review

Genetically modified mice for the study of apolipoprotein B

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Abstract Over the past five years, several laboratories have used a variety of transgenic and gene-targeted mice to study apoB. These studies have helped in *1*) generating new mouse models suitable for investigating the genetic and environmental factors affecting atherogenesis; *2*) providing systems for investigating apoB structure/function relationships; *3*) understanding the regulation of apoB gene expression in the intestine; *4*) delineating a critical role for apoB expression in mouse embryonic development; *5*) yielding insights into the "physiologic rationale" for the existence of the two different forms of apoB, apoB-48 and apoB-100, in mammalian metabolism; and *6*) providing basic insights into mechanisms involved in the human apoB deficiency syndrome, familial hypobetalipoproteinemia.**—Kim, E., and S. G. Young.** Genetically modified mice for the study of apolipoprotein B. *J. Lipid Res.* 1998. **39:** 703–723.

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The B apolipoproteins, apoB-100 and apoB-48, play central roles in lipoprotein metabolism and plasma lipid transport (1, 2). ApoB-100 is essential for the assembly of VLDL in the liver, while apoB-48 is required for chylomicron formation in the intestine. In addition, apoB-48 and apoB-100 are key components in all classes of lipoproteins considered to be atherogenic, including LDL, IDL, chylomicron and VLDL remnants, and Lp[a] (1, 2).

Both apoB-100 and apoB-48 are derived from a common structural gene (3–5). ApoB-100 contains 4536 amino acids, while apoB-48 contains the amino-terminal 2152 amino acids of apoB-100 (1, 2). The synthesis of apoB-48 results from the enzymatic editing of a single codon of the apoB mRNA, which converts codon 2153 (CAA, specifying glutamine) into a premature stop codon (UAA). Virtually all of the apoB produced by the intestine is apoB-48, as a result of the abundant expression of APOBEC-1, the catalytic component of the apoB mRNA-editing complex. In human liver, APOBEC-1 is absent, thus only apoB-100 is synthesized. However, in some rodents, such as rats and mice, a sizable fraction of the hepatic apoB transcripts are edited, causing the liver to secrete both apoB-48 and apoB-100-containing lipoproteins (6–10).

Over the past 5 years, several laboratories, including our own, have used both conventional transgenic mice and gene-targeted mice to investigate various aspects of apoB biology. This review will summarize what has been learned from these studies.

APOB TRANSGENIC MICE

Many lines of evidence have indicated that high plasma levels of apoB-100 and LDL cholesterol are risk factors for atherosclerosis (11–13). To study the role of apoB-100 in lipid metabolism as well as in atherogenesis, several investigators have developed mice expressing human apoB-100 (14–16).

ApoB transgenic mice generated with a minigene expression vector

Soon after the apoB cDNA and gene were cloned, Blackhart, Yao, and McCarthy (17) generated an apoB-

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; IDL, intermediate density lipoproteins; HDL, high density lipoproteins; Lp[a], lipoprotein[a]; FPLC, fast performance liquid chromatography; CETP, cholesteryl ester transfer protein; RT-PCR, reverse transcription polymerase chain reaction; BAC, bacterial artificial chromosome; YAC, yeast artificial chromosome; MTP, microsomal triglyceride transfer protein; FHβ, familial hypobetalipoproteinemia; ES, embryonic stem.

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100 minigene expression vector from cDNA and genomic clones. The 5′ portion of the minigene was constructed from cDNA clones, while the 3′ end consisted of an ~12-kb genomic clone that spanned from the middle of exon 26 to a *Kpn*I site located several kb 3′ to the apoB gene's polyadenylation signal. Although this minigene and various derivatives yielded human apoB expression in cultured hepatoma cells (17), they did not work well for generating transgenic mice. Despite enormous effort and the production of dozens of transgenic founder mice, only one transgenic line expressing apoB was ever developed (16). Unfortunately, the levels of human apoB in the plasma of those mice were extremely low, making it impossible to study the metabolic consequences of apoB overexpression. However, the transgenic mice did prove to be useful for demonstrating that the "apoB mRNA-editing codon" of the human apoB transcript was edited efficiently by the mouse apoB mRNA-editing machinery (16).

The reason that minigene vectors did not yield high levels of apoB expression in transgenic mice has never been defined. In general, constructs containing introns yield higher levels of expression in trangenic mice than contructs containing only cDNA sequences (18, 19), particularly when the cDNA sequences are more than 2 kb in length (20). Although the apoB minigene contained three introns (introns 26, 27, 28), they were located near the 3′ end of the minigene, perhaps too far away to have a salutary effect on transgene expression. Also, the absence of introns 1–3 from the minigene vector might have been responsible for the low levels of expression because there is evidence to suggest that regulatory sequences may exist within those introns (21, 22).

ApoB transgenic mice generated with a P1 bacteriophage clone (p158)

Achieving high levels of human apoB expression in transgenic mice depended on an alternate strategy that used large fragments of genomic DNA spanning the entire human apoB gene. Cloning genomic DNA fragments sufficiently large to span the apoB gene became routine in the early 1990s after Sternberg and Pierce (23, 24) introduced the P1 bacteriophage vector. Our laboratory, as well as Callow and collaborators (15), was successful in identifying a single P1 clone (p158) that contained an 80-kb insert spanning the entire human apoB gene. In addition to the ~43 kb structural gene, p158 contained 19 kb of 5′ flanking sequences and 17.5 kb of 3′ flanking sequences (14, 25). Microinjection of the insert from p158 into fertilized mouse eggs resulted in dozens of transgenic founder mice. Virtually all of the transgenic mice expressed substantial amounts of human apoB in the plasma, with the plasma levels appearing to be proportional to the transgene copy number (14, 15). In transgenic lines with more than 10 copies of the transgene, the plasma levels of human apoB in chow-fed hemizygous mice were 60 to 80 mg/dl, similar to those in normolipidemic humans (14).

Lipid metabolism in transgenic mice overexpressing human apoB. The plasma of the p158-apoB transgenic mice contains large amounts of both human apoB-100 and human apoB-48. The presence of human apoB-48 in the plasma is due to the fact that ~70% of the human apoB transcripts in the mouse liver are edited (26). As judged by Western blot analysis, the ratio of apoB-100 to apoB-48 in the plasma of chow-fed transgenic mice is ~5:1, although this ratio increases to ~10 or 15:1 after an overnight fast. On a chow diet, both male and female human apoB transgenic mice have higher plasma levels of cholesterol than littermate control mice [144.2 ± 7.4 mg/dl in female transgenic mice vs. 103.4 ± 4.2 mg/dl in nontransgenic controls; $P < 0.0001$, as a result of a significantly increased mass of low density lipoproteins (LDL)] (**Fig. 1**, panel A) (26). Interestingly, the plasma triglycerides are also significantly elevated, due almost entirely to the fact that the LDL of the transgenic mice is strikingly enriched in triglycerides (Fig. 1, panel B).

The triglyceride enrichment of LDL in the human apoB transgenic mice is perplexing. One possibility is that the triglyceride-rich LDL actually represent nascent lipoproteins rather than remnant particles. For example, overexpression of apoB in the setting of a fixed amount of neutral lipid synthesis might lead to the production of triglyceride-rich LDL particles. Alternatively, the triglyceride enrichment of LDL might relate to differences in lipoprotein processing within the plasma. We initially hypothesized that the triglyceride-rich LDL might be due to the fact that mice lack cholesteryl ester transfer protein (CETP). In humans, CETP is thought to transfer triglycerides from the apoB-containing lipoproteins to HDL (27, 28); therefore, the absence of CETP in the mouse might reasonably be expected to cause triglyceride accumulation within the LDL. However, this potential explanation now appears unlikely as transgenic mice expressing high levels of both human apoB and human CETP manifest triglyceride-enriched LDL (29). In addition, transgenic rabbits overexpressing human apoB also have triglyceride-rich LDL (30), even though this species expresses CETP. Other possibilities were that the triglyceride-rich LDL might reflect inadequate levels of hepatic lipase activity or, in some way, might result from the fact that hepatic lipase circulates in the plasma of mice (31). However, these hypotheses also are unlikely, as the LDL triglyceride/cholesterol ratio remains high (~1.2:1) in transgenic mice expressing both human

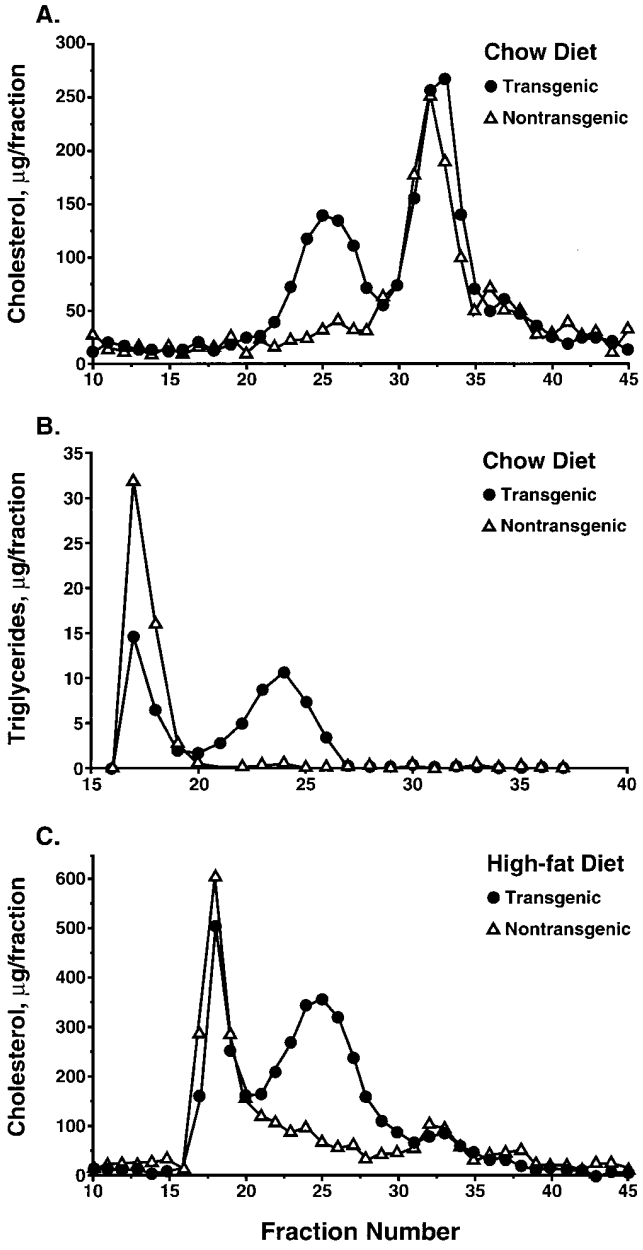

Fig. 1. Distribution of lipids in the plasma of female human apoB transgenic mice and nontransgenic mice, as assessed by Superose 6 chromatography. A: Distribution of cholesterol on a chow diet. B: Distribution of triglycerides on a chow diet. C: Distribution of cholesterol on a synthetic high-fat diet. Modified, with permission, from Linton et al. (14) and Purcell-Huynh et al. (26).

apoB and high levels of human hepatic lipase (32). Triglyceride enrichment of LDL does not appear to be a property of human apoB expression because the same finding was noted with overexpression of mouse apoB (33). Thus, at the current time, the reason that LDL in the human apoB transgenic mice are enriched in triglycerides remains mysterious. Triglyceride enrichment of LDL has also been noted in LDL receptor-deficient mice that synthesize exclusively apoB-100, a situation where apoB clearance is defective and apoB production rates are presumably normal (M. Véniant and S. Young, unpublished observations).

