Recent Progress in Understanding Apolipoprotein B

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The two isoforms of apolipoprotein (apo) B, apo B-48 and apo B-100, are important proteins in human lipoprotein metabolism. Apo B-48, so named because it appears to be about 48% the size of apo B-100 on sodium dodecyl sulfate (SDS)–polyacrylamide gels, is synthesized by the intestine in humans. Apo B-48 is necessary for the assembly of chylomicrons and therefore has an obligatory role in the intestinal absorption of dietary fats. Apo B-100, which is produced in the liver in humans, is required for the synthesis and secretion of very low density lipoproteins (VLDL). Low density lipoproteins (LDL), which contain about two thirds of the cholesterol in human plasma, are metabolic products of VLDL. Apo B-100 is virtually the only protein component of LDL. Elevated concentrations of apo B-100 and LDL cholesterol in plasma are recognized risk factors for developing atherosclerotic coronary artery disease.

Because of the central roles of the two isoforms of apo B in lipoprotein metabolism and the atherogenic potential of apo B–containing lipoproteins, apo B has been a prime target for study by investigators in the lipoprotein and arteriosclerosis field. Over the past 4 years, significant progress has been made in understanding apo B, including the determination of the primary structure of the two isoforms of apo B, the organization of the apo B gene, and the delineation of the important functional domains of apo B. A substantial amount of heterogeneity in the apo B gene has been documented, and multiple apo B gene mutations that affect the plasma cholesterol level have been reported. Recent developments in these areas will be included in this review. However, not all of the important developments within the apo B field can be reviewed within the space limitations of this article; several of these topics deserve special attention. In the past few years, progress has been made in understanding the kinetics of apo B synthesis and secretion by cells and also the cellular pathways for the catabolism of apo B-containing lipoproteins; these topics have been recently reviewed. Additionally, in the past 5 years it has become apparent that biological modifications of apo B-containing lipoproteins may be important in understanding their atherogenicity. This exciting area of apo B research has been recently reviewed by Steinberg and coworkers. Another rapidly developing area of apo B research has been the structure and functional significance of Lp(a), an apo B-100–containing lipoprotein that appears to be a strong risk factor for coronary artery disease. Progress in understanding Lp(a) has been recently reviewed by Utermann.

Overview of Roles of Apo B-48 and Apo B-100 in Lipoprotein Metabolism

Apo B-48 has an obligatory structural role in the assembly of chylomicrons in the intestine (Figure 1A). Existing evidence suggests that only one apo B-48 molecule is integrally associated with each chylomicron particle; unlike the other apolipoprotein components of chylomicrons, it remains associated with the particle and does not exchange onto other lipoprotein particles. Whether chylomicrons from normal adult human subjects might occasionally contain apo B-100 instead of apo B-48 is under investigation in several laboratories. Recently, Dul-laart et al presented evidence for some apo B-100 production in the intestine. However, in our own immunocytochemical studies of the duodenal and jejunal enterocytes of organ donor patients, we found abundant amounts of intracellular apo B-48 but no evidence of apo B-100 synthesis (J. Boyles, S. Young; unpublished results). After secretion from enterocytes, chylomicrons enter the lymph and are delivered to the bloodstream through the thoracic duct. In the circulation, chylomicrons are rapidly sequestered along the capillary endothelium, principally that of skeletal muscle and adipose tissue, where the enzyme lipoprotein lipase hydrolyzes 80–90% of the particles' triglyceride mass. The resultant lipoproteins,
A. Chylomicron/Apo-B48 Metabolism

which are called chylomicron remnants, are then transported to the liver, where they are probably further metabolized by hepatic lipase and are ultimately bound and taken up by a remnant receptor that recognizes apo E (Figure 1A). Whether the receptor involved in chylomicron remnant uptake is the LDL receptor, a recently described LDL receptor–related protein, or another protein is a matter for active investigation. The existing evidence suggests that apo B-48 does not participate in the particle's interaction with the hepatic remnant receptor. Once internalized into the hepatocyte, the particles are directed to the lysosome, where they are digested. The cholesterol derived from remnant lipoproteins is esterified and stored as cholesteryl ester, used for synthesis of bile acids, or used in the synthesis of hepatic lipoproteins. Virtually all chylomicron remnants are removed from plasma as relatively large particles that have a density of less than 1.019 g/ml; consequently, apo B-48 is not normally detectable in the LDL (d=1.019–1.063 g/ml).

The normal residence time of chylomicrons in the plasma is only 5–10 minutes. As a consequence of this rapid catabolism, the concentration of apo B-48

B. VLDL/Apo-B100 Metabolism

Figure 1. Panel A: Schematic of metabolism of apolipoprotein (apo) B-48–containing lipoproteins. Panel B: Schematic of metabolism of apo B-100–containing lipoproteins. See text for discussion of this figure and Reference 8 for overview of metabolism of apo B. ER, endoplasmic reticulum; VLDL, very low density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein.
in the plasma is very low, probably only a few micrograms per milliliter, and perhaps only 0.1% that of apo B-100. Despite the very low levels of apo B-48 in the plasma, it is invariably detectable on a stained SDS-polyacrylamide gel of a VLDL fraction obtained from a fasting donor. Apo B-48 concentrations within the chylomicon and VLDL fractions increase after a fat-rich meal relative to that of apo B-100, as determined by relative staining intensity of the two bands on SDS-polyacrylamide gels. Recently, Simons et al. examined the amount of apo B-48 relative to apo B-100 on stained gels of the postprandial lipoproteins of control subjects and subjects with coronary artery disease. They found that the apo B-48-to- apo B-100 ratio was significantly increased in patients with coronary artery disease, even when age and other lipid discriminators, such as triglycerides and cholesterol, were included in the analysis. The metabolic basis of their observation on apo B-48 levels—whether the finding is due to increased secretion or decreased catabolism of apo B-48-containing lipoproteins—is not known; however, the finding is consistent with long-standing theories that postprandial lipoproteins may be atherogenic. No immunoassay for apo B-48 in plasma has yet been developed because no one has been able to develop an antibody that is specific for apo B-48 (i.e., an antibody that would bind to apo B-48 but not to the more abundant apo B-100).

Apo B-100-containing lipoproteins are assembled within hepatocytes, secreted into the space of Disse, and ultimately enter the circulation through the hepatic vein (Figure 1B). Most of the apo B-100 is secreted from the liver on triglyceride-rich VLDL particles, which also contain apo E and various apo Cs; kinetic data from turnover studies on normolipidemic subjects suggest that there is probably very little independent secretion of apo B-100 on smaller, denser particles of the LDL fraction. Unlike other apolipoprotein constituents of the particle, the apo B-100 of VLDL particles does not exchange between lipoprotein particles, so radiolabels attached to apo B-100 can be used to trace the metabolic fate of the particle in plasma. After release of nascent VLDL particles into the bloodstream, the initial phase of their metabolism resembles that of chylomicrons. The triglyceride-rich core of VLDL particles is hydrolyzed by lipoprotein lipase along the capillary endothelium. As the particle’s core is reduced in volume, the apo B-100 and apo E on the surface of the VLDL particle probably change their conformation in such a way that they can serve as ligands for uptake of the particle by hepatic receptors. Probably one half of all VLDL particles is removed from the circulation in the liver through interaction with the LDL receptor (also known as the apo B,E receptor); the other half remains in the circulation and is further metabolized to denser particles of the LDL fraction. The half-life of VLDL particles in the plasma is heterogeneous, ranging from minutes to hours. Existing evidence suggests that large VLDL particles, which may contain a substantial number of apo E molecules, are quickly removed from the circulation, whereas smaller VLDL particles tend to be metabolized more slowly, with a greater likelihood of being metabolized to the smaller, denser particles of the intermediate density lipoprotein (IDL) and LDL fractions. By the time the particles are metabolized to the size and density of the LDL range, they have become enriched in cholesteryl esters, and virtually the only remaining protein component is apo B-100. The average residence time of LDL is 2–3 days; about 80% of LDL is removed from the circulation by the interaction of apo B-100 with the LDL receptor and the remainder by nonreceptor pathways. Approximately one half of the LDL is removed from human plasma by the liver; the other half is removed by extrahepatic tissues. Once LDL is bound and internalized by cells, it is directed to the lysosome, where both its protein and lipid components are digested (Figure 1B).

Apo B-100 concentrations can be measured by a variety of immunoassays, and normal plasma levels range from 60 to 120 mg/dl. More than 90% of the apo B-100 within the plasma of normolipidemic individuals is contained within the LDL fraction; hence, there is normally little difference between the total plasma apo B and LDL apo B levels. The LDL size and chemical composition are known to be heterogeneous in the population, and some of this heterogeneity is probably due to genetic factors. A variety of data indicate that the plasma of patients with coronary artery disease tends to contain an increased number of small, dense LDL particles that have a decreased cholesteryl ester-to-apo B-100 ratio. The metabolic basis for this finding is incompletely understood but is under active investigation. These patients may have relatively normal LDL cholesterol concentrations yet elevated apo B-100 levels. This observation has led some investigators to recommend measuring apo B levels in addition to LDL cholesterol levels in patients at risk for coronary artery disease.

