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Expression of human apolipoprotein A-I in transgenic mice results in reduced plasma levels of murine apolipoprotein A-I and the appearance of two new high density lipoprotein size subclasses

(apolipoprotein A-I regulation/high density lipoprotein regulation)

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ABSTRACT **In Western societies high density lipoprotein (HDL) levels correlate inversely with the risk for coronary heart disease. The primary protein component of both human and mouse HDL is apolipoprotein A-I (apoAI), which comprises >70% of HDL protein and 30% of HDL mass. Human HDLs include particles of several distinct size subpopulations, whereas HDLs from inbred C57BL/6 mice contain a single population of particles. To study the regulation of apoAI expression and its role in HDL assembly, we created transgenic C57BL/6 mice containing the human apoAI gene. Two independent lines of transgenic mice with approximately twice the normal plasma levels of total apoAI were studied. The level of mouse apoAI is reduced >4-fold in both transgenic lines, comprising only 4% of total plasma apoAI levels in one transgenic line and 13% in the other. We demonstrate that the mechanism responsible for the decrease in mouse apoAI is posttranscriptional. Parallel to the replacement of mouse with human apoAI, the single HDL species normally present in the plasma of C57BL/6 is replaced by two HDL subclasses similar in size to human HDL_{2b} and HDL_{3a}. The changes in murine apolipoprotein levels and HDL subclass size are inherited by all transgenic offspring of the two founder animals. These results suggest a dominant role of apoAI in determining the HDL particle size distribution and a mechanism involving expression of human apoAI transgenes that alters the plasma levels of mouse apoAI.**

Several lines of evidence indicate that decreased concentrations of plasma high density lipoprotein (HDL) are correlated with increased atherosclerosis (1, 2), and elevated HDL levels are correlated with protection against coronary heart disease and increased longevity (3). The protein components of HDL include apolipoprotein A-I (apoAI; 70%), apoAII (20%), and small amounts of apoE and apoC. In addition to apoAI being the primary protein component of HDL, a central role for this apolipoprotein in the synthesis and assembly of HDL is suggested by the tight correlation between plasma apoAI and plasma HDL levels (4).

The existence of several discrete HDL size subclasses in human plasma has been confirmed in many studies (1, 5). Early studies by Gofman *et al.* (6) categorized the major subpopulations as HDL_1, HDL_2, and HDL_3. More recently, HDL subclass sizes have been further subdivided into HDL_{2a}, HDL_{2b}, HDL_{3a}, HDL_{3b}, and HDL_{3c} size groupings (7). There is also heterogeneity of apolipoprotein composition among human HDL subpopulations. Some HDL particles contain apoAI and apoAII whereas others (predominantly HDL_{2b} and HDL_{3a}) contain apoAI without apoAII (8). Recent characterization of HDL in inbred strains of mice has revealed that of the 11 strains tested all contained a monodisperse population of HDL particles (9). Mechanisms to explain the presence of multiple major HDL subpopulations in human plasma versus the single population in mouse plasma include differences in their apolipoproteins, in the activities of enzymes and lipid transfer proteins that interact to alter the lipid composition of HDL, and in diet or other environmental factors affecting plasma lipid transport.

The roles of HDL in cholesterol transport and in the development of atherosclerosis add to the importance of understanding the processes that determine plasma levels and structure of these lipoproteins. Evidence from human apoAI turnover studies (10, 11) and a study comparing differences in apoAI and HDL levels in nonhuman primates (12) suggest that the apoAI synthetic rate contributes to determining plasma HDL levels. However, other work suggests that differences in HDL concentrations are more closely related to the fractional catabolic rate of apoAI (13). Despite these conflicting results, it is likely that apoAI gene expression plays at least some role in determining HDL levels. Such a role is suggested by the finding of linkage disequilibrium in or near the apoAI gene with plasma apoAI and HDL levels (14) and by the recent observation of increased plasma HDL levels in outbred lines of transgenic mice expressing high levels of human apoAI (15). Dietary factors are also believed to affect apoAI expression and resulting HDL levels (12) although the mechanisms for these effects have not been established. In light of the complicated interactions determining apoAI and HDL levels the transgenic mouse system offers an attractive genetically defined setting where controlled studies of the regulation of apoAI and HDL plasma levels can be performed *in vivo*.