When fed a high-fat diet, the human apoB transgenic mice, like nontransgenic littermate controls, developed high levels of cholesteryl ester-enriched VLDL. However, unlike the nontransgenic mice, the transgenic mice had high levels of LDL (Fig. 1, panel C). In the setting of the high-fat diet, the LDL fraction was enriched in cholesteryl esters and contained very low levels of triglycerides (similar to the LDL in humans). As a result of the elevated LDL levels, the total cholesterol levels in the transgenic mice were higher than in nontransgenic controls (312 ± 17 mg/dl in female transgenic mice vs. 230 ± 19 mg/dl in nontransgenic females; $P < 0.0001$) (26). Interestingly, feeding the human apoB transgenic mice a high-fat diet resulted in an enormous increase in the plasma levels of apoB-48 (which was distributed rather evenly throughout the VLDL, LDL, and HDL) (26). In contrast, human apoB-100 levels did not increase and, if anything, were actually reduced by the high-fat diet. This differential effect of the high-fat diet on apoB-48 and apoB-100 levels was also observed in nontransgenic mice (26, 34). This finding cannot be explained by differences in apoB mRNA editing, inasmuch as the extents of mRNA editing were identical on the chow and high-fat diets (26). Although the mechanism responsible for the high apoB-48 levels in animals on the high-fat diet is not currently understood, it is important to note that the magnitude of the increase will probably be influenced by the mouse strain. In earlier studies, Lusis and coworkers (35) reported that the plasma levels of mouse apoB-48 and apoB-100 in animals on chow and high-fat diets are strikingly different in various inbred strains of mice. Consistent with those findings, Blanche et al. (36) have studied human apoB transgenic mice generated with FVB/N mice, and did not find significantly increased apoB-48 levels in mice on a high-fat diet.

The high levels of LDL in the human apoB transgenic mice are almost certainly caused by a combination of increased synthesis rates and decreased clearance rates. Compared with nontransgenic mice, the LDL levels in the high-expressing human apoB transgenic mice are increased by 10- to 20-fold. The fact that the highest expressing transgenic lines had more than 10 copies of the human apoB gene (14, 15) suggested that apoB secretion by the liver might be increased by 10-fold or more. However, this appears not to be the case. We recently prepared primary hepatocytes from hemizygous transgenic and nontransgenic mice main-

tained on a chow diet and found that the total amount of apoB secretion was increased by only 2- to 3-fold in transgenic hepatocytes compared with nontransgenic hepatocytes (unpublished observations, M. Véniant, J. Borén, and S. Young). Retarded clearance of human LDL almost certainly plays a major role in causing the high levels of LDL in the human apoB transgenic mice. Human LDL bind to the mouse LDL receptor with low affinity (37) and are cleared from mouse plasma much more slowly than human LDL (38).

Interestingly, we found that the synthesis and secretion of mouse apoB was equivalent in primary hepatocytes prepared from transgenic and nontransgenic mice, implying that the overexpression of human apoB did not have a detectable effect on mouse apoB synthesis and secretion, at least under the experimental conditions that we used (unpublished observations, M. Véniant, J. Borén, and S. Young). The latter result is consistent with our earlier observation that mouse apoB levels were equivalent in nontransgenic and human apoB transgenic mice (26).

An unexplained phenotype of the human apoB transgenic mice is low HDL cholesterol levels. The reduction in HDL cholesterol levels was observed in both male and female transgenic mice and on both a high-fat diet and chow diet (26). Reduced HDL cholesterol levels were also observed in human apoB transgenic rabbits (30) and mouse apoB transgenic mice (33), suggesting that reduced HDL cholesterol levels may be a metabolic feature of apoB overexpression. The mechanism for reduced HDL levels in the setting of apoB overexpression is perplexing, inasmuch as low HDL levels are clearly a feature of reduced apoB gene expression [heterozygosity for an apoB gene knockout mutation (39, 40)].

Atherosclerosis in human apoB transgenic mice. Human apoB transgenic mice, even mice that have been backcrossed several times to C57BL/6 (an atherosclerosis-susceptible strain), do not develop atherosclerotic lesions when fed a chow diet (26). In view of the fact that the total cholesterol levels in those animals were less than 150 mg/dl, the absence of atherosclerotic lesions is not particularly surprising. In contrast, the same transgenic mice maintained on a high-fat diet (41) for 18 weeks developed much higher plasma cholesterol levels (312 ± 17 mg/dl) and had extensive atherosclerotic lesions in the proximal aortic root (>160,000 μm^2 per cross-section of the aorta). The extent of atherosclerosis was more than 11-fold greater than in nontransgenic littermates, which had total cholesterol levels of 230 ± 19 mg/dl (26) (**Fig. 2**). Female transgenic mice have about 5-fold more atherosclerotic lesions than male mice. After 6 months on a high-fat diet, lesions in the human apoB transgenic mice are even larger and contain fibrous caps, necrotic cores with large pools of extracellular lipid, and abundant cholesterol clefts, similar to those reported in chow-fed apoE-deficient mice (42, 43).

Callow and collaborators (44) also have examined

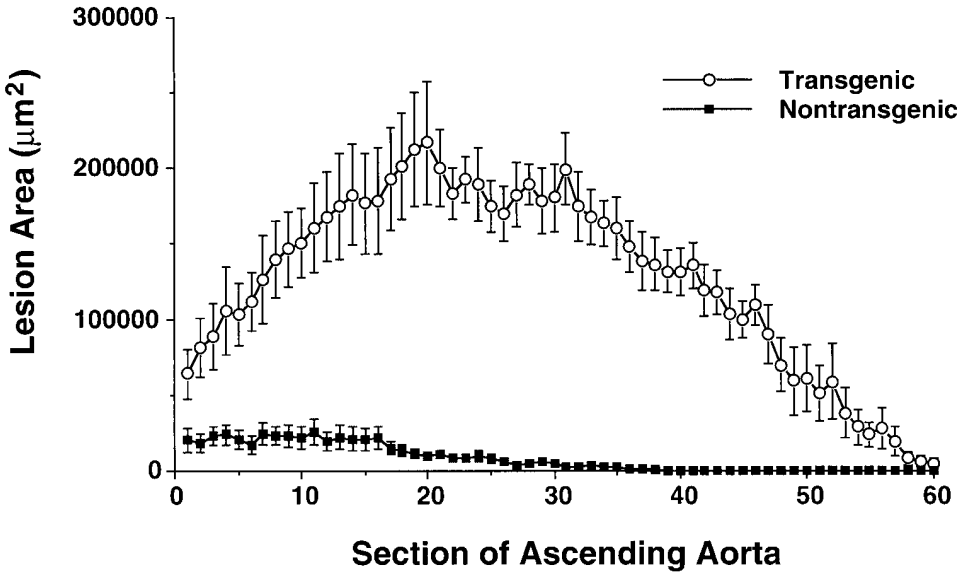

Fig. 2. Comparison of the extent of atherosclerotic lesions in female human apoB transgenic mice (generated with the P1 bacteriophage clone, p158) and nontransgenic littermate controls after 18 weeks on a high-fat diet. Mean lesion areas (and standard errors) for 60 progressive sections spanning the proximal 1200 μm of the mouse ascending aorta are shown. Section 1 is in the proximal aortic sinus where the aortic valve leaflets first appear. Reproduced, with permission, from Purcell-Huynh et al. (26).

atherosclerosis in human apoB transgenic mice (on a mixed genetic background of C57BL/6, SJL, and FVB). After 18 weeks on a high-fat diet, the lesions in the high-expressing human apoB transgenic mice were smaller than those observed in our studies (~14,000 μm^2 per aortic cross-section), presumably reflecting the influences of a different genetic background.

The amount of atherosclerosis in the human apoB transgenic mice on a high-fat diet is probably less than that in apoE-deficient mice fed a chow diet, although a side-by-side comparison of atherosclerosis in the two animal models has never been performed. If the use of a chow diet is judged to be essential for a particular atherosclerosis study, analyzing atherosclerosis susceptibility in apoE-deficient mice obviously holds a major advantage over the human apoB transgenic mice. On the other hand, for certain genetic studies of atherosclerosis susceptibility, the use of a high-fat diet may be satisfactory. For these studies, the use of human apoB transgenic mice might hold an advantage over the apoE-deficient mice in that the atherosclerosis experiments can be set up with less breeding as severe atherosclerosis is observed without breeding the transgene to homozygosity. Another advantage of human apoB transgenic mice is that their lipoprotein profile more closely resembles that observed in humans (i.e., high levels of apoB-100-containing LDL). In apoE-deficient mice, the plasma levels of apoB-100 are actually reduced (34), and most of the cholesterol is in apoB-48-containing VLDL (45–47).

Recently, Hobbs and coworkers have bred the human apoB transgene onto the background of LDL receptor deficiency (H. Hobbs, unpublished observations). The LDL receptor–deficient human apoB transgenic mice ($HuBTg^{+/+}Ldlr^{-/-}$) had total cholesterol levels of 700–800 mg/dl, much higher than in LDL receptor–deficient mice lacking the human apoB transgene ($Ldlr^{-/-}$), and nearly all of the cholesterol was in apoB-100-containing LDL. The plasma triglycerides were also significantly increased. The lipoprotein profile of the $HuBTg^{+/+}Ldlr^{-/-}$ mice was basically similar to that observed in humans with familial hypercholesterolemia, except that the LDL of the mice were enriched in triglycerides. Interestingly, the $HuBTg^{+/+}Ldlr^{-/-}$ mice developed severe aortic atherosclerosis on a chow diet and thus are a very attractive alternative to apoE-deficient mice for investigators who are interested in using chow-fed animals to study the environmental and/or genetic factors affecting atherogenesis.