Apo B-100 can become associated with apo(a), a large glycoprotein structurally related to plasminogen, to form a unique lipoprotein [Lp(a)]. Lp(a) particles have about the same lipid composition as LDL particles, but because of the addition of apo(a) they are larger and denser than LDL particles. Apo B-100 and apo(a) dissociate from each other when subjected to reducing agents, and it is generally assumed that a disulfide bond links the two proteins. The molecular weight of apo(a) is quite heterogeneous in the population and is genetically determined. The plasma concentration of Lp(a) is strongly controlled by genetic factors and markedly varied, from virtually undetectable amounts to 100 mg/dl. The concentration of Lp(a) in plasma is strongly related to the risk of developing coronary artery disease. The cause of its atherogenicity is under intense investigation; the atherogenicity may relate
in part to the potential of Lp(a) in inhibiting thrombolytic mechanisms.\textsuperscript{7}

**Structure of Apo B-100**

In 1986 four groups reported the complete nucleotide sequence for the apo-B complementary DNA (cDNA), along with the deduced amino acid sequence.\textsuperscript{29-32} The cDNA is 14,121 nucleotides in length, with 5' and 3' untranslated regions of 128 and 304 base pairs (bp), respectively. The cDNA codes for a 27-amino-acid hydrophobic signal peptide, which is cotranslationally cleaved, followed by a mature protein of 4,536 amino acids. Boerwinkle and Chan\textsuperscript{33} have confirmed the existence of a 3-amino-acid insertion/deletion polymorphism in the apo B signal peptide, which is of no known functional significance. The sequence of the apo B-100 polypeptide chain is unique, although computer-assisted searches have identified remote but statistically significant internal amino acid repeats within apo B-100.\textsuperscript{34} Computer sequence alignment programs have also identified remote but statistically significant amino acid homologies with vitellogenin and other apolipoproteins.\textsuperscript{34,35} The sequence homologies with other apolipoproteins were almost always within sequences predicted to be capable of forming amphipathic helices. Apo B is known to be a glycoprotein, with about 4-9\% of its mass as carbohydrate linked to asparagine.\textsuperscript{36} There are 19 potential N-linked glycosylation sites on apo B; by direct sequencing, Yang et al\textsuperscript{37} found that all but three of these are glycosylated. Apo B-100 is reportedly posttranslationally modified by covalently bound fatty acids.\textsuperscript{38,39} Weisgraber and Rall\textsuperscript{40} have shown that there are seven different regions of the apo B-100 molecule that strongly bind to heparin.\textsuperscript{40} Heparin-binding sites on apo B may serve to promote the binding of triglyceride-rich lipoproteins to the capillary endothelium, where their lipid cores are digested by lipoprotein lipase. Apo B contains 25 cysteine residues, and these are distributed in an asymmetric fashion within the molecule – 12 occur within the first 500 residues. Sixteen of the 25 cysteines are known to exist in disulfide form, including all 12 in the first 500 amino acids.\textsuperscript{41} Recently, Coleman et al\textsuperscript{42} reported that there are two free cysteines in LDL–apo B-100 located at positions 3,734 and 4,190. Either of these carboxyterminal cysteines (or both) could potentially form a disulfide bond with apo(a) to form Lp(a).

While recently investigating a subject whose plasma lipoproteins contained both apo B-100 and an amino terminal–truncated isoform of apo B (apo B-46), we found apo(a) exclusively on the apo B-100 particles (A. Scanu, S. Young, unpublished observations). These data are consistent with one of the carboxy-terminal cysteines being involved in the formation of Lp(a).

Apo B-100 contains numerous hydrophobic domains throughout its length that are believed to be important in lipid binding.\textsuperscript{3} In addition, De Loof et al\textsuperscript{44} and others\textsuperscript{59} have identified nine amphipathic helices, each one 22 amino acids in length, that are similar to amphipathic \( \alpha \)-helices found in the putative lipid-binding domains of other apolipoproteins. Nearly all of the apo B-100 sequences having the capacity to form amphipathic \( \alpha \)-helices are located in the carboxyterminal half of the apo B-100 molecule. In addition, apo B contains multiple proline-rich sequences predicted to form amphipathic \( \beta \)-sheets and \( \beta \)-turns; these are located throughout the apo B sequence except for the aminoterminal 1,000 amino acids. Although amphipathic \( \beta \)-sheets are not found in other apolipoproteins, these structures are thought to have high lipid-binding potential. Thus, apo B has many potential lipid-binding regions throughout its length, a result thought to be adequate for explanation of the fact that apo B never exchanges between lipoprotein particles, in contrast to the other apolipoproteins, which have one or two putative lipid-binding domains and readily exchange between lipoproteins. Chen and coworkers have reported experimental evidence that many different apo B peptides are capable of binding to lipoprotein particles.\textsuperscript{43} They digested LDL particles with proteases in the presence of SDS and then removed the SDS by dialysis, allowing the apo B peptides to recombine with lipid. Recombinant lipoproteins having the size and physical characteristics of LDL formed spontaneously after removal of the detergent. Peptides throughout the length of apo B-100 were identified in these recombinant particles. Although these investigators did not rigorously exclude peptide-to-peptide interactions among multiple hydrophobic fragments of apo B, their study tends to confirm the prediction of multiple lipid-binding domains. If many sequences throughout the entire length of apo B are important for the binding of lipid, then truncated apo B proteins would be predicted to form denser, lipid-poor particles. Indeed, studies performed by Young et al\textsuperscript{44} and Graham et al\textsuperscript{45} have shown that shorter isoforms of apo B seem capable of binding less lipid. Young and coworkers found that a truncated apo B of 2,046 amino acids (apo B-46) was found primarily in the VLDL and LDL fractions;\textsuperscript{46} a truncated apo B of 1,728 amino acids (apo B-37) was also found in the d<1.006 g/ml fraction of both fasting and postprandial plasma, but a large portion of the apo B-37 was contained in the HDL fraction;\textsuperscript{47} a truncated apo B of 1,425 amino acids (apo B-31) was found only in the dense subfractions of HDL and in the d>1.21 g/ml fraction.\textsuperscript{44} So the shorter the apo B protein, the denser and more lipid poor the particle. Furthermore, the discovery of apo B-37 but not apo B-31 in the VLDL fraction suggests that apo B sequences between 1,425 and 1,728 amino acids may be crucial for forming buoyant, triglyceride-rich VLDL particles.\textsuperscript{44}

A significant contribution to our understanding of the disposition of apo B on lipoprotein particles has been made by Yang and coworkers.\textsuperscript{37} These investigators have determined the regions of the apo B molecule on LDL particles that were “accessible” to
trypsin and therefore readily releasable from LDL particles [the trypsin-releasable (TR) peptides] and the portions that were accessible only after the LDL particles had been delipidated and trypsinized (the trypsin nonreleasable [TN] peptides). Some peptides were contained in both the TR and TN fractions; these were designated mixed (MX) peptides. Altogether, they sequenced more than 88% of the apo B-100 protein, with many peptides being repetitively sequenced. On the basis of the trypsin releasability and nonreleasability of various regions of the molecule, they identified five broad domains of apo B on LDL particles (Figure 2): domain I, amino acids 1–1,000, predominantly TR; domain II, amino acids 1,001–1,700, alternating regions of TR and TN peptides, with a significant proportion of MX peptides; domain III, amino acids 1,701–3,070, occasional TR peptides but primarily a TN region; domain IV, amino acids 3,071–4,100, mainly TR and MX peptides; and domain V, amino acids 4,101–4,536, almost exclusively TN peptides. In general, the more hydrophobic the region, the less likely it was to be accessible to trypsin. The authors were careful to point out that the factors governing trypsin releasability versus nonreleasability are not completely understood, and they explicitly cautioned against the assumption that the trypsin releasability versus nonreleasability implies a lipoprotein-surface versus lipoprotein-core location for the peptide. However, the latter assumption clearly represents a tempting hypothesis. Recently, Yang and coworkers48 purified and sequenced 13 tryptic peptides of apo B-100 that spontaneously associated with three different phospholipids. All of the peptides were highly hydrophobic; predictive algorithms revealed no evidence for amphipathic helices, and no spectroscopic evidence for β-sheet structure was found. Five of the peptides were “surface” ones (trypsin accessible), and these were located throughout the polypeptide chain. The remainder were core peptides (trypsin inaccessible), and most of these were clustered in the carboxyterminal 33 amino acids of apo B-100.