To examine regulation of the apoAI gene and the role of apoAI in the assembly of HDL particles, we created transgenic mice that express high levels of human apoAI in the inbred strain C57BL/6. Total plasma apoAI levels were elevated 1.5- and 2-fold in two lines of transgenic mice. Analysis of endogenous mouse apoAI plasma and mRNA levels indicated that high-level expression of the human apoAI transgene results in a severalfold reduction in mouse apoAI plasma levels whereas mouse apoAI tissue mRNA levels were minimally changed in the transgenic compared to control mice. Along with the replacement of mouse with human apoAI in the plasma of the transgenic animals, the single HDL species normally present in C57BL/6 has been replaced by two populations of HDL particles similar in size to human HDL_{2b} and HDL_{3a}.

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Abbreviations: apoAI, apoAII, etc., apolipoprotein A-I, apoprotein A-II, etc.; HDL, high density lipoprotein; RID, radial immunodiffusion.

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MATERIALS AND METHODS

Animals. The fertilized eggs used in the microinjection experiments were from the matings of C57BL/6 mice (obtained from Bantin & Kingman, Fremont, CA). The transgenic and control mice used in the apolipoprotein, lipid, and RNA studies were all 2-month-old males.

Preparation of DNA for Injection and Creation of Transgenic Animals. The human apoAI gene injected into the fertilized mouse eggs was contained on an 11.0-kilobase (kb) *Eco*RI fragment that, in addition to the 1.8-kb structural gene, contains 5.5 kb of 5′ and 3.5 kb of 3′ flanking DNA. (The human apoAI genomic clone was a gift of S. Karanthanasis, Childrens Hospital, Boston.) Microinjection of fertilized eggs was performed essentially as described by Gordon *et al.* (16). Eggs were reimplanted into pseudo-pregnant female mice by oviduct transfer (17).

Preparation and Analysis of DNA. Tail DNA (5 μg) was digested with restriction enzyme *Pst* I, separated on a 1% agarose gel, and transferred to nylon filters. The filters were hybridized with a 2.2-kb *Pst* I fragment of the human apoAI gene which had been ^{32}P-labeled by random priming (18).

Preparation and Analysis of RNA. Total RNA extracted from the tissues of 2-month-old male animals was homogenized in guanidine thiocyanate (19) and pelleted through a 5.7 M cesium chloride gradient.

Transcription of the human apoAI and mouse apoAI genes was studied by Northern blot analysis. The human apoAI probe was a 2.2-kb genomic *Pst* I fragment. The mouse apoAI probe was a 345-base-pair mouse 3′ cDNA. [The mouse apoAI cDNA clone was a gift of R. Elliott, Roswell Park Memorial Institute, Buffalo, NY (20).] All Northern blots were reprobed with a rat cytochrome oxidase II cDNA sequence (21). RNA isolated from HepG2 cells (22) was used as a control for the presence of human apoAI.

Apolipoprotein and Lipoprotein Measurements. Blood from 2-month-old males was collected after an overnight fast into tubes containing anticoagulant and antimicrobial agents [2 mM EDTA/gentamycin sulfate (50 μg/ml)/0.05% sodium azide] and centrifuged in a microcentrifuge for 5 min at 4°C. Plasma was separated and stored for 1 week or less at 4°C before analysis. Total lipoproteins ($d \leq 1.20$ g/ml) were isolated by sequential preparative ultracentrifugation in a Beckman 50.3 rotor (40,000 rpm, 24 hr, 10°C). Both mouse and human HDLs were isolated in the density fraction 1.063–1.21 g/ml (23).

ApoAI from mice and humans was purified from delipidated HDL by gel-sieving (1.5 × 200 cm; Sephacryl S-200; Pharmacia) and ion-exchange (DEAE-Sepharose; Pharmacia) column chromatography. Purified apoAI from both species electrophoresed as a single Coomassie blue R250-staining band of 24–28 kDA in SDS/PAGE. Purified apoAI was assayed for protein by a modified method (24) of Lowry *et al.* (25) by using human serum albumin as a calibrator (Cohn fraction V; Sigma) and stored at −70°C. Antisera to mouse and human apoAI were raised in New Zealand White rabbits.

