorly to the mouse LDL receptor (39). Thus, retarded clearance of human apo-B100 from transgenic mouse plasma may play a role in the high plasma concentrations of human apo-B100.

The human apo-B-transgenic mice display a different phenotype than a mouse line lacking the LDL receptor described by Ishibashi et al. (40); on a normal diet, mice homozygous for the LDL receptor deficiency had a significant accumulation of



**Figure 7.** Superose 6 chromatography studies demonstrating the distribution of cholesterol (*A*), triglycerides (*B*), and human apo-B100 (*C*) in transgenic mouse and nontransgenic samples. The Superose 6 chromatographic analysis of plasma was performed as described in Methods (36, 54). Cholesterol and triglycerides were measured enzymatically, and the human apo-B100 content was assessed by the direct-binding sandwich RIA described in Methods. Fractions 16–21 contain VLDL-sized lipoproteins; fractions 22–27, LDL-sized lipoproteins; fractions 28–34, HDL-sized lipoproteins. (*A* and *B*) ( $\diamond - \diamond$ ) Average values for five separate chromatography studies of the plasma of five nontransgenic littermates of founders 1101 and 1102. (*A–C*) ( $\blacksquare - \blacksquare$ ) Founder 1101 and ( $\bullet - \bullet$ ) founder 1102. (*C*) (+---+) Distribution of human apo-B100 in the plasma of founder 1097. The Superose 6 chromatography fractions from founder 1102 were subjected to electrophoresis on 3–12% SDS-polyacrylamide gels, and a Western blot was performed using  $^{125}\text{I}$ -antibody Cl.4. Apo-B100 was visible in fractions 22–27, with a peak in fractions 23–26. Apo-B48 was visible in fractions 21–33, without a distinct peak in any of the fractions (data not shown).

cholesterol and apo-B100 in the IDL and LDL fractions. This pattern contrasts with the human apo-B100 distribution in our high-expressing transgenic mice, which was confined almost exclusively to the LDL density fraction. The difference in the phenotypes of the two mice probably relates to the fact that the LDL receptor plays an important role in the apo-E-mediated clearance of IDL. The clearance of IDL is undoubtedly defec-

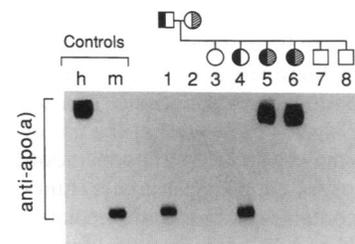


**Figure 8.** Density distribution of human apo-B100 in transgenic mouse plasma and human plasma. Discontinuous salt gradient ultracentrifugation of mouse and human plasma samples was performed as described in Methods. After ultracentrifugation, the salt gradients were unloaded into 19 fractions of 280  $\mu\text{l}$ ; the apo-B100 content of each fraction was assessed in duplicate using the sandwich RIA described in Methods. In this figure, the average  $^{125}\text{I}$  cpm for each of the 19 fractions is plotted against density. ( $\circ - - - \circ$ ) Distribution of human apo-B100 in founder M11 (25  $\mu\text{l}$  of plasma initially loaded onto gradient); ( $\bullet - \bullet$ ) founder 1102 (15  $\mu\text{l}$  of plasma loaded); ( $\square - \square$ ) normal human plasma (25  $\mu\text{l}$  of plasma loaded).

tive in the LDL receptor-knockout mice, whereas it may be relatively normal in the human apo-B-transgenic mice.

In the human apo-B-transgenic mice, it is noteworthy that the LDL was enriched in triglycerides, a finding that contrasts with LDL from human plasma, which contains only small amounts of triglycerides (1). A likely explanation for the increased triglyceride content of the transgenic LDL is that mice, unlike humans, lack cholesteryl ester transfer protein (CETP), a plasma protein that transfers triglycerides from apo-B-containing particles to HDL in exchange for cholesteryl ester (41). It would be expected that transgenic mice expressing both human apo-B and CETP would have LDL that is more enriched in cholesteryl ester, but depleted in triglycerides. Alternatively, the human apo-B100-containing, triglyceride-enriched LDL may not represent remnants of VLDL metabolism, but rather lipoproteins synthesized and secreted de novo as LDL. The relative availabilities of apo-B and lipid in the hepatocytes of human apo-B-transgenic mice conceivably could result in the secretion of nascent triglyceride-rich particles that have LDL size and density.