Recently, Phillips and Schumaker49 examined the apo B-100 of LDL particles by electron microscopy (EM) after the particles had been treated with glutaraldehyde and their lipid extracted; the images thus produced strongly suggested that apo B-100 may indeed encircle LDL particles, as shown in Yang et al’s schematic (Figure 2). New EM techniques have recently been developed to visualize apo B on the surface of LDL particles.50 The binding of apo B–specific monoclonal antibodies whose epitopes have been precisely localized can also be directly visualized (V. Schumaker, personal communication). These studies should improve our understanding of the relative location of various regions of apo B on LDL particles.

For several years, it has been observed that the LDL receptor–binding region of apo B-100 is in the carboxyterminal portion of the molecule. The receptor-binding region of apo B-100 has been recently studied in detail by Milne and coworkers.51 They determined the location of the epitopes of more than 30 apo B–specific monoclonal antibodies and which of the antibodies were capable of inhibiting the binding of LDL particles to the LDL receptor. The antibodies with epitopes located between amino acids 2,980 and 3,780 completely block the specific binding of LDL to the LDL receptor when bound to LDL. Antibodies that bind to epitopes immediately flanking this region partially block binding of LDL to its receptor, whereas monoclonal antibodies binding elsewhere in the molecule had for the most part little or no receptor-blocking activity. A summary of the efforts of Milne and coworkers is shown in Figure 3. Other data also implicate the carboxyterminal region of apo B-100 in binding to the LDL receptor. First, a lipoprotein containing a truncated apo B isoform lacking the carboxyterminal region does not bind to the LDL receptor.52 Second, the carboxyterminal domain of apo B implicated in the studies of Milne et al contains three different heparin-binding regions50 that have clusters of positively charged amino acids; sequences within one of the heparin-binding domains (residues 3,359–3,367) have sequence homology with the region of apo E known to interact with the LDL receptor.29 Yang and coworkers53 have presented evidence that a peptide containing these amino acids but no other apo B peptide could bind to the LDL receptor. The regions of apo B containing the clusters of positively charged amino acids are known to be evolutionarily well conserved.51 Positively charged
regions of apo B are thought to bind to the many negatively charged amino acid residues within the cysteine-rich repeats of the ligand-binding domain of the LDL receptor.\textsuperscript{5} Chemical modification of the positively charged residues of apo B block its binding to the LDL receptor.\textsuperscript{54} Finally, as discussed below, a missense (Arg $\rightarrow$ Gln) mutation in the codon for apo B-100 amino acid 3,500 is associated with defective binding of LDL to the LDL receptor.

**Apo B Gene and Control of Its Expression**

The apo B gene, which is located on the p23-ter region of chromosome 2,\textsuperscript{55-58} spans approximately 43 kb and contains 29 exons and 28 introns; two of the exons, exons 26 and 29, are extremely long (7,572 and 1,906 bp, respectively) (Figure 4). Exon 26, which codes for amino acids 1,379-3,903, is threefold longer than any previously reported exon of any mammalian gene. Whether the two large exons arose from fusion of smaller exons is unknown. Six repetitive sequences are present in the introns of the gene.\textsuperscript{59} There is an AT-rich hypervariable region within 200 bp of the polyadenylation site that consists of a variable number of 15-bp hypervariable elements.\textsuperscript{60,61} There are at least 14 different 3′ hypervariable region alleles, and about 75% of the population is heterozygous for different alleles at this site.\textsuperscript{62,63} Another tandem repetitive sequence, with at least three commonly occurring alleles, has been recently identified within intron 20 of the apo B gene (H. Hobbs, personal communication). The number of

**Figure 3.** Schematic of proposed structure of the putative binding domain of apolipoprotein (apo) B. Regions (specified by position numbers of apo B-100 amino acid residues) that contain the epitopes of monoclonal antibodies that totally or partially block binding of low density lipoprotein (LDL) to the LDL receptor are indicated by solid and open boxes above or below polypeptide chain, respectively. The two short stretches of positively charged amino acids are indicated by open boxes within polypeptide chain; regions of amphipathic $\beta$-sheet structure are depicted by zigzag line. The locations of cysteines at residues 3,167 and 3,297, which are linked by disulfide bond (S-S), are indicated, as is the location of the Arg$\rightarrow$Gln amino acid change at residue 3,500, a substitution that is associated with hypercholesterolemia (see text). The cysteines at residues 3,167 and 3,297 are not well conserved in apo B sequences of other mammalian species.\textsuperscript{64} The epitopes for antibodies MB47 and B1B3 are shown in close proximity because these antibodies have been shown to compete with each other for binding to LDL. Reproduced, with slight modifications, with permission.\textsuperscript{54}

**Figure 4.** Schematic of mechanism of apolipoprotein (apo) B-48 synthesis from the apo B gene. The apo B gene contains 29 exons, which are illustrated by black lines and bars at top of figure. The location of the two large exons (26 and 29) are indicated. Within the apo B gene and hepatic apo B messenger RNA (mRNA), codon 2,153 (CAA) specifies glutamine. However, in the intestine, the first base of this codon (nucleotide 6,666) is edited to U, producing a UAA termination codon. A subset of human apo B mRNAs are 7-8 kb in length because of the use of alternate polyadenylation signals, but virtually all human intestinal apo B mRNAs, regardless of length, contain the edited base and therefore yield apo B-48.\textsuperscript{80,81}
repetitive elements at the hypervariable regions can be rapidly and accurately assessed with polymerase chain reaction–based techniques; therefore, the utility of these regions for epidemiological and family studies is large.

The apo B gene contains a TATA box and a CAT box within the 60 bp upstream from the transcriptional start site. Das and coworkers\(^6\) have reported that 261 bp upstream from the transcriptional start site was sufficient for liver-specific expression; sequences located between -128 and -86 contained a positive element acting to increase expression.\(^6\) In the latter positive element, Levy-Wilson et al\(^6\) reported the existence of a DNase I-hypersensitive site and a 13-bp sequence that is perfectly conserved between the mouse and human promoters.\(^6\) Sequences between -86 and -70 contained another strongly positive element; recent studies have shown that a protein designated AF-1 binds to these sequences. AF-1 has also been shown to bind to promoters of other apolipoprotein genes.\(^6\) Mutations within this AF-1–binding sequence have been shown to reduce transcription rates by 50-fold. C/EBP, another hepatic transcription factor, binds to an element between -69 and -52; site-directed mutagenesis of this element reduced transcriptional activity twofold to fourfold.\(^6\) These sequences are not likely to be the only important protein-binding sites; Levy-Wilson et al\(^6\) have documented the existence of DNase I-hypersensitive sites (which tend to correlate with protein binding) as far as 700 bp upstream from the transcriptional start site.

The factors and sequence elements that are responsible for terminating apo B gene expression in tissues other than liver and intestine are not fully understood. One factor could be DNA methylation. Levy-Wilson and Fortier\(^7\) have shown that the promoter of the apo B gene is undermethylated in intestinal and liver cell lines but methylated in cells not transcriptionally active. Whatever the mechanism for terminating apo B transcription in other cell lines, it is probably not invariably absolute. We have recently amplified the apo B cDNA from fibroblast RNA.\(^8\) Thus, there is probably low-level “illegitimate transcription”\(^9\) of the apo B gene in tissues other than liver and intestine.

There is little evidence that the apo B messenger RNA (mRNA) level is significantly regulated by dietary factors in mammals. Long-term cholesterol feeding has reportedly resulted in only minor changes in apo B mRNA levels in mice, rats, and rabbits.\(^70\)–\(^72\) Recently, Sorci-Thomas et al\(^73\) investigated apo B mRNA levels in African Green monkeys fed low- or high-cholesterol diets in the presence of saturated or polyunsaturated fats. Ingestion of cholesterol and saturated fats markedly increased plasma apo B and LDL cholesterol levels, but no increases in hepatic apo B mRNA levels were found in the animals. However, the high-cholesterol-diet group did have a 50% decrease in mRNA for the LDL receptor. The authors concluded that the plasma levels of apo B-containing lipoproteins were governed primarily by factors controlling the removal of these lipoproteins from the plasma rather than by factors controlling their synthesis.