Mouse apoAII, apoE, and apoC were similarly isolated from HDL (apoAII) and very low density lipoproteins of $d <$ 1.006 g/ml (apoE and apoC). The purified individual apolipoproteins were coupled to keyhole lympet hemacyanin prior to immunization of New Zealand White rabbits for production of antisera.

Human and mouse plasma apolipoproteins were quantified by single radial immunodiffusion (RID) assays incubated at 37°C for 24 hr using buffer (0.05 M Tris·HCl, pH 7.4/1 mM EDTA/0.05% NaN_3) containing 8 M urea as a diluent for all samples, except for apoE where samples were diluted in RID buffer containing 1% Triton X-100. RID immunoprecipitates were stained with Coomassie blue R-250 for apoAI, apoAII, and apoE and with a silver stain for apoC.

Lipoproteins were analyzed by nondenaturing PAGE and by scanning densitometry according to Nichols *et al.* (26). Immunoblot analyses of lipoproteins separated by PAGE were accomplished as described (27, 28).

Lipid Analyses. Plasma lipids (cholesterol and triglycerides) were measured colorimetrically using a microtiter plate reader and commercially available assay reagents. Total plasma cholesterol and HDL–cholesterol were measured enzymatically by incubating 30 μl of plasma diluted in saline with 150 μl of cholesterol reagent (High Performance Cholesterol Kit; Boehringer-Mannheim). HDL-cholesterol was measured in polyethylene glycol (PEG, 8 kDa; Polysciences)-treated plasma. Equal volumes (35 μl) of plasma and PEG solution [20% (wt/vol) in 0.2 M glycine (pH 10)] were mixed and, after 5 min at room temperature, the mixture was centrifuged in a microcentrifuge, and cholesterol in the supernatant was measured. Plasma triglycerides were measured enzymatically (Triglyceride Reagent Set; Seragen) and corrected for free glycerol in the plasma (Triglyceride Blank Blend; Craig Bioproducts, Steamwood, IL). The latter measurements were accomplished by incubating 25 μl of diluted plasma with 100 μl of reagent. All reaction products were spectrophotometrically quantitated at 490 nm on a microplate reader (model 580; Dynatech).

RESULTS

Production of Human ApoAI Transgenic Mice. An 11-kb human genomic DNA fragment containing the apoAI gene (Fig. 1) was injected into fertilized C57BL/6 eggs and later Southern blot analysis identified two transgenic mice. Separate transgenic lines from these two founder animals were established in the C57BL/6 background and designated A2 and A16. These transgenic animals contained ≈21 and 5 copies, respectively, of the human apoAI gene, as calculated from densitometric scanning of Southern blot autoradiographs (Fig. 1).

Human ApoAI Expression. To examine the expression of the human transgene in the mouse, total RNA was isolated from various tissues of A2 transgenic and control C57BL/6 animals and examined by Northern blot analysis using a human apoAI probe (Fig. 2*A*). High levels of the human mRNA were found in the liver of the transgenic mouse. Of the

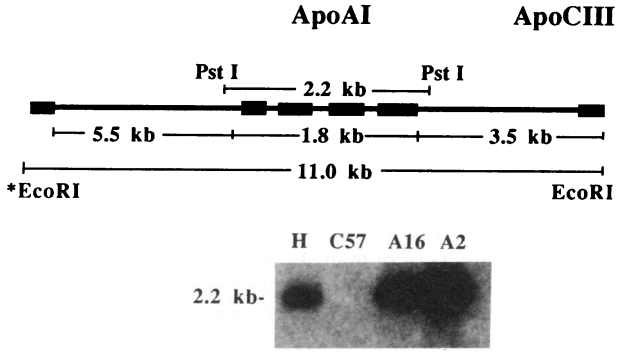

FIG. 1. (*Upper*) Restriction map of the 11-kb human apoAI genomic DNA fragment used for microinjection. This fragment contains the entire apoAI gene and, in addition, contains 2.5 kb downstream from the 3′ end of the apoAI gene, a portion of the 3′ end of the apoCIII gene. The apoAI and apoCIII genes are transcribed from opposite DNA strands. The unlabeled thickened bar at the 5′ end of the map represents a 300-base-pair segment from the pSV2 cloning vector in which the indicated **Eco*RI site is located. (*Lower*) Autoradiograph of a Southern blot containing 5 μg of human (H), C57BL/6 (C57), A2, and A16 DNA digested with *Pst* I and probed with the 2.2-kb *Pst* I apoAI fragment.