Using transgenic mice to understand apoB gene expression in the intestine

Although the 80-kb insert from p158 made it possible to generate transgenic mice expressing high plasma levels of human apoB, the tissue pattern of transgene expression was distinctly abnormal. The human apoB gene was expressed at high levels and homogeneously (without variegation) in the liver, whereas intestinal transgene expression was absent (26, 48–50). For some transgenic lines, even very sensitive techniques such as RNase protection assays and reverse transcriptase-polymerase chain reaction (RT-PCR) were not capable of detecting human apoB gene expression in the intestine (26). The absence of intestinal transgene expression in the p158 transgenic mice became quite obvious when the p158 human apoB transgenic mice were mated with the apoB knockout mice. The human apoB transgenic mice that were homozygous for the apoB knockout mutation ($HuBTg^{+/o}Apob^{-/-}$) lacked all apoB synthesis (mouse or human) in the intestine and consequently developed severe intestinal fat malabsorption (50) (these mice are discussed in detail below).

The absence of transgene expression in the intestines of p158-transgenic mice was quite unexpected. Earlier cell culture studies with transiently transfected reporter gene constructs had suggested that the sequences required for intestinal apoB gene expression were contained within the proximal promoter sequences of the apoB gene (51, 52). The simplest explanation for the absence of intestinal apoB gene expression in the p158-transgenic mice was that the DNA sequences controlling apoB expression in the intestine were not located within the proximal promoter, but instead were very distant from the structural gene (so far away that they were not contained within p158). However, another formal possibility existed: that the DNA sequences controlling intestinal expression were contained within p158, but that these human sequences were not recognized by the mouse transcriptional machinery. To test this possibility, mouse apoB transgenic mice with an 87-kb P1 clone (p649) spanning the mouse apoB gene (including 33 kb of 5′ flanking sequences and 11 kb of 3′ flanking sequences) were generated (33). Although the mouse apoB transgene was expressed at high levels in the liver, two independent experimental approaches (a transgene-specific RNase protection assay and genetic complementation studies with the knockout mice) demonstrated that transgene expression was absent in the intestines (33).

The absence of intestinal transgene expression in both the p158 and the p649 transgenic mice provided firm evidence that the DNA sequences controlling intestinal apoB expression were simply located a great distance from the structural gene. To examine this issue further, 145- and 207-kb bacterial artificial chromosomes (BACs) spanning the human apoB gene were used to generate additional lines of human apoB transgenic mice (53). The 207-kb BAC contained 120 kb of sequences 5′ to the gene and 35 kb 3′ to the gene, while

the 145-kb BAC contained 70 kb 5′ and 22 kb 3′ to the gene. Both of these BACs directed high levels of human apoB expression in the intestines and livers of transgenic mice (**Fig. 3**). Moreover, the pattern of transgene expression appeared to be appropriate: both in situ hybridization and immunohistochemical studies revealed that the BACs were expressed within the villus enterocytes of the duodenum and jejunum, a pattern identical to that observed with the endogenous mouse apoB gene. These studies with the BAC transgenic mice indicated that appropriate expression of the apoB gene in the intestines is controlled by distant DNA sequences contained within the BACs but absent from p158 or p649.

Further experiments established that the element controlling apoB gene expression in the intestine is located more than 30 kb 5′ to the structural gene (54). When p158 was coinjected with 70 kb of apoB 5′ flanking sequences, the transgenic mice expressed the human apoB transgene in the intestine. However, when p158 was coinjected with 22 kb of apoB 3′ flanking sequences, intestinal expression was absent (54).

ApoB expression in the heart

An intriguing aspect of the p158 human apoB transgenic mice was that the human apoB gene was expressed in the heart (14, 15), a site where the apoB gene is assumed to be silent. The apoB mRNA in the heart could be detected easily on a short exposure of a Northern blot or an RNA slot blot, and quantification of these blots revealed that the levels of the apoB mRNA in the heart were about 4% of those in the liver. In view of the fact that p158 did not confer a normal pattern of apoB gene expression (e.g., human apoB expression in the intestine was absent), we initially assumed that the heart expression of the apoB gene represented a "transgenic artifact," perhaps because p158 did not contain all of the 5′ and 3′ flanking sequences that would normally function to silence apoB expression in inappropriate tissues.

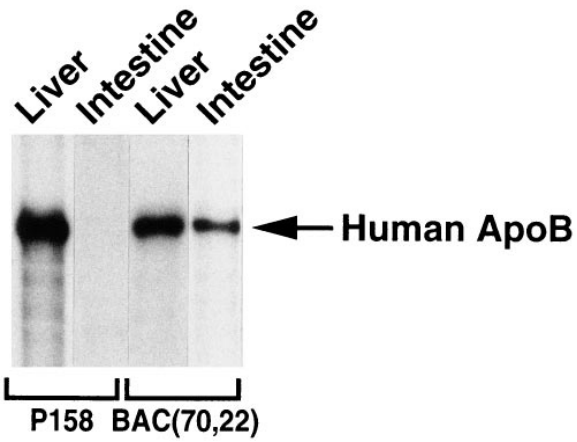

Fig. 3. RNase protection assay illustrating human apoB expression in the liver and duodenum of human apoB transgenic mice generated with p158, BAC(120,35), and BAC(70,22) (54). BAC(120,35) contains 120 kb of 5′ flanking sequences and 35 kb of 3′ flanking sequences; BAC(70,22) contains 70 kb of 5′ flanking sequences and 22 kb of 3′ flanking sequences. The RNase protection assay was performed with a 121-bp human apoB riboprobe. Both BAC transgenes conferred expression in the liver and intestine, whereas the 80-kb P1 clone (p158) yielded expression only in the liver.

Subsequent studies have shown that this assumption was incorrect. When we examined transgenic mice generated with the 207-kb and 145-kb BACs (where intestinal expression was normal), we found that the human apoB transcript was invariably present in the heart (unpublished observations, L. Nielsen and S. Young). Those data led us to consider the hypothesis that heart apoB expression was actually specified by the regulatory elements of the apoB gene. To test this possibility, we have obtained multiple human heart biopsies and documented that the apoB gene is also expressed in the human heart at about the same levels as in the human apoB transgenic mice (unpublished observations, L. Nielsen and S. Young).

Finding the apoB gene expression in the heart led us to consider the possibility that the human heart is actually a lipoprotein-producing organ. To test this possibility, we have performed [^{35}S]methionine metabolic labeling/immunoprecipitation studies with both transgenic mouse hearts and human heart tissue and have documented that significant amounts of human apoB-containing lipoproteins (primarily of the LDL and IDL density ranges) are secreted by the heart (unpublished observations, J. Borén, M. Véniant, and S. Young). Because apoB secretion from hepatocytes and enterocytes requires microsomal triglyceride transfer protein, we suspected that the heart would also express that gene. Indeed, MTP is expressed in the heart (unpublished observations, L. Flynn, M. Raabe, and S. Young). Lipoprotein production by the heart is not unique to humans; apoB-containing lipoproteins are also secreted from the hearts of nontransgenic mice.

Why the heart synthesizes and secretes apoB-containing lipoproteins, and whether this finding is important in heart physiology, is not yet clear. The heart lipolyzes large amounts of triglycerides for fuel, but the ability to store triglycerides within the parenchyma of the heart is obviously not unlimited. Lipoprotein production by the heart possibly may allow the heart to unload excess triglycerides (and perhaps other lipids as well) back into the circulation. The latter hypothesis is particularly attractive and potentially testable with a variety of transgenic mouse experiments.

MICE EXPRESSING MUTANT FORMS OF HUMAN APOB

Even though the apoB cDNA and gene were cloned and sequenced more than a decade ago, progress in understanding many aspects of apoB structure/function relationships has been slow. There are two reasons for the slow progress. First, the introduction of mutations into an ~20-kb apoB minigene is not a trivial task from a technical perspective. Second, even after mutant minigenes have been constructed, the cell-culture systems for expressing mutant forms of human apoB-100 have been sub-optimal. It is a prodigious feat to isolate a few μg of purified human apoB-100-containing lipoproteins from transfected rat hepatoma cells, and this amount is generally inadequate to pursue structure/function studies seriously.

The ability to express large amounts of human apoB-100 in the p158-human apoB transgenic mice (14, 15) suggested a new approach for studying apoB structure/function: mutating clone p158, making transgenic mice, and then purifying and analyzing the mutant human apoB protein. However, at that time, there were no obvious strategies for performing site-directed mutagenesis on a genomic clone as large as p158 (i.e., the total length of p158 is ~96 kb).

Two techniques have recently emerged for efficiently mutating large genomic clones. One method is to mutate large genomic clones in a yeast artificial chromosome (55, 56). This strategy is based on the fact that DNA in yeast chromosomes can be modified efficiently by homologous recombination (57–59) and is complemented by the emergence of techniques for generating transgenic mice with YAC DNA (60–62). Because of the high efficiency of homologous recombination in yeast, the introduction of mutations with gene-targeting techniques can be accomplished easily within a time frame of several weeks. After the modification is completed, the YAC DNA can be purified from a pulsed-field agarose gel and microinjected into fertilized eggs to generate transgenic mice (55). A second technique for manipulating large genomic clones is *RecA*-assisted endonuclease (RARE) cleavage (63). For example, through RARE cleavage, specific small *Eco*RI and *Hin*dIII restriction fragments can be removed from p158. After the use of conventional site-directed mutagenesis to introduce mutations into the *Hin*dIII or *Eco*RI fragments, the mutant restriction fragments can be ligated back into the P1 construct to regenerate a full-length mutant p158.

Both the YAC gene-targeting system and the RARE cleavage strategies have worked efficiently for mutating p158. The YAC system has allowed us to generate dozens of transgenic founders, with an efficiency equal to that obtained with short fragments of DNA from plasmids. All of the techniques for implementing this YAC system, including the methods for preparing YAC DNA for microinjection, have been described in reviews (48, 56). The RARE cleavage strategy, which is also very efficient, holds an advantage over the YAC method in that it is easier to prepare high-quality microinjection DNA from P1 clones than from YACs. On the other hand, the RARE cleavage strategy requires more DNA sequence information (i.e., the DNA sequences surrounding specific *Hin*dIII and *Eco*RI sites) and is probably not as well suited for introducing certain types of complex mutations into large clones. Another drawback of the RARE cleavage system, at least in our hands, is that the efficiency of RARE cleavage can be quite low at certain *Hin*dIII and *Eco*RI sites within the apoB gene.