No studies on human hepatic or intestinal apo B mRNA levels in response to diet have been performed. However, Pullinger et al\(^74\) recently studied the apo B mRNA levels and synthesis rate in a human hepatoblastoma cell line under a variety of metabolic conditions. Although they found that the secretion of apo B into the medium could be modulated by insulin and fatty acids, they found little or no evidence for acute regulation of apo B mRNA levels. They concluded that the apo B gene was constitutively expressed in these cells and that differences in apo B secretion rates under different conditions must be due to cotranslational or posttranslational processes. This conclusion was supported by the observation that the apo B mRNA half-life was quite long (16 hours). In a study of rat intestinal synthetic rates and mRNA levels under different conditions, Davidson and coworkers\(^75\) reached similar conclusions. The apo B mRNA level did not change under conditions in which synthesis rates varied significantly. The cotranslational and posttranslational factors governing apo B synthesis and secretion rates are currently not fully understood; however, several investigators working with different systems have reported evidence that intracellular lipid availability may be an extremely important factor in the rate of apo B secretion from cells.\(^76\)–\(^78\) Several investigators engaged in the study of the kinetics of apo B secretion from cells have recently reported evidence that a portion of the intracellular apo B may be degraded but not secreted and have suggested that metabolic conditions (i.e., lipid availability) may influence the portion of the intracellular apo B pool that is secreted.\(^76\) Whether the rate of apo B secretion is physiologically regulated in vivo by this type of mechanism is an important issue that deserves intensive investigation, as it could have a bearing on strategies to control oversecretion of apo B.

Mechanism of Apo B48 Formation

In addition to the structural relation of apo B-48 to apo B-100, the mechanism for apo B-48 formation was almost simultaneously determined in 1987 by Chen et al\(^80\) and Powell et al\(^81\) and later by Hospatankar et al.\(^82\) These investigators demonstrated that apo B-48 is produced from the apo B-100 gene by a novel mechanism involving mRNA editing. In sequencing the apo B cDNA isolated from human intestinal cDNA libraries, they found that the intestinal apo B cDNA contained a T at nucleotide 6,666, in contrast to the C found at the position in the liver apo B cDNA clones (Figure 4). The substitution of the T for a C at nucleotide 6,666 yields an in-frame stop codon (TAA) that replaces the CAA codon specifying apo B-100 amino acid Gln at position 2,153. The location of the stop codon predicts that apo B-48 found in plasma would contain the amino-
terminal 2,152 amino acids of apo B-100; this prediction was strongly supported by the protein-sequence efforts of Chen et al.\(^8\) as well as by the studies of Hardman et al.\(^6\) This substitution at nucleotide 6,666 was present in the intestinal mRNA\(^8\) but not in intestinal genomic DNA,\(^8\) so it was clearly the result of a specific form of posttranscriptional editing of the intestinal apo B mRNA. Apo B mRNA editing to create an in-frame stop codon has been shown to occur in rat and rabbit intestinal apo B mRNA\(^8\) and rat liver, which is known to produce both apo B-48 and apo B-100.\(^8\)\(^,\)\(^8\) A fraction of the human intestinal apo B mRNA is 7–8 kb in length because of the use of alternate polyadenylation signals after the edited nucleotide (Figure 4). The process in humans appears to be developmentally regulated; intestinal organ cultures drawn from fetuses in early gestation primarily make apo B-100, whereas the adult intestine makes apo B-48 and very little, if any, apo B-100.\(^8\)\(^,\)\(^8\) The efficiency of apo B mRNA editing in rat liver has recently demonstrated modulation by fasting/ refeeding\(^8\) and thyroid hormone.\(^8\)

The exact mechanism for apo B mRNA editing remains unclarified, but the most plausible hypothesis is that a tissue-specific enzyme recognizes a specific sequence within the apo B mRNA and deaminates position 4 of cytosine-6,666, creating a uracil residue.\(^8\)\(^,\)\(^8\) A reasonable hypothesis suggests that a specific RNA editing process would require conservation of the nucleotide sequence surrounding nucleotide 6,666 through evolution. Indeed, sequencing studies of the apo B gene from several species have shown that the sequence of the region flanking nucleotide 6,666 is highly homologous (90%) among the mouse, rat, rabbit, and human genes.\(^8\)\(^,\)\(^8\) However, Chen et al.\(^8\) have recently used site-directed mutagenesis techniques to introduce 22 different mutations into a nine-base region flanking nucleotide 6,666. Unexpectedly, they discovered that most of the constructs were edited, implying that the editing mechanism may be at least partially lax. Two groups have reported data on the length of the apo B sequence required for apo B editing. Bostrom and coworkers\(^9\) inserted two lengths of the apo B cDNA flanking nucleotide 6,666 into the protein-coding sequence of an apo E expression vector. When a 354-bp segment of apo B cDNA flanking nucleotide 6,666 was inserted into the expression vector and the expression vector transfected into an intestinal carcinoma cell line, editing of the mRNA at nucleotide 6,666 occurred. However, no editing was observed when a 63-bp portion of apo B cDNA flanking nucleotide 6,666 was inserted into the expression vector. Davies and coworkers\(^8\) have also examined the question of sequences required for the apo B editing process. They transfected constructs containing eight different lengths of apo B cDNA flanking nucleotide 6,666 (ranging in size from 26 to 2,385 bases) into McArdle 7777 cells, a rat hepatoma cell line that synthesizes and secretes apo B-48. They found that mRNA editing occurred with each of the constructs, revealing that as few as 26 nucleotides surrounding base 6,666 were sufficient for mRNA editing to occur. The discrepancy between Davies’s and Bostrom’s results could very well be due to the confounding effects of the apo-E sequences in Bostrom’s constructs. Davies et al.\(^8\) reported that an RNA-folding computer program predicted that the edited nucleotide exists in a conserved eight-nucleotide loop in an mRNA stem loop structure.

Driscol and coworkers\(^8\) have demonstrated that the RNA editing mechanism can be observed in vitro with cytoplasmic extracts from McArdle 7777 cells. They inserted various lengths of the apo B cDNA surrounding base 6,666 into appropriate plasmid vectors, and various lengths of apo B RNA were synthesized in vitro. Editing of the synthetic RNAs containing as few as 55 bases of the apo B sequence was demonstrated with cytoplasmic extracts. Editing was specific for RNA but not DNA and was destroyed by proteinase K, suggesting that the editing activity involves a protein. Results of gel retardation assays using extracts from an apo B-48–producing cell line have suggested the presence of a factor that binds specifically to the appropriate region of the apo B mRNA.\(^9\) Efforts to purify this activity are under way in several laboratories. Scott and coworkers have recently developed transgenic mice containing a 2–3-kb construct spanning the apo B-48 editing site.\(^9\) Apo B mRNA was identified in multiple tissues, and substantial amounts of editing activity were documented in the intestine, spleen, and lung. Lower levels of editing activity were documented in the liver, brain, and heart. The physiological rationale for editing in this broad spectrum of tissues is not known, but these data certainly present the possibility that editing might occur for gene products other than apo B.

Editing of the intestinal apo B mRNA to create an in-frame stop codon was unprecedented in the molecular biology of mammals, and its existence raises many questions.\(^8\)\(^,\)\(^8\)\(^,\)\(^8\) Is the C at base 6,666 simply deaminated to produce a U, or is it possible that the base is modified in some other way so that it is read by the ribosome and reverse transcriptase as a U? Outside the nine-base region examined by Chen et al.,\(^8\) what are the nucleotide specifications of the editing process? What process signals the use of alternate polyadenylation sites after the edited nucleotide in the human intestine? Is RNA editing the result of a single protein or a complex comprising several proteins (and perhaps RNAs)? Does the RNA editing process exist only for the apo B mRNA, or is it a mechanism that is used for other transcripts in other tissues? These questions are important, and at least some of the answers will probably be forthcoming in the next 2–3 years. However, a more fundamental question for the lipoprotein metabolism field should also be considered. Why (in mammalian physiology) is there a need to make two forms of apo B, and why has the process been so conserved?
through evolution? Currently, there is no clear answer to this question. Apo B-100 seems perfectly capable of participating in the assembly of large, triglyceride-rich particles. What physiological role does apo B-48 have in the intestine that would not be equally well served by apo B-100? Perhaps apo B-48 particles can accommodate more apo E molecules than can apo B-100 particles, resulting in more efficient and rapid delivery of dietary fats to the liver. Scott et al.\(^9\) recently postulated that apo B-48 may have evolved for the purpose of efficient delivery of antioxidants to the liver, where they may protect nascent hepatic lipoproteins from free radical attack.