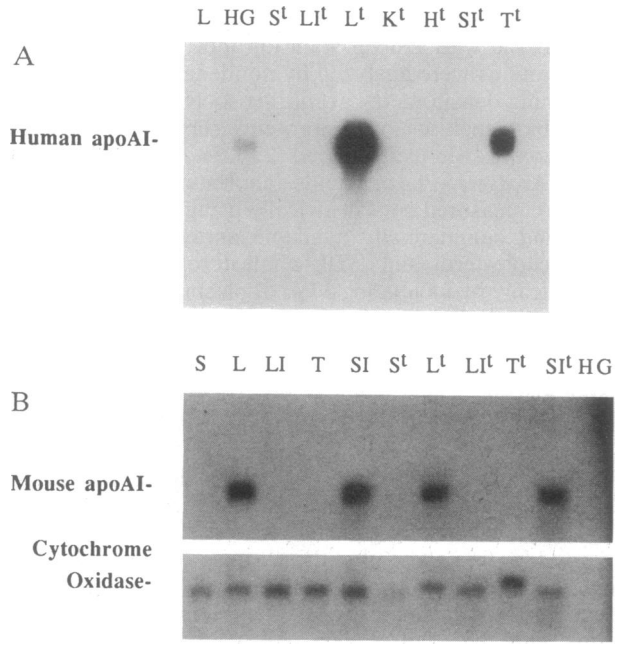

FIG. 2. Northern blot analysis of human apoAI (*A*) and mouse apoAI (*B*) expression. Tissues studied included spleen (S), liver (L), large intestine (LI), kidney (K), heart (H), small intestine (SI), testes (T), and the human liver-derived HepG2 cell line (HG). The superscript t denotes material derived from transgenic mice. (*A*) Northern blot analysis of RNA derived from the liver of a nontransgenic mouse, HepG2 cells, and the indicated transgenic tissues probed with human apoAI. (*B*) Northern blot analysis of RNA derived from the indicated nontransgenic tissues, transgenic tissues, and HepG2 cells probed with mouse apoAI. This filter was reprobed with rat cytochrome oxidase II and the results are shown.

remaining tissues tested for human apoAI expression only the testes was positive with a level ≈15% that of the liver. Testicular expression of apoAI has been shown to occur at low levels in humans (29) and African Green monkeys (12).

Mouse ApoAI Expression. Endogenous mouse apoAI mRNA levels were also studied by Northern blot analysis using a mouse apoAI probe (Fig. 2*B*). The tissue distribution of mouse apoAI expression is identical in transgenic and control animals. The level of mouse apoAI expression in the liver and small intestine of these animals was quantified by scanning autoradiographs of the same Northern blot probed with the mouse apoAI cDNA and then reprobed with cytochrome oxidase II cDNA. The ratio of intensity of murine apoAI to cytochrome oxidase II mRNA signals in the liver and small intestine of the transgenic versus the control mice differed by <20%. These studies were repeated with RNA isolated from several A2 and A16 animals and similar results were obtained.

Apolipoprotein Plasma Levels. To further examine the function and effects of human apoAI transgene expression *in vivo*, human and mouse apoAI and mouse apoAII were quantified in plasma by RID assay (Table 1). The anti-human and anti-mouse apoAI sera demonstrated <1% cross-species reactivity. Total apoAI (murine plus human apoAI) was increased >2-fold and 1.5-fold in transgenic lines A2 and A16, respectively, compared to C57BL/6 controls. This change in both transgenic lines was the result of a decrease in the absolute amount of mouse apoAI compared to control animals, compensated for by high-level expression of human apoAI transgenes. Plasma levels of mouse apoAI were reduced ≈95% in transgenic line A2 and 85% in the A16 line compared with controls. The ratio of mouse to human apoAI is 3% in the plasma of transgenic line A2 and 11% in that of transgenic line A16. The plasma levels of mouse apoAII in the transgenic versus C57BL/6 control mice were reduced ≈45%. The HDL-cholesterol levels in the transgenic mouse lines were also measured and noted to increase almost 2-fold in both lines whereas triglyceride levels remained largely unchanged between the transgenic and control mice.