Recent apoB structure/function issues that have been examined through the manipulation of large apoB genomic clones include the structure of the receptor-binding region of apoB-100 (64, 65) and the structural features of the apoB molecule that are important in its covalent interaction with apo[a] in Lp[a] assembly (55, 56, 66, 67).

Identifying structural features of apoB-100 that are required for Lp[a] assembly

We used the YAC system to identify the structural features of apoB-100 that are important for Lp[a] assembly, particularly the cysteine residue that participates in the disulfide bond with apo[a]. Because prior studies (68–70) suggested that Lp[a] formation depended upon the presence of the carboxyl terminus of apoB-100, we tested whether the last cysteine residue of apoB-100, cysteine-4326, was involved in the disulfide bond with apo[a]. After the human apoB gene (the insert from p158) was cloned into a yeast artificial chromosome (YAC), the apoB gene was mutated (Cys4326 Gly) by homologous recombination with a "pop-in, pop-out" sequence-insertion gene-targeting vector (55) (**Fig. 4**), and transgenic mice expressing the mutant human apoB were generated. The mutant apoB lacked the ability to bind to apo[a] to form Lp[a] (56), either in in vitro assays of Lp[a] formation or in vivo in transgenic mice expressing both apo[a] and the mutant human apoB. These studies strongly suggested that apoB cysteine-4326 was the site of attachment for apo[a].

More recently, this same YAC mutagenesis/transgenic mouse system was used to define other apoB sequences (aside from cysteine-4326) that might be important for Lp[a] formation (66) such as the sequences carboxyl terminal to cysteine-4326. To address that issue, transgenic mice expressing truncated forms of human apoB, apoB-95 (4330 amino acids) and apoB-97

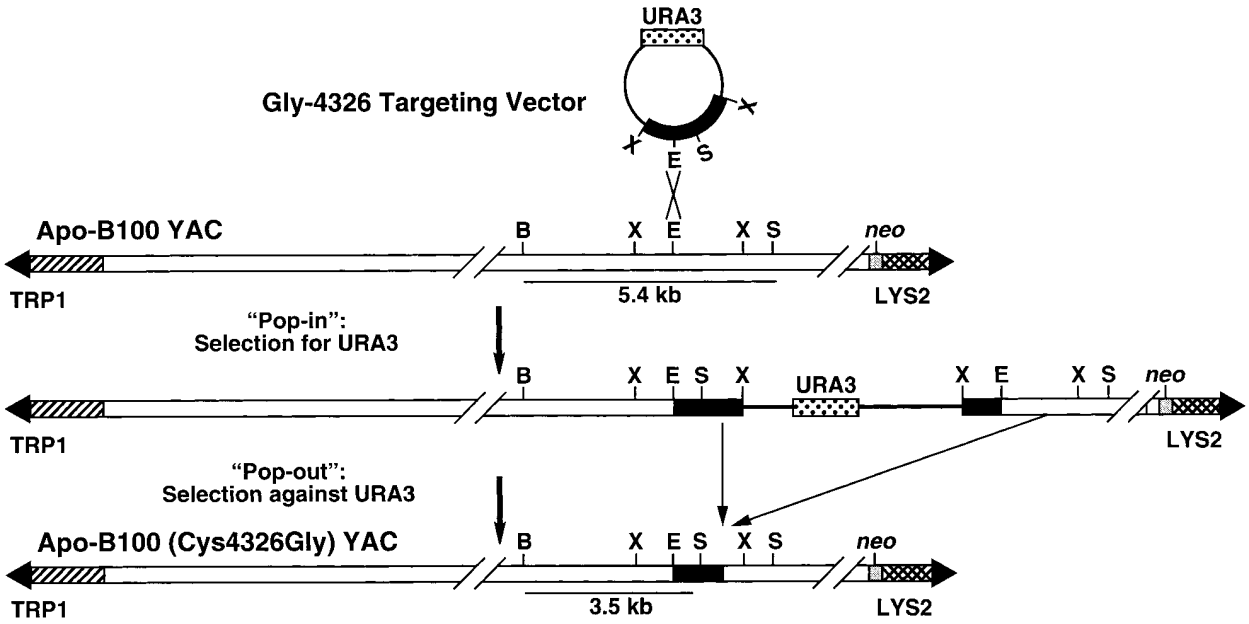

Fig. 4. A strategy for introducing the Cys4326Gly mutation into a 108-kb human apoB yeast artificial chromosome (YAC). A sequence-insertion gene-targeting vector for mutating the YAC was constructed by cloning a 2.8-kb *Xba*I fragment from the 3′ end of the apoB gene into the yeast integrating vector, pRS406. The codon for cysteine-4326 was then changed to a glycine by site-directed mutagenesis. A new *Stu*I site was created in the process. The targeting vector was linearized at the *Eco*RI site in exon 29 of the apoB gene and introduced into spheroplasts containing the 108-kb apoB YAC. Transformants (which acquired the URA3 gene) were initially selected on plates lacking uracil (101). To identify targeted clones after the "pop-in" step, yeast colonies were analyzed by pulsed-field gel electrophoresis and Southern blot analysis (the new *Stu*I site changed the length of a *Bam*HI–*Stu*I fragment) (55). For the "pop-out" step, yeast harboring a targeted YAC were grown overnight in a selective medium that permits growth of cells that have lost the URA3 gene. Colonies were subsequently analyzed by pulsed-field gel electrophoresis and Southern blot analysis. The YAC DNA containing the Cys4326Gly mutation was then microinjected into mouse eggs to generate multiple lines of transgenic mice expressing the mutant form of human apoB. Reproduced, with permission, from McCormick et al. (55).

(4397 amino acids), were generated, and the ability of these truncated apoBs to form Lp[a] was tested (66). These studies revealed that bona fide Lp[a] was formed with apoB-95, but slowly and inefficiently (**Fig. 5**). In contrast, Lp[a] formed rapidly with apoB-97, although not quite as rapidly as with apoB-100. These studies demonstrated that the sequences carboxyl terminal to cysteine-4326, particularly residues 4331–4397, play an important role in apoBs initial interaction with apo[a]. Expression of additional mutant apoBs in transgenic mice will undoubtedly lead to a more complete understanding of the structural features of apoB that are required for Lp[a] assembly.

Callow and Rubin (67) also have demonstrated that cysteine-4326 is required for Lp[a] assembly by mutating p158 and then expressing mutant constructs in transgenic mice. Using the RARE cleavage strategy, they generated transgenic mice expressing mutant forms of human apoB that lacked either apoB cysteine-3734 or apoB cysteine-4326. Their studies revealed that the mutant human apoB lacking cysteine-3734 formed Lp[a] normally, whereas the apoB lacking cysteine-4326 did not form Lp[a] (67).

Analysis of the structure of the LDL receptor-binding region of apoB-100

More recently, Borén and collaborators (64, 65, 71) have used RARE cleavage to investigate the apoB sequences that are important for the binding of apoB-100 to the LDL receptor. They generated transgenic mice expressing mutant forms of human apoB in which the arginine at residue 3500 was replaced with several other amino acid residues. In humans, two different amino acid substitutions at residue 3500 [Arg3500Gln (72) and Arg3500Trp (73)] disrupt binding to the LDL receptor and cause the hypercholesterolemic syndrome, familial ligand-defective apolipoprotein B-100. As expected, the LDL isolated from transgenic mice expressing a mutant form of apoB (Arg3500Gln) bound poorly to the LDL receptor of cultured human fibroblasts. Interestingly, the LDL from mice expressing another mutant human apoB (Arg3500Lys) also bound poorly to the LDL receptor, implying that normal binding to the LDL receptor requires more than just a positively charged amino acid at residue 3500. Other transgenic mouse expression studies have demonstrated that a

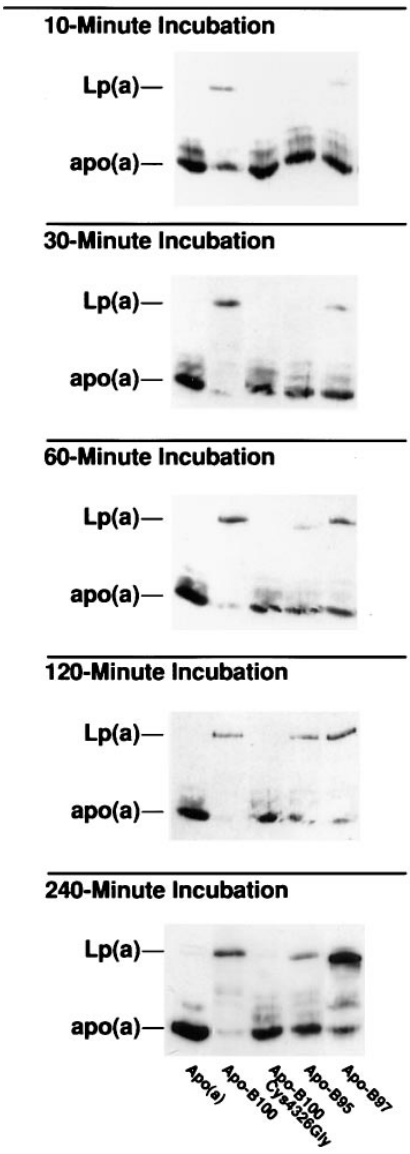

Fig. 5. Kinetic analysis of Lp[a] formation with human apoB-95, apoB-97, and apoB-100. The ability of different apoB proteins to bind covalently to apo[a] and form Lp[a] was assessed with a Western blot assay described by Chiesa et al. (102). The plasma from a human apo[a] transgenic mouse (102) was incubated with plasma samples from a transgenic mouse expressing wild-type human apoB-100 (102), a transgenic mouse expressing a mutant human apoB-100 (Cys4326Gly) (55), a human apoB-95 transgenic mouse (66), and a human apoB-97 transgenic mouse (66), according to methods described previously (55, 68). Each incubation contained an identical amount of apoB, as judged by a monoclonal antibody–based human apoB radioimmunoassay. Samples were removed from the incubations after 10, 30, 60, 120, and 240 min and subjected to electrophoresis on 4% polyacrylamide/SDS gels under nonreducing conditions. The separated proteins were transferred to a nitrocellulose membrane for Western blot analysis with a horseradish peroxidase–conjugated human apo[a]–specific monoclonal antibody IgG-a5 (103, 104). Lane 1 shows the plasma from the apo[a] transgenic mouse; lane 2, the incubation mixture containing wild-type human apoB-100; lane 3, the incubation mixture containing human apoB-100 (Cys4326Gly); lane 4, the incubation mixture containing the mutant human apoB-95; and lane 5, the incubation mixture containing the mutant human apoB-97. Reproduced, with permission, from McCormick et al. (66).

stretch of positively charged amino acids between residues 3358 and 3370 [the so-called "B" site (74, 75)] is essential for high-affinity binding to the LDL receptor. That stretch of amino acids shares significant homology with the segment of apoE molecule that binds to the LDL receptor (apoE amino acids 141–153) (74, 75). Further analysis of human apoB transgenic mice expressing other mutant apoBs will undoubtedly lead to a more complete definition of the apoB sequences that are involved, directly or indirectly, in interacting with the LDL receptor.