**Genetic Variation in Apo B Gene**

Even before the cloning and sequencing of the cDNA and gene for apo B, strong evidence existed that apo B was a polymorphic protein. From the early 1960s, it has been observed that antibodies reacted with human LDL in the sera of multiply transfused thalassemic patients. Family studies showed that the antigens bound by these antibodies were present in the sera of some individuals but not others and that the presence of the antigens was a trait that was inherited in a simple Mendelian fashion. By 1974 five different antithetical pairs of LDL antigens \([\text{Ag}(x/y), \text{Ag}(a/d), \text{Ag}(c/g), \text{Ag}(t/z), \text{Ag}(h/i)]\) had been described; no new antigens have since been described.\(^94\) When clones for the apo B cDNA became available, a family study demonstrated that one of the Ag polymorphisms was in strong linkage disequilibrium with a commonly occurring restriction fragment length polymorphism (RFLP) in the apo B gene,\(^1\) lending considerable support to the idea that the Ag polymorphisms resulted from inherited structural variations in the apo B gene. Based on various combinations of these five pairs of antigens, many different LDL antigenic phenotypes are clearly possible; with the use of these antisera, a large number of different LDL phenotypes has actually been documented in human populations. The large number of LDL phenotypes has been shown to be due to at least 15 different LDL (or apo B) haplotypes.\(^94\) The frequency of different LDL phenotypes and haplotypes differs in various ethnic populations.\(^94\)

In the past 3 years, several investigators have shown that several of the same Ag antigens originally recognized by human polyclonal antisera could also be recognized by apo B–specific murine monoclonal antibodies. Young et al.\(^17\) showed that monoclonal antibody MB19, which was originally developed by Curtiss and Edgington,\(^96\) detects a commonly occurring two-allele polymorphism in apo B; it binds to apo B allotypes MB19, and MB19, with high and low affinity, respectively. Subsequently, the polymorphism detected by antibody MB19 was shown to be identical to the Ag(c/g) polymorphism.\(^98,99\) Antibody MB19 has been a very useful reagent in determining the concentrations of a particular apo B allotype produced by a mutant apo B allele.\(^100\) In addition, immunoaffinity columns using antibody MB19 have been useful techniques for separating the apo B allotypes in the plasma of MB19 heterozygotes (S. Young, J. Witzum, L. Curtiss, T. Innerarity, unpublished observations). More recently, a mouse monoclonal antibody (H1163) that detects the Ag(h/i) antigen pair has been developed and characterized.\(^101\) DNA polymorphisms associated with the MB19 and H1163 immunochemical polymorphisms have been identified.\(^102,103\)

With the cloning of the apo B cDNA and gene, data demonstrating the genetic heterogeneity in apo B have rapidly accumulated. Many RFLPs have been defined (Table 1). Furthermore, many nucleotide sequence differences have been documented. A list of these sequence differences, originally compiled by Ludwig et al.\(^104\) has been recently updated and expanded by Yang et al.\(^37\) to include 75 sequence differences. As Yang and associates discussed, some of these differences are undoubtedly the result of DNA sequencing errors; however, 12 of the differences have been documented by more than one laboratory and therefore probably represent bona fide nucleotide sequence polymorphisms. Ten of these 12 sequence changes result in amino acid substitutions. Several of the amino acid substitutions correlate with the Ag phenotypes and are easily detectable because they change restriction endonuclease sites in the apo B gene (Table 1).

With the identification of apo B gene RFLPs, a number of different investigators performed “association” studies, that is, studies designed to determine whether some commonly occurring apo B sequence polymorphisms were associated with a given clinical phenotype, such as hypercholesterolemia, hypertriglyceridemia, or coronary artery disease. Such studies have been based on the supposition that the DNA sequence polymorphisms accounting for the RFLP might change a crucial amino acid, alter the metabolism of apo B, and thereby cause a particular clinical phenotype or on the more likely supposition that the DNA sequence polymorphism would not cause the phenotype but would be in linkage disequilibrium with an as-yet undiscovered causative DNA polymorphism.\(^105\) The most widely evaluated RFLP has been the XbaI polymorphism (Table 1). Interestingly, the DNA sequence change altering this restriction site does not result in an amino acid substitution and therefore could not by itself account for a phenotype change. Many investigators have found the XbaI polymorphism to have significant clinical associations. In a study of nonfasting serum from men attending a London heart study clinic, Law et al.\(^106\) found that individuals homozygous for the presence of the XbaI site had slightly increased cholesterol and triglyceride levels compared with men with normal levels. Shortly afterward, the same positive association between the XbaI site and lipid concentrations was observed by Talmud et al.\(^107\) in a random series of normolipidemic men in London. An association between elevated cholesterol and apo B levels and the presence of the XbaI site was noted in Norwegian
TABLE I. Restriction Fragment Length Polymorphisms in the Apolipoprotein B Gene*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Location</th>
<th>Frequency of rare allele</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvuII</td>
<td>4 kb upstream of exon 1</td>
<td>0.2 (site absent)</td>
<td>Reference 155</td>
</tr>
<tr>
<td>MspI</td>
<td>Apo B gene site promoter, −265 bp from cap site</td>
<td>0.21 (site absent)</td>
<td>Reference 156</td>
</tr>
<tr>
<td>ApaLI</td>
<td>Exon 4; cDNA nucleotide 417</td>
<td>0.36 (site absent)</td>
<td>Presence of site associated with Thr18, Ag(g), low-affinity MB19 allotype. Absence of site associated with Ile19, Ag(c), and high-affinity MB19 allotype.97,102,157</td>
</tr>
<tr>
<td>HinII</td>
<td>Intron 4, 3,334 bp 3' to exon 3</td>
<td>0.12 (site present)</td>
<td>References 116, 155, and 158</td>
</tr>
<tr>
<td>PvuII</td>
<td>Intron 4,523 bp 5' to exon 5</td>
<td>0.08 (site present)</td>
<td>References 116, 155, and 158</td>
</tr>
<tr>
<td>AluI</td>
<td>Exon 14; cDNA nucleotide 1,981</td>
<td>0.48 (site absent)</td>
<td>Presence of site associated with Ala299, Ag(a), and low-affinity H1163 allotype. Absence of site associated with Val299, Ag(d), and high-affinity H1163 allotype.103</td>
</tr>
<tr>
<td>BalI</td>
<td>Intron 20, 146 bp 5' to exon 21</td>
<td>0.50</td>
<td>Reference 155</td>
</tr>
<tr>
<td>XbaI</td>
<td>Exon 26; cDNA nucleotide 7,674</td>
<td>0.4–0.5 (site present)</td>
<td>No associated amino acid substitution.159</td>
</tr>
<tr>
<td>MspI</td>
<td>Exon 26, cDNA nucleotide 11,040</td>
<td>0.12 (site absent)</td>
<td>Presence of site associated with Arg501 and Ag(i). Absence of site associated with Glu551 and Ag(h).160,161</td>
</tr>
<tr>
<td>EcoRI</td>
<td>Exon 26; cDNA nucleotide 12,670</td>
<td>0.20 (site absent)</td>
<td>Presence of site associated with Glu44,454 and Ag(t). Absence of site associated with Lyn54,164 and Ag(z).56,162</td>
</tr>
</tbody>
</table>

* cDNA, complementary DNA.

** A rare TaqI polymorphism,112 rare EcoRV and Stnl polymorphisms,163 and uncommon Rsal polymorphisms164 have also been reported. Size of polymorphisms created by 3’ hypervariable region can be detected with many different restriction enzymes.50,61

Apo B haplotypes and atherosclerotic disease was observed in a South Wales population.116 In a case-control study, Hegele et al found that alleles lacking the XbaI sites, alleles lacking the EcoRI site, and alleles containing more repeats in the 3’ hypervariable region were all found at higher frequencies in patients with coronary artery disease compared with that of controls.114 Monsalve et al117 found a similar association between the XbaI and EcoRI polymorphisms and the presence of peripheral vascular disease. In a recent study of an Austrian population, Friedl et al119 also found an increased frequency of alleles with more repeats in the 3’ hypervariable region in a coronary artery disease population. However, in Seattle there appeared to be an increased frequency of shorter 3’ hypervariable region alleles in a coronary artery disease population compared with that of controls, and there was no difference in the frequency of the XbaI and EcoRI RFLPs between controls and coronary artery disease patients.112 Additionally, no association between the XbaI and EcoRI polymorphisms and the presence of coronary artery disease could be demonstrated in a small London population studied by Ferns and coworkers.120

Several reasons are obvious for some of the different results obtained in these association studies. First, several studies were quite small, involving less than 100 and occasionally less than 50 subjects, and the possibility of overlooking a significant relation is increased in small studies. Second, in some studies, the control group was poorly defined and probably not comparable with the hyperlipidemic or athero-
sclerotic disease groups. Finally, different ethnic groups were evaluated in the different studies, and apo B haplotype frequencies are known to be different in different ethnic populations. The, it is quite plausible that in Finland one of the apo B haplotypes containing the XbaI site contains an as-yet-undefined “mutation X” that causes high cholesterol levels. Linkage of mutation X to the XbaI site could explain the fact that Finnish subjects possessing an apo B allele with the XbaI site have higher cholesterol levels. However, mutation X may be extremely rare in South Wales, and the association between the XbaI polymorphism and cholesterol levels would therefore be expected to be negative.