Table 1. Lipid and apolipoprotein values

Molecule	C57BL/6	A2	A16
ApoAI (mouse)	112.1 ± 10.2	6.5 ± 2.2	17.3 ± 8.5
ApoAI (human)	0	245.3 ± 9.6	156.1 ± 4.0
ApoAII (mouse)	45.7 ± 8.1	25.1 ± 2.4	24.3 ± 4.6
Total cholesterol	77.8 ± 7.1	127.1 ± 11.1	110.7 ± 3.5
HDL-cholesterol	64.1 ± 8.2	118.8 ± 10.3	109.7 ± 24.0
Triglycerides	33.2 ± 5	46.0 ± 11.4	35.3 ± 17.1

Data are presented as mg/dl (mean ± SEM). Seven C57BL/6 animals and five transgenic animals of each line were used.

Size Characterization of HDL Particles in the Plasma of Transgenic Mice. To analyze the size distribution of mouse HDL, plasma lipoproteins were separated on nondenaturing 4–30% polyacrylamide gradient gels, and computer assisted densitometry was used for determining particle sizes (Fig. 3). HDL isolated from C57BL/6 control animals consists of a single size population of particles with a mean diameter of 9.5 ± 0.2 nm, in agreement with previous sizes reported for HDL from this inbred strain (29). The two transgenic founder animals and their offspring contain two distinct HDL subpopulations. The peak diameters of the larger HDL particles in the two transgenic lines are between 10.2 and 10.8 nm and the smaller HDL particles are between 8.4 and 8.6 nm (Fig. 3). These diameters fall within the size ranges for human HDL_{2b} and HDL_{3a} (7), as illustrated in Fig. 3. The predominant population of HDL particles of nontransgenic C57BL/6 mice is no longer present in the plasma of the transgenic animals, and the HDL profiles of the two transgenic founder animals as well as their transgenic offspring are all remarkably similar.

Association of Apolipoproteins with HDL Particles. The human and mouse apoAI distribution among HDL particles in the plasma of transgenic mice was examined by Western blot analysis of HDLs separated on nondenaturing 4–30% polyacrylamide gradient gels using antibodies to human and mouse apoAIs. After hybridization to either anti-mouse or anti-human apoAI antibodies, the blots were scanned and the sizes of the lipoproteins associated with these apolipoproteins were determined (Fig. 4). Human apoAI in the transgenic mice (Fig. 4*A*) is found in two major discrete bands of similar size to the two HDL species observed by protein staining of HDL (Fig. 3). However, in comparison with the results for protein-stained HDL, the height of the apoAI peak for the species of larger diameter (HDL_{2b}) is increased relative to the height of the peak of smaller diameter. Possible explanations for this finding include a relatively greater immunoreactivity of the apoAI in the larger versus the smaller HDL species, differential transfer to nitrocellulose of the two HDL species, or an increase in the ratio of apoAI to other proteins in the larger HDL. The anti-mouse apoAI antibody, which does not cross-react with human apoAI, recognizes to various degrees the same sized subpopulations of HDL particles in transgenic mice (Fig. 4*B*). A much weaker signal was seen with the anti-mouse apoAI antibody than with the anti-human apoAI antibody, consistent with the much lower concentration of mouse apoAI than human apoAI in plasma from the transgenic mice. In data not shown, mouse apoAII, apoE, and apoC are also evenly distributed among the two major transgenic HDL subspecies.