Expression of additional mutant apoBs in transgenic mice will certainly be useful for understanding other apoB structure/function issues, including an analysis of the apoB sequences that are required for lipoprotein assembly. Furthermore, these strategies should also be useful for studying the promoter or enhancer sequences that are thought to be important in regulating apoB gene expression (see discussion of intestinal expression of apoB).

USING GENE-TARGETING TECHNIQUES TO UNDERSTAND APOB

Gene targeting in embryonic stem cells has made it possible to generate mice lacking specific gene products and containing subtle mutations in any gene of interest. For the apoB field, gene-targeted mouse models have provided new insights into the role for apoB in mammalian development, clarified the roles of apoB-48 and apoB-100 in lipoprotein metabolism and atherogenesis, and provided mechanistic insights into the human apoB deficiency syndrome, familial hypobetalipoproteinemia (FHβ).

ApoB-70 and apoB-81 mice

In 1993, Homanics and co-workers (76) used a sequence-insertion gene-targeting vector to interrupt the 3′ portion of exon 26 of the mouse apoB gene, generating a mutant apoB allele that yielded a truncated apoB, apoB-70. Because the targeted mutation did not affect the apoB mRNA-editing site, the apoB-70 allele also yielded apoB-48. The phenotype of the apoB-70 mice

was similar to that of humans with the apoB deficiency syndrome, FHβ. In humans, FHβ is caused by a variety of mutations (almost all point mutations) that interfere with the synthesis of a full-length apoB molecule (69). In many instances, the human mutations led to the production of a truncated apoB, which is invariably found in very low concentrations in the plasma of affected subjects. In human FHβ heterozygotes, the plasma concentration of the truncated apoB is typically less than 5–10% of that of the full-length apoB-100 (produced by the normal allele). In the apoB-70 mice, the plasma levels of the truncated apoB were similarly reduced: the apoB-70 level in the IDL/LDL of heterozygous mice was only ~10% that of apoB-100. Further evaluation of the apoB-70 mice revealed that apoB-70 mRNA levels in the intestine and liver were reduced by 75% and 60%, respectively, compared to wild-type transcripts. The reduction in the mutant apoB mRNA levels in these animals was not surprising, inasmuch as the apoB-70 transcript was structurally abnormal, terminating within the plasmid sequences that had been inserted into the exon 26 coding sequences.

Analysis of homozygous apoB-70 mice provided a seminal insight: that apoB synthesis is important in mouse development (76, 77). Homozygous apoB-70 mice manifested neurodevelopmental abnormalities, including exencephalus and hydrocephalus, and approximately 50% died in utero. Of the homozygotes that survived until weaning, 32% manifested hydrocephalus, whereas fewer than 1% of heterozygous and wild-type mice manifested similar abnormalities. The severe developmental abnormalities were unexpected and intriguing because they have never been reported in humans with homozygous FHβ. Interestingly, the plasma levels of α-tocopherol in adult homozygous apoB-70 mice were only 35% of those in wild-type mice (76), and hydrocephalus and exencephalus are characteristic features of vitamin E–deficient rat embryos (78, 79). At this point, it is not clear whether vitamin E deficiency plays a role in the developmental abnormalities in the apoB-70 mice, as supplementation of the maternal diets from heterozygous apoB-70 intercrosses with α-tocopherol failed to mitigate the developmental abnormalities of the homozygous apoB-70 mice (77). The developmental abnormalities in the apoB-70 mice may be dependent on the genetic background of the mice. After the apoB-70 mice were backcrossed to strain C57BL/6, the developmental abnormalities became more severe, and it was no longer possible to obtain live homozygous animals (unpublished observations, N. Maeda).

Several other characteristics of the apoB-70 mice were noteworthy. First, in addition to low levels of apoB-containing lipoproteins, the plasma of apoB-70 mice had low levels of HDL (76). In fact, most of the reduction in total plasma cholesterol levels in the apoB-70 mice could be accounted for by low HDL cholesterol levels. The mechanism for the low HDL levels in the apoB-70 mice has never been fully delineated. Second, the plasma of homozygous apoB-70 mice manifested chylomicronemia (as judged by agarose gel electrophoresis), even in plasma samples taken after a prolonged (20 h) fast. "Fasting chylomicronemia" has been noted in human FHβ homozygotes (80) and has been presumed to be due to an imbalance between the amounts of apoB and lipids available for lipoprotein production (i.e., diminished apoB production in the setting of normal production of neutral lipids would be predicted to yield large, chylomicron-sized particles) (69). In the homozygous apoB-70 mice, the mean size of VLDL particles was not significantly different from wild-type mice. However, the percentage of VLDL particles with a diameter greater than 70 nm was increased in homozygous mice compared with wild-type controls (1.8% vs. 0.6%, respectively) (76). Large VLDL particles have also been observed in humans with homozygous hypobetalipoproteinemia (80).

In a recent study, Toth and coworkers (81) used a sequence-insertion vector to generate a mutant apoB allele that yielded apoB-81 (and apoB-48). Although the apoB mRNA levels in the apoB-81 mice were not measured, they were probably low, inasmuch as the structure of the apoB-81 transcript would have been very abnormal, terminating within plasmid sequences (similar to that in the apoB-70 mice). Like the apoB-70 mice, the homozygous apoB-81 mice manifested low plasma levels of cholesterol. In addition, they had decreased intrauterine viability and an increased incidence of exencephalus and hydrocephalus, although the frequency of these abnormalities was less than in the apoB-70 mice.

ApoB knockout mice

To generate a mouse model in which apoB production was absent, Farese and coworkers (39) used gene-targeting in ES cells to disrupt the 5′ portion of the mouse apoB gene. In heterozygous apoB knockout mice (*Apob*$^{+/-}$) fed a low-fat chow diet, the plasma levels of apoB were reduced by ~70% (39), and total plasma cholesterol levels were reduced by ~19%. As was the case for the apoB-70 mice, most of the reduction in the plasma cholesterol levels in the *Apob*$^{+/-}$ mice could be accounted for by decreased HDL levels. On a high-fat diet, the *Apob*$^{+/-}$ mice were protected from developing hypercholesterolemia (39), largely because the increase in VLDL-IDL cholesterol levels was blunted. On the high-fat diet, the reduced VLDL and IDL levels in the *Apob*$^{+/-}$ mice were probably due to diminished lipoprotein synthesis by the liver rather than

to impaired fat absorption by the intestine. First, we observed no accumulation of cytosolic fat in the intestinal enterocytes of the *Apob*$^{+/-}$ mice on the high-fat diet. Second, we measured intestinal cholesterol absorption in *Apob*$^{+/-}$ and *Apob*$^{+/+}$ mice on the high-fat diet (n = 6 in both groups), and found that the percent cholesterol absorption was no different in the *Apob*$^{+/-}$ mice (31%) and the *Apob*$^{+/+}$ mice (36%) (82).

The *Apob*$^{+/-}$ mice were intercrossed to produce homozygous apoB knockout mice (*Apob*$^{-/-}$). Most of the *Apob*$^{-/-}$ embryos died early during embryonic development (approximately embryonic day 10 to 12) (39). In the few *Apob*$^{-/-}$ embryos that survived until late in gestation, there were developmental abnormalities in the central nervous system and cranium that were similar to (but more severe) than those observed in the apoB-70 mice (76). In a recent study, Farese and coworkers (83) examined the hypothesis that the developmental abnormalities in the *Apob*$^{-/-}$ mice might be due to a deficiency in lipoprotein production by the yolk sac during development. In wild-type embryos, the rough endoplasmic reticulum and Golgi apparatus of the visceral endoderm cells of the yolk sac contained numerous lipoproteins, but in the *Apob*$^{-/-}$ embryos, yolk sac lipoprotein production was absent. In addition, the visceral endoderm cells of the *Apob*$^{-/-}$ yolk sacs accumulated numerous large cytosolic fat droplets. These findings suggested that the *Apob*$^{-/-}$ yolk sacs were probably deficient in their ability to transfer lipid nutrients to the developing embryo. In support of that theory, the 9.5-day *Apob*$^{-/-}$ embryos had reduced stores of cholesterol and virtually absent levels of α-tocopherol (83).

Huang and coworkers (40) have independently generated apoB knockout mice. Their *Apob*$^{+/-}$ mice also manifested reduced levels of apoB-containing lipoproteins, total and HDL cholesterol, and plasma apoA-I. The basis for the low HDL cholesterol and apoA-I levels in the *Apob*$^{+/-}$ mice was examined by determining the fractional catabolic rates for isotopically labeled HDL-cholesteryl esters and apoA-I. The fractional catabolic rates for both HDL-CE and apoA-I in heterozygous and wild-type mice were similar, suggesting that the reduced HDL cholesterol and apoA-I plasma levels in *Apob*$^{+/-}$ mice were due to decreased HDL-CE and apoA-I transport (production) rates.

Interestingly, there were several differences between the apoB knockout mice of Huang and coworkers (40) and of Farese and coworkers (39). First, in Huang's studies, all of the *Apob*$^{-/-}$ embryos died by the ninth day of gestation, whereas a few of our *Apob*$^{-/-}$ embryos survived until later in gestation (39, 83). In addition, the majority of the Huang's heterozygous mice died during development or manifested developmental defects such as exencephalus and hydrocephalus, while none of the heterozygotes died in our studies. Second, 60% of the male *Apob*$^{+/-}$ mice in Huang's studies were infertile despite normal mating behavior. Further studies revealed that sperm from Huang's heterozygous knockout males failed to fertilize eggs, either in vivo or in vitro (40). Infertility has not been problematic in our *Apob*$^{+/-}$ male mice or in any of our other gene-targeted mice with subtle mutations in the apoB gene.