What have the apo B RFLP association studies taught us? First, despite the aforementioned methodological concerns, the significant RFLP associations in multiple studies suggest that genetic variation at the apo B locus can influence lipids and attendant coronary artery disease risk. Theoretically, this phenomenon is interesting and informative. However, because conflicting data on specific associations have been obtained in different populations, it seems equally clear that determination of individual RFLPs in heterogeneous populations for assessing the genetic risk of coronary artery disease is not worthwhile. Second, these studies have taught us that future efforts to assess the effect of the apo B locus on hyperlipidemia and atherosclerosis need to involve larger numbers of patients as well as carefully defined controls, and genetic variation at the apo B locus will need a more sophisticated definition. Rather than observing associations with a single RFLP, future studies must use a combination of genetic markers that include multiple RFLPs and a precise assessment of the number of repeats in the 3′ hypervariable domain so as to define apo B haplotypes. Precise definition of haplotypes will require many family studies. Because a substantial number of apo B haplotypes are likely to be defined, large population studies will be required to discern associations between an isolated apo B haplotype and a clinical phenotype. If an association between a given haplotype and a clinical phenotype is found, then the observation should be confirmed in another subgroup of the same population and also in family studies.

If future studies provide successful identification of an apo B haplotype associated with a clinical phenotype, every effort should be made to understand the observation on a biochemical level. For example, when an apo B haplotype is associated with altered levels of cholesterol, a key goal should be to determine whether the apo B haplotype is associated with an altered plasma concentration of the apo B allotype. That certain apo B alleles may be associated with an altered concentration of an apo B allotype was initially demonstrated by Young et al in 1986 and more recently in studies by Gavish et al. If a haplotype is found to be associated with altered levels of a given apo B haplotype, every effort should be made to identify the precise apo B gene mutation accounting for this finding. Given the size of the apo B gene, the magnitude of these studies would not be trivial. However, new techniques for screening mutations may ultimately facilitate this process.

Mutations in Apo B Gene That Cause Low Blood Cholesterol Levels

For many years, it has been recognized that a phenotype characterized by low plasma levels of LDL cholesterol can be inherited within families in a simple Mendelian fashion. The phenotype was designated familial hypobetalipoproteinemia because of the low plasma concentrations of LDL, a class of lipoproteins that migrate in the β-position on agarose electrophoresis. In the past 3 years, a variety of laboratories have shown that specific apo B gene mutations interfering with the translation of a full-length apo B-100 molecule (i.e., nonsense and frame-shift mutations) can cause the syndrome of familial hypobetalipoproteinemia (Table 2).

Subjects who inherit one normal and one mutant apo-B allele, and are therefore heterozygous for familial hypobetalipoproteinemia, are usually asymptomatic. It makes intuitive sense that these individuals, who possess only one normal apo B allele, would have plasma LDL cholesterol and apo B concentrations of about one half those found in normal subjects. In fact, most hypobetalipoproteinemia heterozygotes have LDL cholesterol and apo B concentrations closer to one quarter or one third those of normal (~30–40 mg/dl). The explanation for the less-than-half normal apo B and LDL cholesterol levels observed in many of these subjects is as yet not fully understood. Although one could argue that this finding could partially be the result of selection bias, the presence of very low cholesterol values in affected members of an extended kindred argues against this possibility. It is possible that the hepatic LDL receptor activity is upregulated in these individuals, resulting in an enhanced clearance rate for the VLDL and LDL particles produced by the normal allele. Alternatively, it is possible that the buoyant, triglyceride-rich, apo B–containing lipoproteins produced by the normal apo B allele contain the full amount of apo E produced by two normal apo E alleles. A double dose of apo E per lipoprotein particle would be expected to increase the hepatic clearance of VLDL and IDL particles derived from the normal allele, thereby reducing LDL production rates from the normal allele. These possible explanations for the very low cholesterol levels of familial hypobetalipoproteinemia heterozygotes could potentially be tested by lipoprotein turnover studies and biochemical analysis of the lipoproteins of affected subjects.

Although the prevalence of atherosclerotic disease in well-characterized familial hypobetalipoproteinemia heterozygotes has never been systematically studied, it is reasonably presumed that because of their low LDL cholesterol levels, they would be at low risk for developing coronary heart disease.
Indeed, several investigators have reported data suggesting that heterozygotes may be protected from the development of atherosclerosis and may therefore have increased longevity. Thus, a single copy of an apo B gene containing a premature stop codon might be beneficial for a North American adult with a diet rich in saturated fats and cholesterol!

It is rare that an individual inherits two copies of a defective apo B allele, causing hypobetalipoproteinemia. The clinical phenotype of familial hypobetalipoproteinemia homozygotes (or compound heterozygotes) is quite variable, and the phenotype probably depends on whether the mutant apo B alleles are incapable of producing any apo B (null apo B alleles) or whether one or both of the mutant alleles are capable of producing small amounts of a full-length or truncated apo B. When a subject inherits two null apo B alleles, the clinical syndrome is often severe and essentially indistinguishable from that of abetalipoproteinemia. Abetalipoproteinemia is a rare autosomal recessive syndrome in which apo B is apparently synthesized in the liver but in which there is an apparent defect in the synthesis and secretion of apo B-containing lipoproteins. The molecular basis for abetalipoproteinemia is unknown, but genetic studies have shown that it does not result from a defect in the apo B gene. Familial hypobetalipoproteinemia homozygotes that possess two null alleles, as well as subjects with abetalipoproteinemia, have no conventional VLDL and LDL particles in their plasma; they typically have very low total cholesterol levels (<50 mg/dl) and triglyceride levels of only a few milligrams per deciliter. These subjects have a variety of significant clinical problems, including steatorrhea and mental retardation. Most of the medical problems are caused by the inability to synthesize chylomicrons and failure to absorb dietary fats and fat-soluble vitamins. An appropriate diet and treatment with the fat-soluble vitamins, in particular vitamin E, can favorably influence many of these clinical problems. Several investigators have described familial hypobetalipoproteinemia homozygotes who had symptoms of intestinal malabsorption despite the apparent ability of at least one apo B allele to synthesize trace amounts of apo B; in these cases, the small amount of apo B synthesis was apparently inadequate for intestinal fat absorption.

In several cases, familial hypobetalipoproteinemia homozygotes or compound heterozygotes have been essentially asymptomatic. One such case was identified and characterized by Steinberg and coworkers in 1979. They investigated a 67-year-old man who had extremely low levels of LDL cholesterol (<3 mg/dl) but normal triglyceride levels. Notably, the triglyceride levels increased normally after a fat-rich meal. Small amounts of apo B were detected in the man's triglyceride-rich lipoproteins. He had no symptoms of fat malabsorption, although metabolic ward studies did document slightly increased amounts of fat in the stool. This constellation of findings was recognized as unique; it was quite obvious that he did not have the clinically severe form of the aforementioned homozygous hypobetalipoproteinemia. In 1986 Young, Witz-
tum, and coworkers\textsuperscript{47,100} showed that the patient and two of his siblings were compound heterozygotes for familial hypobetalipoproteinemia, possessing one mutant apo B allele yielding small amounts of a truncated apo B protein (apo B-37) and a second mutant apo B allele producing small amounts of apo B-100.\textsuperscript{47,100} Recent studies have strongly suggested that the latter allele yields small amounts of apo B-100, small amounts of a truncated apo B species (apo B-86), and normal amounts of apo B-48. Apo B-100, apo B-48, and apo B-37 were all prominent protein components of the patient’s d<1.006 g/ml fraction, both after a fat-rich meal and after an overnight fast. The ability of both the patient’s mutant apo B alleles to produce apo B, thereby participating in the formation of triglyceride-rich lipoproteins, explains the normotriglyceridemic phenotype and the absence of clinically significant fat malabsorption. In 1981 Malloy and coworkers\textsuperscript{131} reported on and extensively characterized another subject with normotriglyceridemic hypobetalipoproteinemia. This subject was homozygous for a mutant apo B allele that yielded a truncated apo B species (apo B-50).\textsuperscript{132} That mutant allele also produced apo B-48. Both apo B-48 and apo B-50 were present in the subject’s VLDL fraction, and the subject had no fat malabsorption.\textsuperscript{132} Krul and coworkers\textsuperscript{133,134} have recently described a family in which three siblings were compound heterozygotes for familial hypobetalipoproteinemia. These subjects were normotriglyceridemic and had no symptoms of fat malabsorption. In these subjects, one mutant apo B allele produced a truncated apo B species (apo B-40), and the second yielded another truncated apo B species (apo B-89). Thus, the generative capacity of the mutant apo B alleles to produce small amounts of apo B (even a truncated isoform) that can be assembled into VLDL and chylomicron particles seems to be critical for the asymptomatic, normotriglyceridemic phenotype.