The levels of mouse apo-B in transgenic mice expressing large amounts of human apo-B were not decreased compared with nontransgenic mice. This result contrasts with observa-



**Figure 9.** Apo(a) distribution in plasma of transgenic mice. 1  $\mu\text{l}$  of plasma from an apo(a) transgenic mouse (lane 1), an apo-B transgenic mouse (lane 2), and their offspring (lanes 3–8) was loaded onto a 4% nondenaturing gel. A total of 0.2  $\mu\text{l}$  of human plasma (h) and 1  $\mu\text{l}$  of apo(a) transgenic mouse plasma (m) was included as controls. After electrophoresis, immunoblotting was performed using an apo(a)-specific mAb, 1A<sup>2</sup>.  $\blacksquare$ , apo(a) transgene-positive;  $\bullet$ , human apo-B transgene positive.

tions in human apo-AI-transgenic mice, where the levels of mouse apo-AI are markedly decreased (42). In the apo-B-transgenic mice, the steady-state concentration of the mouse apo-B must be determined by its rate of secretion from cells and its clearance from the plasma. As yet, we have not measured these factors. However, it will be interesting to determine whether the high human apo-B synthesis and secretion rates affect the intracellular synthesis and secretion rates for mouse apo-B, as it is widely assumed that the amount of lipid available per apo-B molecule may affect the percentage of newly synthesized apo-B molecules that are secreted from cells (43).

The ratios of human apo-B100 to human apo-B48 in two animals with high expression levels (1101 and 1102) were relatively high (~5:1), whereas the apo-B100/apo-B48 ratios were low in founders with low plasma levels of apo-B100. Lusis et al. (16) previously have reported marked differences in apo-B100/apo-B48 ratios in different inbred strains of mice, and, because our founders did not share an identical genetic background, caution is warranted in ascribing specific explanations for the different apo-B100/apo-B48 ratios. A comprehensive study involving multiple mice in multiple lines of human apo-B-transgenic mice will be needed to elucidate the mechanisms underlying the different apo-B100/apo-B48 ratios.

Coexpression of human apo-B and apo(a) in transgenic mice resulted in the production of an Lp(a) particle indistinguishable from human Lp(a). This result confirms that the lack of association of apo(a) with lipoproteins in the apo(a)-transgenic mice is due to structural differences between mouse and human apo-B100. In the double transgenic mice, all of the apo(a) in the plasma was associated with the plasma lipoproteins. Interestingly, it appears that expression of human apo-B increases the concentration of apo(a) in the plasma of the transgenic mice. Expression of human apo-B may have the effect of increasing the synthesis and/or secretion of apo(a) by hepatocytes. Alternatively, the higher plasma apo(a) concentration in the apo(a) × apo-B mice may be due to a decrease in the catabolism of Lp(a) when compared with free apo(a). Studies to elucidate the underlying mechanism responsible for the increased apo(a) levels in the Lp(a) mice are in progress. Moreover, the pathologic factors that cause Lp(a) to be atherogenic can now be studied in these animals.

The human apo-B-transgenic mice reported in this paper were generated using the insert from a P1 bacteriophage clone, p158. P1 bacteriophages package 80–100-kb segments of DNA, making this vector system attractive for recovering the ~45-kb apo-B gene (44). We are unaware of a precedent for generating transgenic mice by microinjecting P1 DNA, although significantly larger fragments of DNA derived from yeast artificial chromosomes (up to 250 kb) recently have been microinjected into murine zygotes to generate transgenic mice (45, 46). Clone p158 may prove to be useful for future studies of apo-B structure and function. Sternberg and coworkers have used transposons to produce random interruptions of P1 bacteriophage clones (47), and they have suggested that the use of transposons to interrupt exons may be useful for the generation of a series of truncated proteins for analyzing structure/function relationships. P1 clones containing transposon-mediated interruptions of apo-B exons 26 and 29 might be useful for the study of apo-B, as these large exons code for important functional regions of the apo-B100 molecule, including the receptor-binding region and the attachment site for human apo(a) (44, 48).

In future studies, it will be interesting to determine whether the transgenic mice expressing human apo-B100 and high levels of LDL cholesterol are susceptible to atherosclerosis. The studies of Paigen et al. (48, 49) have demonstrated that certain strains of mice, such as C57BL/6J, develop arterial lesions in response to a high-fat diet. The susceptibility of C57BL/6J mice to atherosclerosis depends upon the presence of several atherosclerosis susceptibility genes, including one, designated *Ath-1*, that is associated with low HDL cholesterol levels in response to a high-fat diet (48, 50). Because LDL appears to be particularly atherogenic in many mammalian species, including humans, it would be reasonable to speculate that the apo-B-transgenic mice might be susceptible to atherosclerosis in the setting of several different genetic backgrounds, perhaps even genetic backgrounds lacking the previously identified atherosclerosis susceptibility genes. It also will be interesting to determine whether the human apo-B100 mice will develop spontaneous atherosclerosis on a chow diet, as was described recently for mice lacking apo-E (51, 52).

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