The reason(s) for the differences in the two independent lines of apoB knockout mice is not clear. Subtle genetic strain differences may influence the phenotype, perhaps as a result of the fact that different ES cells (isolated from different 129 substrains) were used to generate the mice. In support of this explanation, Maeda observed that the genetic strain appears to affect the frequency of embryonic lethality in homozygous apoB-70 mice. Other explanations for the phenotypic differences are possible, although none that we have considered seem compelling. For example, the nature of the knockout mutation may underlie the different phenotypes. In Huang's experiments, a sequence-replacement vector was used to replace the first three exons of the apoB gene with a PGK-*neo* cassette. In our mice, a sequence-insertion vector was used to insert more than 10 kb of sequences (including an RNA polymerase II-*neo* cassette) into the 5′ portion of the gene; this mutant allele is predicted to allow the translation of the first four exons of the gene (spanning apoB amino acids 1–101). Even if this protein were synthesized and secreted (and we have never identified such a protein with immunochemical techniques), 101 amino acids would not be expected to be lipidated or support lipoprotein formation. Nevertheless, it is a formal possibility that the synthesis of this extremely short apoB protein could underlie the phenotypic differences. Another formal possibility is that the different targeted mutations might have different effects on the transcription of an adjacent gene. Along these lines, Olson and co-workers (84) have suggested that the PGK-*neo* cassette might affect the transcription of neighboring genes. Finally, it is a formal possibility that low levels of "leakiness" in our knockout mice might underlie the phenotypic differences in homozygotes. In knockout mice generated with a sequence-insertion vector (such as ours), intrachromosomal recombination [which would be expected to occur in ~1 in 10^6 or 10^7 somatic cells (85)] could restore a wild-type allele. Thus, approximately one in 10^6 or 10^7 intestinal or liver cells in *Apob*$^{-/-}$ embryos might have an *Apob*$^{+/-}$ genotype. Although this type of genetic leakiness could influence the phenotype of homozygous knockout embryos, it is very difficult to imagine how this type of leakiness could explain the major phenotypic differences in heterozygous mice.

Mating apoB knockout mice and human apoB transgenic mice to generate mice lacking apoB expression in the intestine

After the development of the apoB knockout mice (39), our first goal was to determine whether the human apoB transgene (14) would prevent the lethal developmental abnormalities associated with the apoB knockout mutation. Because the p158 transgene conferred high levels of apoB expression in the yolk sac during development, one would expect that homozygous apoB knockout mice carrying a copy of the human apoB transgene (HuBTg$^{+/o}$*Apob*$^{-/-}$) would develop normally. In fact, HuBTg$^{+/o}$ *Apob*$^{-/-}$ mice exhibited normal size and vitality at birth and were free of developmental abnormalities (39, 50).

Even though HuBTg$^{+/o}$*Apob*$^{-/-}$ mice were indistinguishable from wild-type mice at birth, they manifested gross abnormalities during the suckling phase. Because neither p158 nor the endogenous apoB gene was expressed in the intestines of HuBTg$^{+/o}$*Apob*$^{-/-}$ mice, they lacked the ability to synthesize chylomicrons and therefore developed fat malabsorption and growth retardation (50). Within hours after their first meal, the intestines of HuBTg$^{+/o}$*Apob*$^{-/-}$ mice became white and bloated, reflecting a reduced capacity to absorb lipid nutrients from maternal milk. Upon microscopic analysis, the absorptive enterocytes of the small intestines were nearly completely filled with cytosolic fat (**Fig. 6**), a pathologic finding that is virtually identical to the intestinal pathology in humans with homozygous hypobetalipoproteinemia or abetalipoproteinemia (86). Approximately two-thirds of the HuBTg$^{+/o}$*Apob*$^{-/-}$ mice became emaciated and died during the suckling phase. However, surviving mice that were weaned onto a chow diet grew rapidly and ultimately achieved normal size (50). Adult HuBTg$^{+/o}$*Apob*$^{-/-}$ absorbed d-xylose normally. However, [^{14}C]cholesterol absorption was undetectable, and the mice had elevated urinary mevalonate levels, almost certainly reflecting increased levels of de novo cholesterol biosynthesis. Only small amounts of orally administered retinyl palmitate were absorbed, and the appearance of retinyl palmitate in the plasma was delayed. The intestinal absorption of triglycerides was not measured but probably was not zero, based on the observation that the animals did not manifest severe essential fatty acid deficiency. The plasma levels of α-tocopherol were quite low, approximately 10% of those in littermate controls.

The development of the HuBTg$^{+/o}$*Apob*$^{-/-}$ mice has made it possible to assess how the absence of chylomicron metabolism affects the plasma lipoproteins, particularly the levels of apoB-containing lipoproteins

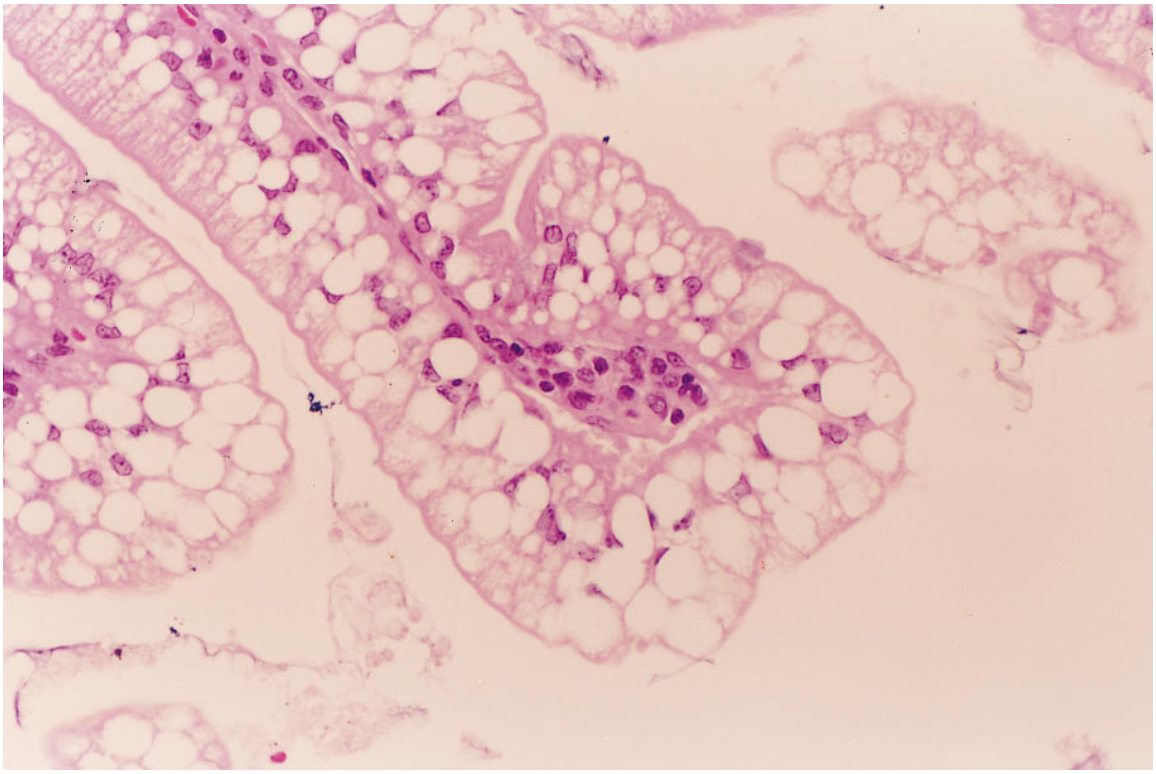

Fig. 6. Hematoxylin and eosin-stained sections of the duodenum of a 10-week-old HuBTg$^{+/o}$*Apob*$^{-/-}$ mouse.

derived from the liver. Because the human apoB transgene was expressed at high levels in the livers of HuBTg$^{+/o}$*Apob*$^{-/-}$ mice, the plasma of those animals contained liver-derived apoB-48- and apoB-100-containing lipoproteins. Interestingly, the plasma levels of apoB-100 and LDL cholesterol in the HuBTg$^{+/o}$*Apob*$^{-/-}$ mice were identical to those in human apoB transgenic mice that synthesized chylomicrons normally (HuBTg$^{+/o}$ *Apob*$^{+/+}$) (50). These studies indicated that the inability to secrete chylomicrons does not affect the plasma levels of liver-derived lipoproteins, at least in mice on a chow diet. It is likely that the livers of adult HuBTg$^{+/o}$ *Apob*$^{-/-}$ mice have sufficient lipids to maintain normal levels of lipoprotein secretion, either because these animals have elevated levels de novo lipogenesis and/or because these animals absorb some lipids from the intestine independently of chylomicrons.

In contrast to their "normal" levels of LDL, the HDL levels of HuBTg$^{+/o}$*Apob*$^{-/-}$ mice were only ~50% of those in HuBTg$^{+/o}$*Apob*$^{+/+}$ mice (50). The mechanism for the low HDL levels in the HuBTg$^{+/o}$*Apob*$^{-/-}$ mice is unknown, but we suspect that this finding may be caused by low levels of intestinal apoA-I secretion. Interestingly, HDL cholesterol levels are also reduced by ~50% in humans with abetalipoproteinemia (87).

ApoB-48-only and apoB-100-only mice

To gain insights into why mammals synthesize two forms of apoB and to assess whether the two apoB proteins have intrinsic differences in their capacity to promote atherosclerosis, we used gene targeting in embryonic stem (ES) cells to generate mice that synthesize exclusively apoB-48 (apoB-48-only mice) or exclusively apoB-100 (apoB-100-only mice) (34). To produce the apoB-48-only mice, "pop-in, pop-out" gene targeting (88, 89) was used to replace the "apoB-48 editing codon" (codon 2153) of the mouse apoB gene with a TGA stop codon. To produce apoB-100-only mice, codon 2153 (CAA, specifying glutamine) was changed to a CTA-leucine codon. This mutation has the effect of being a "nonstop" mutation, as editing of the CTA codon does not produce a stop codon (i.e., UTA specifies leucine). As expected, mice that were homozygous for the apoB-48-only mutation (*Apob*$^{48/48}$) synthesized exclusively apoB-48, whereas mice that were homozygous the apoB-100-only mutation (*Apob*$^{100/100}$) synthesized only apoB-100 (**Fig. 7**).

Lipid metabolism in apoB-48-only and apoB-100-only mice. The *Apob*$^{48/48}$ mice had slightly lower levels of LDL cholesterol than wild-type mice, whereas the *Apob*$^{100/100}$ mice had slightly higher levels (34). The *Apob*$^{48/48}$ mice also had lower plasma triglyceride levels than wild-type mice, while the *Apob*$^{100/100}$ mice had significantly higher triglyceride levels.