The causes of low plasma levels of LDL cholesterol in nonsense and frameshift mutations of the apo B gene are incompletely understood, and the explanation may prove to be different for the different apo B mutations. A consistent finding with the mutations yielding truncated apo B isoforms is that the absolute concentration of the truncated apo B protein in plasma is very low, in the range of 2–10% of the concentration of apo B-100 produced by a normal apo B allele. Apo B-37 is present in very low levels (<3 mg/dl) in the plasma of affected subjects, despite a demonstration that it is incapable of binding to the LDL receptor.\textsuperscript{52} Similarly, apo B-31 and apo B-46 are present in low levels in plasma, although they lack the region of apo B that binds to the LDL receptor. The inability of the mutant lipoproteins to bind to the LDL receptor might be expected to cause them to accumulate in plasma and have a high rather than low concentration. This logic has produced a hypothesis that the low plasma levels of the truncated apo B species may be explained by low rates of synthesis and secretion from hepatocytes and enterocytes. Recent studies by our laboratory on the postprandial lipoproteins of apo B-46 heterozygotes could be interpreted as supporting this hypothesis. After a fat-rich meal, there was a significant increase in the amount of apo B-48 in the plasma chylomicrons and VLDL subfractions of apo B-46 heterozygotes, but there was no detectable increase in the amount of apo B-46 (S. Young and D. Chappell, unpublished observations). In an earlier study involving normal volunteers, our laboratory showed that both apo B alleles are normally expressed in the intestine and that both equally contribute to the production of chylomicrons and VLDL found in the blood after a fat-rich meal.\textsuperscript{135} Failure to observe an increase in the amount of apo B-46 in plasma after a fat-rich meal strongly implies the possibility of decreased secretion of apo B-46 by the intestine. However, this evidence is not definitive because one could propose that the low levels of apo B-46 are a consequence of extremely rapid catabolism of apo B-46–containing chylomicrons. Ultimately, lipoprotein turnover studies and careful study of the synthesis and secretion of truncated apo B isoforms in cell culture are necessary to understand the metabolism of the truncated apo B species and why these apo B mutations cause low plasma cholesterol levels. Graham and coworkers\textsuperscript{45} have recently reported cell culture expression of apo B-39, and we have expressed two other truncated apo B species (apo B-31 and apo B-37) found in families with hypobetalipoproteinemia (Z. Yao, M. Linton, S. Young, B. Blackhart, B. McCarthy, unpublished observations). However, the efficiency of synthesis, intracellular transport, and secretion of these truncated proteins compared with that of the full-length proteins have yet to be studied. The mechanism for low LDL and apo B concentrations in the apo B-87 and apo B-89 mutations almost certainly involves another factor. In vitro fibroblast binding studies have demonstrated that unlabeled lipoprotein particles containing these longer truncated isofoms compete more effectively with \textsuperscript{125}I-LDL for binding to the LDL receptor compared with unlabeled apo B-100–containing LDL particles.\textsuperscript{134,136} Thus, the low level of these truncated isofoms in plasma may be explained in part by increased LDL receptor-mediated clearance of the mutant lipoproteins.

Mutations occurring within the portion of the apo B gene encoding the aminoterminal 30% of the protein have not been associated with detectable levels of a truncated apo B protein in plasma in two reported cases.\textsuperscript{128,137} The explanation for this finding has yet to be investigated, but the finding is consistent with two different hypotheses: 1) The length of these very short apo B isoforms falls below the threshold length required for synthesis and secretion of apo B from cells, and 2) these isoforms are normally secreted from cells, yet are removed very rapidly from plasma. Recently, Graham and coworkers\textsuperscript{45} as well as Blackhart and coworkers (B. Blackhart, Z. Yao, B. McCarthy, personal communication) have
observed synthesis and secretion of very short apo B isoforms (apo B-18) in cultured cells that had been transfected with eukaryotic expression vectors containing 5' fragments of the apo B cDNA. The secretion of short apo B isoforms in cell culture suggests that the absence of very short apo B isoforms (apo B-25 and apo B-29) in patients' plasma may not result from decreased synthesis and secretion of these proteins. However, the cell culture studies performed to date are certainly not conclusive. Recently, Baserga and coworkers\textsuperscript{138} have shown that 5' nonsense mutations in the \(\beta\)-globin gene are associated with low levels of \(\beta\)-globin mRNA. Although the basis of this interesting observation is incompletely understood, it is conceivable that some 5' nonsense mutations in the apo B gene are associated with low levels of apo B mRNA, resulting in very low synthetic rates for very short apo B species.

To date, the apo B mutations causing hypobetalipoproteinemia have been nonsense mutations or deletions that interrupt the reading frame of the apo B message. However, it is likely that other types of apo B gene mutations will be discovered that reduce the synthesis of a full-length apo B-100. As noted above, several investigators have reported subjects with hypobetalipoproteinemia who possess an apo B allele associated with markedly reduced amounts of a full-length apo B-100.\textsuperscript{100,108,129} Gavish and coworkers\textsuperscript{121} have recently examined an abnormal apo B allele associated with low plasma cholesterol levels and low levels of the apo B-100 allotype produced by that allele. They characterized this type of abnormal apo B allele by using immunoassays with monoclonal antibody MB19, an antibody that binds to apo B allotypes MB19, and MB19, with markedly different affinities.\textsuperscript{97,135} They identified hypocholesterolemic individuals who were heterozygotes for the MB19 polymorphism yet had a preponderance of the MB19, allotype in their plasma. Ordinarily, the amount of allotypes MB19, and MB19, in the plasma of MB19 heterozygotes is roughly equal.\textsuperscript{97} The metabolic basis for the low cholesterol levels and the relatively lower amount of allotype MB19, in the plasma of the Gavish et al subjects is yet to be determined; their study did not address the issue of whether the allotype MB19, was synthesized at a reduced rate or the allotype was removed from plasma more rapidly. It is probable that this phenotype might ultimately be the result of an allele that yields reduced levels of mRNA and therefore reduced levels of the apo B allotype produced by that allele. This type of abnormal apo B allele might prove to be analogous to mutant \(\beta\)-globin alleles in \(\beta\+\) thalassemia, a disease in which \(\beta\)-globin is made but in reduced amounts. \(\beta\+\) Thalassemia has been shown to result from a variety of mutation types, including intron-exon splicing errors, which result in low \(\beta\)-globin mRNA levels.\textsuperscript{139}

The frequency of mutant apo B alleles that cause hypobetalipoproteinemia in the general population is unknown, and an exact frequency will be difficult to determine. Several investigators' estimation of the frequency of the familial "low cholesterol" phenotype is around 0.1–0.8\% of the population.\textsuperscript{140,141} but it is possible that occasional subjects with the phenotype have defects in other gene products.\textsuperscript{142} Another difficulty in the estimation of the frequency of apo B defects that cause low cholesterol levels is that some subjects with heterozygous hypobetalipoproteinemia do not have abnormal total cholesterol levels. For example, two apo B-46 heterozygotes had total cholesterol levels of 179 and 186 mg/dl, respectively, levels that are within the normal range.\textsuperscript{46} Familial hypobetalipoproteinemia heterozygotes are undoubtedly susceptible to the same "polypgenic" factors that result in broad variation of cholesterol levels in the general population. In rare cases, a hypobetalipoproteinemia heterozygote can inherit another gene that raises plasma cholesterol levels. Hegele and coworkers\textsuperscript{143} reported a subject who inherited single copies of defective apo B and LDL receptor genes. Her lipoprotein profile was in the normal range because the hypercholesterolemic effects of the defective LDL receptor gene were compensated for by the hypocholesterolemic effects of the defective apo B allele. In preliminary studies, Gavish et al\textsuperscript{144} have examined a number of MB19 heterozygotes and found that apo B alleles associated with a reduced plasma level of apo B allotype MB19, might be relatively common in the general population. If apo B alleles significantly affecting the amount of an apo B allotype in the plasma are common, then they might help explain the broad variation of plasma cholesterol levels in the general population, helping to make some physiological sense out of the observations that certain apo B RFLPs are associated with elevated or reduced cholesterol levels.