DISCUSSION

The creation of apoAI transgenic mice in the C57BL/6 background, as described in this study, provides a defined

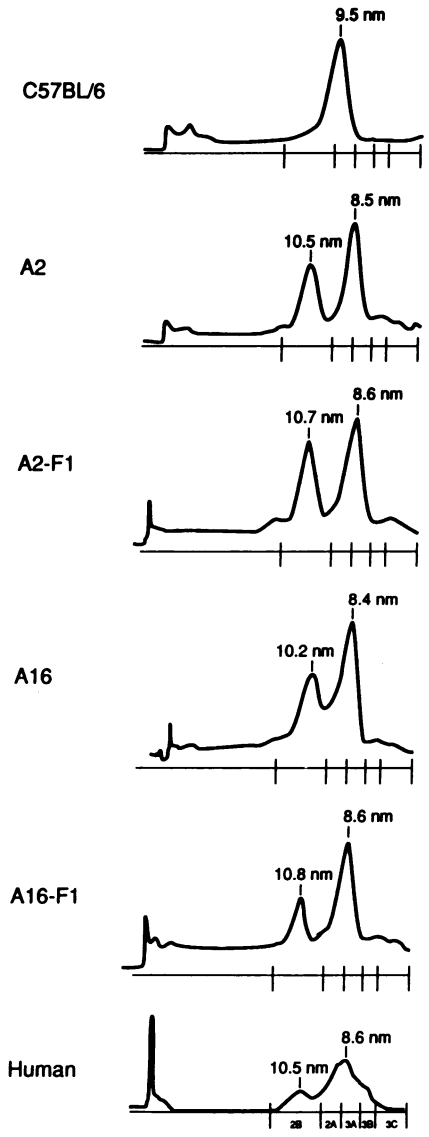

FIG. 3. Distribution by size of HDL particles. Plasma lipoproteins ($d < 1.210$ g/ml) isolated from a normolipidemic human subject, a control C57BL/6 mouse, the two transgenic C57BL/6 founder mice (A2 and A16), and a transgenic offspring of each (A2-F1 and A16-F1) were subjected to nondenaturing PAGE to size-fractionate the HDLs. Computer-assisted densitometry, utilizing coelectrophoresed molecular size calibrators, was used to facilitate determination of lipoprotein particle sizes. The scale at the bottom of the figure gives the particle size ranges of the major human HDL subspecies identified by this procedure (7).

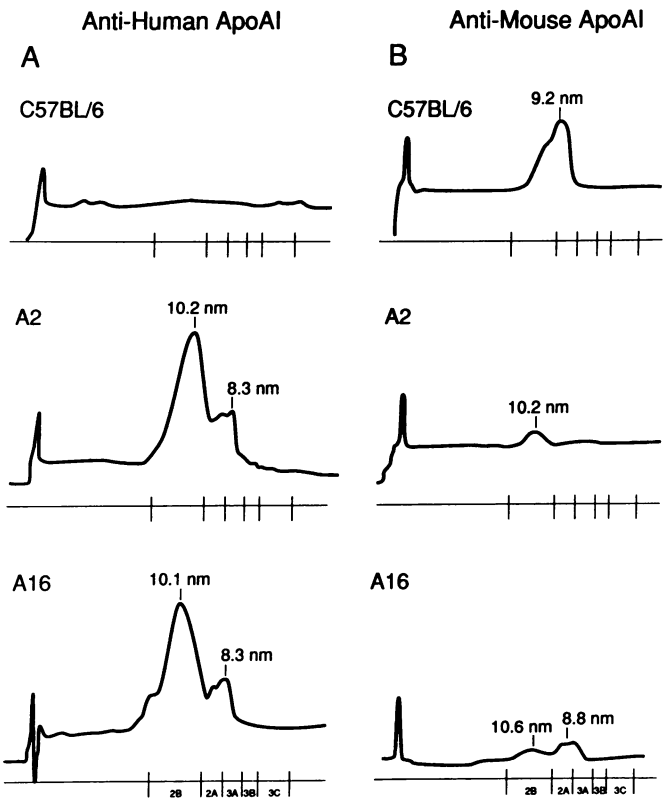

FIG. 4. Size distribution of lipoprotein particles associated with mouse and human apoAI. Lipoproteins separated by nondenaturing PAGE were hybridized with anti-human apoAI antibodies (*A*) and anti-mouse apoAI antibodies (*B*). Computer-assisted densitometry of the hybridized filter, on which the migration distances of the coelectrophoresed molecular size calibrators had been marked, was used to facilitate determination of lipoprotein particle sizes.

genetic setting in which to study the regulation of this gene as well as its influence on the structure of HDL. We have found that the introduction of human apoAI transgenes into mice results in increases in total plasma apoAI due to expression of the human apoAI gene, a marked decrease in mouse apoAI plasma levels, and the replacement of the single mouse HDL species with two distinct HDL subspecies. The observations that high levels of plasma apoAI in the transgenic mice are associated with an elevation of HDL–cholesterol and that the human transgene is expressed primarily in the liver, less so in the testes, and not at all in the small intestine confirm the findings of Walsh *et al.* (15).