To test whether the lower LDL cholesterol levels in the *Apob*$^{48/48}$ mice resulted from increased apoE-mediated clearance of the apoB-48-containing lipoproteins, the *Apob*$^{48/48}$ and *Apob*$^{100/100}$ mice were crossed with apoE-deficient mice (*Apoe*$^{-/-}$) to ultimately generate apoE-deficient apoB-48-only mice (*Apob*$^{48/48}$*Apoe*$^{-/-}$) and apoE-deficient apoB-100-only mice (*Apob*$^{100/100}$*Apoe*$^{-/-}$) (34). The *Apob*$^{48/48}$*Apoe*$^{-/-}$ mice had higher cholesterol levels than *Apob*$^{+/+}$*Apoe*$^{-/-}$ mice, whereas the *Apob*$^{100/100}$ *Apoe*$^{-/-}$ mice had lower levels, reflecting differences in the plasma concentrations of LDL-sized lipoproteins (**Fig. 8**). The lower levels of cholesterol in the *Apob*$^{100/100}$ *Apoe*$^{-/-}$ mice are likely due to the fact that the apoB-100-containing LDL in those animals can be taken up and removed from plasma by the LDL receptor pathway. Interestingly, even though the *Apob*$^{100/100}$ *Apoe*$^{-/-}$ mice had lower plasma cholesterol levels, they nevertheless had higher plasma triglyceride levels than the *Apob*$^{48/48}$*Apoe*$^{-/-}$ or *Apob*$^{+/+}$*Apoe*$^{-/-}$ mice. Another interesting phenotype was that ~25% of the *Apob*$^{100/100}$ *Apoe*$^{-/-}$ mice developed hydrocephalus, while we never observed that abnormality in the *Apob*$^{48/48}$ *Apoe*$^{-/-}$ or *Apob*$^{+/+}$*Apoe*$^{-/-}$ mice.

The apoB-48 and apoB-100 mice have also been crossed with the LDL receptor–knockout mice to generate *Apob*$^{48/48}$*Ldlr*$^{-/-}$ and *Apob*$^{100/100}$*Ldlr*$^{-/-}$ mice (unpublished observations, M. Véniant and S. Young). Interestingly, the *Apob*$^{48/48}$*Ldlr*$^{-/-}$ mice had only slightly greater LDL cholesterol levels than wild-type mice; their LDL levels were much lower than levels in *Ldlr*$^{-/-}$ mice and essentially the same as those in *Apob*$^{100/100}$ *Ldlr*$^{+/+}$ mice. The low LDL cholesterol levels in the *Apob*$^{48/48}$*Ldlr*$^{-/-}$ mice are presumably due to the fact that the LDL receptor-related protein (LRP) provides an efficient pathway for removal of the apoE-rich apoB-

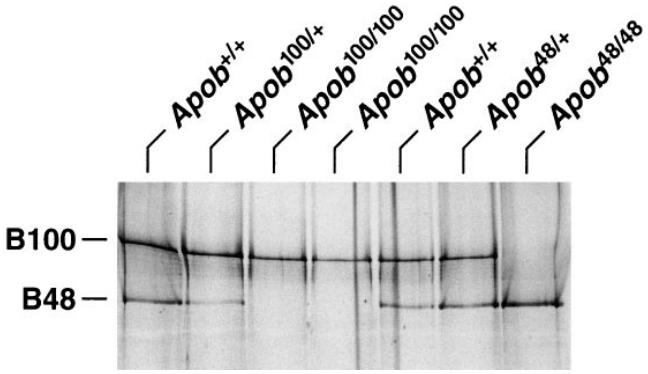

Fig. 7. Sodium dodecyl sulfate/polyacrylamide gel of the d < 1.21 g/ml lipoprotein fraction of wild-type, heterozygous, and homozygous apoB-48-only and apoB-100-only mice. The gel was stained with silver. Reproduced, with permission, from Farese et al. (34).

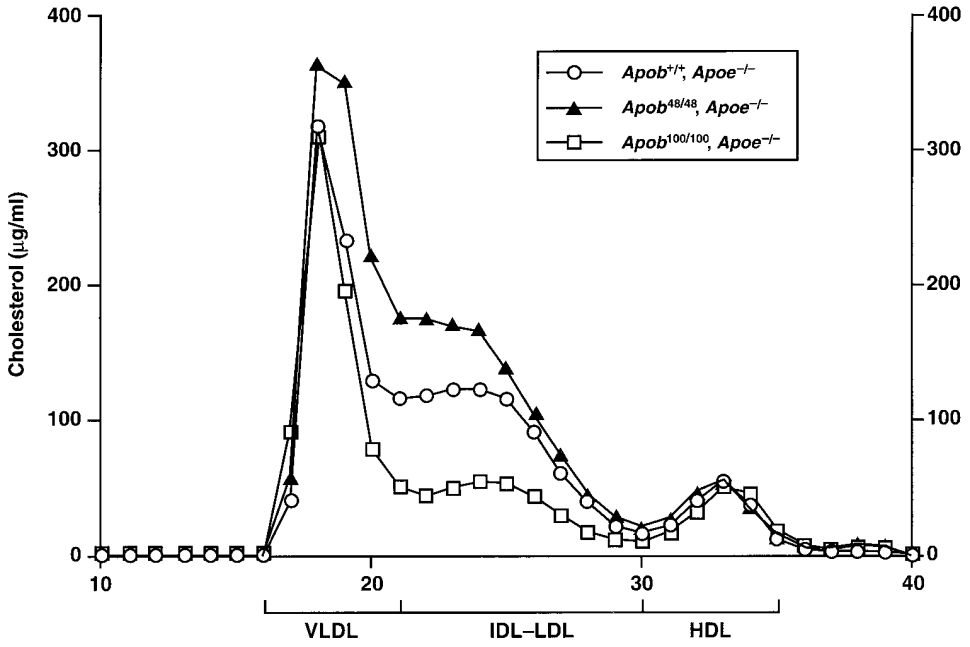

Fig. 8. Distribution of cholesterol in the plasma lipoproteins of *Apob*$^{+/+}$*Apoe*$^{-/-}$, *Apob*$^{100/100}$*Apoe*$^{-/-}$, and *Apob*$^{48/48}$*Apoe*$^{-/-}$ mice. The plasma was fractionated by Superose 6 chromatography, as previously described (26, 39). Reproduced, with permission, from Farese et al. (34).

48-containing lipoproteins. In contrast, the total and LDL cholesterol levels in the *Apob*$^{100/100}$*Ldlr*$^{-/-}$ mice were significantly higher than in *Ldlr*$^{-/-}$ mice. Once again, the *Apob*$^{100/100}$*Ldlr*$^{-/-}$ mice had significantly higher triglyceride levels than the *Ldlr*$^{-/-}$ mice or *Apob*$^{48/48}$*Ldlr*$^{-/-}$ mice. Neither the *Apob*$^{100/100}$*Ldlr*$^{-/-}$ nor the *Apob*$^{48/48}$*Ldlr*$^{-/-}$ mice developed hydrocephalus.

It was interesting that the plasma triglyceride levels were invariably increased in the *Apob*$^{100/100}$ mice, regardless of whether the mice expressed apoE or the LDL receptor. The metabolic basis for the higher triglyceride levels in the apoB-100-only mice has not been determined, but it is tempting to speculate that lipolysis may be less efficient with apoB-100-containing lipoproteins than with apoB-48-containing lipoproteins, either because of intrinsic differences in the apoB molecules or because apoB-48- and apoB-100-containing lipoproteins differ in their content of other apolipoproteins that could affect the extent and speed of lipolysis (e.g., the apoCs). In any case, the consistently higher triglyceride levels in the apoB-100 mice might provide one clue regarding the "physiologic rationale" for two forms of apoB in mammalian metabolism. ApoB-48 could have evolved, at least in part, to ensure a more rapid and complete unloading of triglycerides to peripheral tissues.

Because apoB-48 is present in the intestines of all mammals, it seemed likely that the intestinal synthesis of apoB-100 in the apoB-100-only mice might be suboptimal for the chylomicron assembly and fat absorption. This did not appear to be the case, at least in any easily detectable fashion. By light microscopy, the intestinal histology of the apoB-100-only mice was normal with no abnormal accumulation of fat, even after a bolus of corn oil or after months on a high-fat diet. Electron microscopy demonstrated the presence of large chylomicron particles in endoplasmic reticulum of the enterocytes of apoB-100-only mice. No differences were observed in retinyl palmitate absorption in the apoB-100-only and the apoB-48-only mice. Even though no differences were observed in intestinal fat absorption in the two types of mice, the apoB-100-only mice may have subtle abnormalities of chylomicron metabolism. Genetic and metabolic studies by Ishibashi et al. (90) and Willnow et al. (91) have suggested that the LRP clears apoB-48-containing lipoproteins more efficiently than apoB-100-containing lipoproteins. Based on their studies, it is possible that apoB-100-containing chylomicrons might not be removed as efficiently by the LRP and therefore might accumulate in the plasma, particularly in the setting of LDL receptor deficiency.

A recent study has suggested the possibility that apoB-48 or apoB-100 synthesis might have an effect on body weight (92). The *Apob*$^{48/48}$*Apoe*$^{-/-}$ mice were significantly heavier than the *Apob*$^{+/+}$*Apoe*$^{-/-}$ mice, which in turn were significantly heavier than *Apob*$^{100/100}$*Apoe*$^{-/-}$

mice. Adiposity was not assessed, so it is not clear whether the differences in body weight reflect differences in body fat. The mechanism whereby the length of the apoB protein might affect body weight is unknown and needs further study (e.g., in the setting of apoE expression, LDL receptor deficiency, different mouse strains, a high-fat diet, etc.).

Atherosclerosis in apoB-48-only and apoB-100-only mice. One of the long-standing debates in the lipoprotein field is whether there are intrinsic differences in the atherogenicity of the apoB-48- and apoB-100-containing lipoproteins. To address that issue, susceptibility to atherosclerosis in female $Apob^{100/100}Apoe^{-/-}$, $Apob^{48/48}Apoe^{-/-}$, and $Apob^{+/+}Apoe^{-/-}$ mice (n = 25 in each group) (92) was examined. As noted earlier, the $Apob^{48/48}Apoe^{-/-}$ mice had higher total plasma cholesterol levels than the $Apob^{+/+}Apoe^{-/-}$ mice, while the $Apob^{100/100}Apoe^{-/-}$ mice had lower cholesterol levels than the $Apob^{+/+}Apoe^{-/-}$ mice. Within each group of mice and across all three genotypes, the extent of atherosclerosis correlated strongly with the total plasma cholesterol levels. The $Apob^{48/48}Apoe^{-/-}$ mice had significantly more atherosclerosis than the $Apob^{+/+}Apoe^{-/-}$ mice, which had significantly more than the $Apob^{100/100}Apoe^{-/-}$ mice. However, there was a significant amount of overlap among the three groups of animals, both in terms of total plasma cholesterol levels and the extent of aortic atherosclerosis. In mice from different groups that had similar cholesterol levels, the extent of atherosclerosis was quite similar. Thus, susceptibility to atherosclerosis was dependent on total cholesterol levels, and whether mice synthesized apoB-48 or apoB-100 did not appear to have an independent effect on the extent of atherosclerosis.