**Mutation in Apo B Gene Associated With High Blood Cholesterol Levels**

As reviewed above, the majority of LDL particles are removed from plasma by the interaction of apo B with the cellular LDL receptor. Goldstein, Brown, and coworkers\textsuperscript{5} demonstrated that the gene for mutations in the LDL receptor protein can retard the clearance of LDL and lead to an accumulation of LDL particles in the plasma. A defect in the LDL receptor protein presents a dual difficulty for LDL metabolism. Not only is there delayed receptor-mediated clearance of LDL particles from plasma, but the rate of LDL particle production is increased. LDL production rate is increased because the apo E-mediated hepatic uptake of LDL precursors (e.g., VLDL particles and IDL particles) is delayed as a result of decreased LDL receptor activity; consequently, an increased fraction of these precursor particles is metabolized to LDL. Most patients with familial hypercholesterolemia have total cholesterol levels well above 300 mg/dl. For years lipoprotein investigators speculated that a specific inherited defect in apo B could abolish its ability to bind to the LDL receptor and therefore cause delayed LDL
clearance and an accumulation of LDL particles in the plasma. Such a defect would not be expected to produce the same degree of increase in the plasma LDL concentration as that observed in familial hypercholesterolemia. Although the removal of LDL from the plasma would be retarded because of the defect in the apo B protein on LDL particles, the predicted production rate of LDL would be essentially normal because the apo E–mediated removal of LDL precursor particles would be predicted to proceed at a normal rate.

Over the past 3 years, a genetic defect in apo B that interferes with its capacity to bind to the LDL receptor has been characterized. The seminal observations were made by Vega and Grundy during lipoprotein turnover studies that were designed to understand the metabolic basis for moderate hypercholesterolemia. For their study, they selected subjects with total cholesterol levels of 250–300 mg/dl, deliberately excluding subjects with overt clinical signs of familial hypercholesterolemia. In metabolic ward studies, they compared the fractional catabolic rate of each patient’s autologous LDL with that of homologous LDL obtained from normolipidemic control subjects. In five subjects, the clearance of the patient’s own LDL was significantly slower than that of the control LDL, resulting in the authors’ speculation that these patients had abnormal LDL that was not removed at a normal rate by the LDL receptor. Later, in collaboration with Vega and Grundy, Innerarity and coworkers examined the ability of these five subjects’ LDL to bind to the LDL receptor of cultured fibroblasts. They found that the LDL of one of the five subjects showed defective binding to the LDL receptor, having only about 32% of the normal receptor-binding activity.

Studies with partially delipidated LDL showed an identical binding defect, strongly suggesting that the defect resided in the protein moiety of LDL particles (apo B). An identical binding defect was identified in the LDL of several hypercholesterolemic family members; Innerarity and coworkers proposed that all of the affected family members were heterozygotes for a defect in apo B that prevented its interaction with the LDL receptor, and they designated the disorder familial defective apo B-100.

Subsequently, Soria and coworkers cloned and sequenced a large portion of both apo B alleles from the patient in the above studies and demonstrated that the DNA sequence of the two alleles differed by only a few nucleotides. One of the nucleotide substitutions, which had not been previously reported in any of the prior apo B sequencing efforts, resulted in a substitution of C for A at residue 3,500 (Table 2). Additional studies revealed the same mutation in all of the patient’s affected family members but not in any of those unaffected. The amino acid 3,500 substitution has subsequently been identified in 10 additional hypercholesterolemic unrelated subjects whose LDL was defective in binding to the LDL receptor in fibroblast binding studies. The substitution has never been identified in subjects whose LDL binds normally to the LDL receptor.

These data alone strongly suggest that the observed amino acid substitution at residue 3,500 causes the binding defect. The location of the amino acid substitution certainly adds weight to the argument that the mutation is causative; the substitution occurs close to the epitopes for many monoclonal antibodies that block binding of LDL to the LDL receptor (Figure 3). In fact, recent studies have revealed that one of these antibodies (MB47) binds to the LDL of familial defective apo B-100 heterozygotes with altered affinity compared with its binding to normal LDL. Thus, the current data strongly implicate the amino acid 3,500 mutation in the defective binding of the phenotype. Definitive proof of this will ultimately require the characterization of the apo B–containing lipoproteins produced by cells transfected with a human apo B-100 expression vector and the lipoproteins produced by cells transfected with an apo B expression vector differing only in the codon for amino acid residue 3,500.

To date, all subjects with familial defective apo B-100 have been heterozygous for the mutation; typical total cholesterol levels for these subjects are 250–300 mg/dl—elevated but lower than that of familial hypercholesterolemia heterozygotes. However, a recent study from Europe has demonstrated that some affected subjects may have the extremely elevated cholesterol levels characteristic of patients with familial hypercholesterolemia. As a result of the high cholesterol levels, it is most probable that these individuals will have an increased incidence of atherosclerotic disease; however, the exact frequency of premature atherosclerosis in subjects with this disorder relative to the frequency in normal subjects and other hypercholesterolemic populations remains to be defined. The frequency of the amino acid substitution at the residue 3,500 mutation is unknown, but the screening of miscellaneous populations has suggested that the frequency might be in the same range as that reported for the sum of all the LDL receptor gene mutations—approximately one in every 500 people.

The amino acid 3,500 mutation, as in several of the mutations causing hypobetalipoproteinemia, is caused by a mutation in a CG dinucleotide. CG dinucleotides are mutational hot spots in higher animals, and base substitutions in CG dinucleotides are important in the pathogenesis of many inherited diseases in humans. An important question about the amino acid 3,500 mutation is whether this mutation occurred independently in different human populations, as has been documented with specific thalassemia mutations, or only once in a single “founder” and then subsequently propagated among subgroups in the general population. Ludwig and McCarthy have performed apo B haplotyping studies with the amino acid 3,500 mutation on nine unrelated subjects and their families and have determined that this mutation is invariably found on the
same apo B haplotype. These data suggest that the mutation has probably occurred only once. If there was only one founder for the amino acid 3,500 mutation, then it is probable that future studies will reveal that this mutation will occur with greater regularity in selected countries or ethnic populations.

The region of the apo B molecule interacting with the LDL receptor may be quite large (Figure 3); thus, it seems probable that other apo B amino acid substitutions could interfere with its ability to bind to the LDL receptor, thereby producing the familial defective apo B-100 phenotype. However, to date no other apo B mutation except the amino acid 3,500 mutation has been reported associated with hypercholesterolemia and defective LDL binding. Recently, Ladias and coworkers described a subject with elevated apo B levels and coronary artery disease who had a single nucleotide substitution in the apo B gene that resulted in a substitution of Thr for Arg at apo B-100 amino acid residue 4,019.53 However, in a family study, the mutation did not segregate with elevated apo B levels; thus, the mutation could not account for the clinical findings. Furthermore, the LDL from the proband tended to have slightly increased uptake by fibroblasts rather than the diminished uptake characteristic of the LDL isolated from subjects with familial defective apo B-100.

Summary

For the past 5 years, investigators from many different laboratories have contributed to a greatly increased understanding of two very important lipid-carrying proteins in plasma—apo B-100 and apo B-48. Apo B-100, an extremely large protein composed of 4,536 amino acids, is synthesized by the liver and is crucial for the assembly of triglyceride-rich VLDL particles. Apo B-100 is virtually the only protein of LDL, a cholesteryl ester–enriched class of lipoproteins that are metabolic products of VLDL. The apo B-100 of LDL serves as a ligand for the LDL receptor–mediated uptake of LDL particles by the liver and extrahepatic tissues. The LDL receptor–binding region of apo B-100 is located in the carboxy-terminal portion of the molecule, whereas its lipid-binding regions appear to be broadly dispersed throughout its length. Apo B-48 contains the amino-terminal 2,152 amino acids of apo B-100 and is produced by the intestine as a result of editing of a single nucleotide of the apo B mRNA, which changes the codon specifying apo B-100 amino acid 2,153 to a premature stop codon. Apo B-48 has an obligatory structural role in the formation of chylomicrons; therefore, its synthesis is essential for absorption of dietary fats and fat-soluble vitamins. Both apo B-48 and apo B-100 are encoded on chromosome 2 by a single gene that contains 29 exons and 28 introns.

An elevated level of apo B-100 in the plasma is a potent risk factor for developing premature atherosclerotic disease. In the past 3 years, many different apo B gene mutations that affect the concentrations of both apo B and cholesterol in the plasma have been characterized. A missense mutation in the codon for apo B-100 amino acid 3,500 is associated with hypercholesterolemia. This mutation results in poor binding of apo B-100 to the LDL receptor, thereby causing the cholesteryl ester–enriched LDL particles to accumulate in the plasma. This disorder is called familial defective apo B-100, and it is probably a cause of premature atherosclerotic disease. Familial hypobetalipoproteinemia is a condition associated with abnormally low levels of apo B and cholesterol; affected individuals may actually have a reduced risk of atherosclerotic disease. Hypobetalipoproteinemia can be caused by a number of different apo B gene mutations that interfere with the translation of a full-length apo B-100; the syndrome can also result from apo B alleles that yield reduced amounts of full-length apo B-100. Existing evidence suggests that neither familial defective apo B-100 nor familial hypobetalipoproteinemia is particularly rare; thus, apo B mutations may help explain a small portion of the variation in serum cholesterol levels in the general population. Future research will undoubtedly uncover many more apo B gene mutations affecting cholesterol levels.

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