The reduction of the endogenous mouse apoAI plasma levels in the two transgenic lines is an unexpected consequence of high-level expression of human apoAI transgenes. When the two transgenic lines A2 and A16 were compared, the former, with the higher plasma levels of HDL and human apoAI, has the lower mouse apoAI plasma level. This suggests that it may be the level of HDL or total apoAI that regulates mouse apoAI plasma levels.

The finding that the mouse apoAI mRNA level is reduced by no more than 20% in the transgenic animals indicates that the 90% reduction of murine apoAI in plasma must be occurring primarily through a posttranscriptional mechanism. A variety of steps in the pathway from murine apoAI synthesis to catabolism could be altered in the transgenic mice expressing high levels of human apoAI. The finding that the plasma level of mouse apoAII was also reduced in the apoAI transgenic animals suggests an altered production or metabolism of HDL particles.

The appearance of two HDL subclasses in the transgenic mice that replace the single species present in the control animals is also an unexpected finding. The particle sizes of the two transgenic HDL subclasses as determined by gradient gel electrophoresis are similar to those for human HDL_{2b} and HDL_{3a} (7). Both HDL_{2b} and HDL_{3a} include HDL particles containing apoAI without apoAII (8) and cross-linking studies with these particles have shown that they contain three and four molecules of apoAI, respectively. Although the molar content of human apoAI and other apoproteins in HDL subclasses from the transgenic mice has not yet been determined, immunostaining of human apoAI in HDL transferred from nondenaturing gradient gels (Fig. 4) showed that the apoAI was distributed in two discrete bands corresponding in size to HDL_{2a} and HDL_{3b}. Interestingly, despite the large difference in plasma apoAI levels in the two transgenic strains, the distribution of apoAI among the HDL subclasses was similar, suggesting that the content of apoAI in the major HDL subspecies was fixed and that excess apoAI, particu-

larly in strain A2, was not present in HDL particles. Ultracentrifugation has been shown to displace apoAI from HDL particles (30); therefore, chromatographic techniques will be required to perform a more detailed analysis of the distribution of apoAI in mouse plasma and within HDL subclasses.

Alteration of mouse HDL particle size by expression of human apoAI in the mouse liver could involve the role of apoAI in HDL assembly or the interactions of human apoAI with other proteins and lipids that affect HDL size. Although the sequence of the mouse apoAI protein has not been determined, it is likely that it differs from the human protein, as indicated by our failure to observe significant immunochemical cross-reactivity of human and mouse apoAI or cross-hybridization between the human and mouse apoAI genes on Southern and Northern blots. Spherical particles resembling HDL_{2b} and HDL_{3a} can be formed *in vitro* from human apoAI–phospholipid discoidal structures incubated in the presence of the enzyme lecithin:cholesterol acyltransferase and a source of free cholesterol (31). The present findings are consistent with these *in vitro* results that suggest that the structure of the human apoAI molecule is an important determinant of HDL particle size distribution.

The decrease in endogenous mouse apoAI plasma levels as well as the changes in HDL particle size documented in this study are in contrast to the findings reported by Walsh *et al.* (15). In their study, introduction and expression of human apoAI transgenes in mice resulted in no significant changes in mouse apoAI levels and a predominance of small protein-enriched HDL particles comparable to human HDL_3. Walsh *et al.* (15) introduced the human apoAI transgene into an outbred mouse background and the lack of a mouse-specific apoAI antibody precluded the direct measurement of mouse apoAI levels. The discrepant results might be explained by differences in the genetic backgrounds of the mice studied, differences in the flanking sequences surrounding the human apoAI gene, or differences in the methods employed to study the properties of HDL. Further studies will be required to evaluate these possibilities as well as the influence of factors, such as age, hormonal status, and diet, all of which might influence the expression of the human apoAI gene and HDL structure in transgenic mice.

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