The conclusion that there are probably no major differences in the intrinsic atherogenicity of apoB-48- and apoB-100-containing lipoproteins is subject to several caveats (92). First, because the different groups of animals had different mean cholesterol levels, this conclusion was based on a subset of animals with similar cholesterol levels and on a plot of the "cholesterol versus atherosclerosis" curve that appeared to be very similar in the three groups of animals. A second caveat is that the length of the apoB molecule has secondary effects on lipoprotein size and composition. For example, the VLDL from $Apob^{100/100}Apoe^{-/-}$ mice were much larger than the VLDL from $Apob^{48/48}Apoe^{-/-}$ mice. As a result of these secondary effects, it is very difficult to make specific conclusions regarding the intrinsic atherogenicity of the apoB molecules themselves. For example, the carboxyl-terminal amino acid sequences that are unique to the apoB-100 might cause retention of lipoproteins within the arterial wall (and therefore promote atherogenesis), while the size and composition of apoB-100-containing lipoproteins might mitigate against lipoprotein retention. Although it would be desirable to compare the intrinsic atherogenicities of apoB-48 and apoB-100 under conditions in which every experimental variable (such as plasma cholesterol concentration, lipoprotein size and composition, and diet) is identical, such ideal experimental conditions are not possible. However, an inability to achieve ideal experimental conditions should not discourage further atherosclerosis experiments with these mice, as understanding whether apoB-48 and apoB-100 are intrinsically different in their ability to promote atherosclerosis is fundamental to an understanding of why the apoB-containing lipoproteins are atherogenic.

A gene-targeted mouse model for understanding mechanisms in the human apoB deficiency syndrome, familial hypobetalipoproteinemia

In humans with FHβ, nonsense or frameshift mutations in the apoB gene lead to the synthesis of a truncated form of apoB. In these cases, the concentration of the truncated apoB in the plasma is invariably quite low. Even though many mutations causing FHβ have been identified (69), the mechanism for the low plasma concentrations of the truncated apoB proteins remains obscure. Several lines of evidence have suggested that the truncated apoB might be synthesized at a very low rate, while other lines of evidence suggest that the truncated apoB might simply be removed rapidly from the plasma (69). Because of practical hurdles in measuring apoB synthesis and secretion rates in human subjects with FHβ, we have long believed that an appropriate mouse model could greatly enhance our understanding of mechanisms of FHβ (69, 93).

We suspected that neither the apoB-70 mice nor the apoB-48-only mice provided truly appropriate models for understanding FHβ. Even though the lipoprotein profile in the heterozygous apoB-70 mice was similar to that observed in human FHβ heterozygotes, that mouse model did not provide fundamental insights into mechanisms underlying human FHβ. While virtually all of the cases of human FHβ are caused by point mutations (nonsense mutations or deletions of one to four nucleotides) (69), the apoB-70 mutation was caused by the insertion of a large stretch of foreign DNA into exon 26 of the apoB gene. Thus, the apoB-70 mouse model did not allow one to predict whether the point mutations that typically cause human FHβ would reduce apoB mRNA levels and thereby reduced apoB synthesis rates. In addition, the apoB-70 allele yielded two distinct truncated apoB proteins (apoB-48 and apoB-70) in the liver, adding a layer of complexity to the interpretation of lipoprotein metabolism in these animals. In humans with FHβ, there is no apoB-48 pro-

duction by the liver as the human liver lacks hepatic apoB mRNA–editing activity.

Similarly, the apoB-48-only mice had shortcomings as a model for human FHβ. Those animals had normal levels of the mutant apoB mRNA and actually had higher plasma apoB-48 levels than wild-type mice (34). Because the apoB-48 levels in those animals were not abnormally low, the apoB-48-only mutation did not provide an appropriate model for human FHβ mutations. The absence of reduced apoB synthesis rates or reduced apoB mRNA levels might reflect the placement of a nonsense mutation into a "natural site" within the apoB gene (i.e., the same codon where the apoB mRNA–editing machinery introduces a stop codon into the apoB transcript).

To create an appropriate model of human FHβ and to gain new and definitive insights into mechanisms of the disorder, we used gene targeting to insert an apoB-83 mutation (Leu3798Stop) into the mouse apoB gene (unpublished observations, E. Kim and S. Young). In addition to the Leu3798Stop mutation, the apoB-83-only allele contained a CTA-missense mutation at codon 2153, which abrogates apoB-48 formation without affecting apoB mRNA levels (34). Unlike the apoB-70 and apoB-81 mutations generated by Homanics et al. (76) and Toth et al. (81), the apoB-83-only allele did not alter the length and structure of the apoB transcript and did not produce apoB-48. Unlike the apoB-48-only mutation (34), the nonsense mutation was inserted into an "unnatural" site and therefore did not lead to the production of a "physiologically normal" apoB protein.

In mice heterozygous for the *Apob*^{83} allele, apoB-83 was present in only trace levels in the plasma (~2% of the level of apoB-100 that was produced by the other allele), a profile strikingly similar to that observed in human FHβ heterozygotes with an apoB-83 mutation (94). Analysis of the plasma lipoproteins in the heterozygous apoB-83-only mice revealed that apoB-83 was easily detectable in the VLDL (the ratio of apoB-83 to apoB-100 in the VLDL fraction was 1:10) but was essentially absent in the LDL (where the majority of the apoB-100 in mouse plasma resides), a pattern identical to the distribution of apoB-83 in human FHβ heterozygotes.

Analysis of heterozygous apoB-83-only mice uncovered two mechanisms for the extremely low plasma levels of apoB-83. First, *Apob*^{83} mRNA levels and apoB-83 secretion by hepatocytes were reduced 76% and 72%, respectively. Thus, unlike the nonsense mutation in the apoB-48-only mice, a nonsense mutation at an "unnatural" site can cause low apoB mRNA levels and correspondingly low apoB secretion rates. Although nonsense mutations have been shown to cause low mRNA levels in other gene products (95–99), the studies on the apoB-83-only mice represented the first indication that this mechanism applies to FHβ. In addition to low synthesis rates, apoB-83 was removed rapidly from the plasma. There are several potential explanations for the rapid clearance of apoB-83. One possibility is that apoB-83-containing lipoproteins, like apoB-48-containing lipoproteins, may accommodate more apoE, resulting in a more rapid clearance of lipoproteins from the plasma. An additional possibility is that apoB-83 might actually be more effective than apoB-100 in mediating the uptake of LDL as well as VLDL by the LDL receptor. Krul and co-workers (100) have previously suggested that apoB proteins that lack the carboxyl terminus of apoB-100 might have an increased affinity for the LDL receptor. Enhanced uptake of apoB-83-containing VLDL would explain why apoB-83 was virtually absent from the LDL fraction of *Apob*$^{83/+}$ mice and would also explain the extremely low levels of apoB-83 in the plasma.

Apob$^{83/+}$ mice were intercrossed, and the genotypes of the offspring were examined at weaning. From 20 litters, we identified 102 *Apob*$^{83/+}$ and 44 *Apob*$^{+/+}$ mice but no *Apob*$^{83/83}$ mice. Thus, homozygosity for the apoB-83-only mutation caused a lethal developmental abnormality. Further analysis revealed that *Apob*$^{83/83}$ embryos manifested multiple developmental abnormalities, including exencephalus, hemorrhage from the brain into the amniotic fluid, growth retardation, and herniation of the liver into the umbilical cord, findings similar to those in the apoB-70, apoB-81, and apoB knockout mice. These findings suggest that apoB synthesis rates that are 25–30% of normal fall below the threshold required for normal mouse development.

Studying apoB structure/function with gene-targeting techniques

As noted earlier, the techniques for mutating large fragments of genomic DNA in YACs, BACs, and P1s have allowed investigators to use conventional transgenic mice to study human apoB structure/function. An alternative approach to studying apoB structure/function is to introduce mutations into the endogenous apoB gene via homologous recombination in ES cells. Recently, Toth and coworkers (81) reported the use of this approach to examine the structural features of the apoB-100 molecule that are important in its binding to the LDL receptor. They sought to test whether two stretches of positively charged amino acid residues within the carboxyl terminus of apoB-100 [which have been designated the "A" and "B" sites (74, 75)] might play a significant role in binding to the negatively charged ligand-binding domain of the LDL receptor. They used "pop-in, pop-out" gene targeting to replace the "A" and "B" sites of mouse apoB with more

neutral amino acid sequences from the β-globin gene. In their experiments, codon 2153 was not changed, so wild-type apoB-48 remained a principal product of their mutant allele. Mice homozygous for their mutant apoB allele were free of developmental abnormalities and appeared grossly normal. Interestingly, although eliminating the "A" and "B" sites appeared to reduce triglyceride levels, the mutations did not result in increased total cholesterol or LDL cholesterol levels. These results were somewhat surprising because the experiments of Borén (65) (involving the expression of mutant human apoBs in transgenic mice) have indicated that the "B" site in human apoB-100 is critical for LDL receptor interactions. One interpretation of the studies by Toth and coworkers (81) is that the "A" and "B" sites of mouse apoB-100 are not essential for binding to the mouse LDL receptor. An alternative interpretation is that the targeted mutations did reduce the ability of mouse apoB-100 to bind to the LDL receptor, but that diminished apoB-100 binding to the LDL receptor did not perceptibly perturb lipoprotein metabolism because the apoB-containing lipoproteins were cleared efficiently via apoE-mediated pathways.

SUMMARY

The development of transgenic mice and gene-targeted mice to study apoB has permitted the investigation of numerous issues in apoB biology, including examining the effects of apoB overexpression on lipid metabolism and atherosclerosis, apoB structure/function relationships, defining distant regulatory elements controlling the intestinal expression of apoB, investigating the role for apoB in embryonic development, understanding the specific roles for apoB-48 and apoB-100, and elucidating basic mechanisms underlying the apoB deficiency syndrome familial hypobetalipoproteinemia. We see many opportunities for the continued use of genetically modified mice in apoB research.

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