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T L Innerarity, ... , Y L Marcel, K H Weisgraber

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Research Article

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Structural Relationship of Human Apolipoprotein B48 to Apolipoprotein B100

Thomas L. Innerarity,* Stephen G. Young,* Karen S. Poksay,* Robert W. Mahley,* Richard S. Smith,†
Ross W. Milne,‡ Yves L. Marcel,§ and Karl H. Weisgraber*

*Gladstone Foundation Laboratories for Cardiovascular Disease, Cardiovascular Research Institute, Departments of Pathology and Medicine, University of California, San Francisco, California, 94140; †Johnson and Johnson Biotechnology Center, Inc., San Diego, California, and the ‡Laboratory of Lipoprotein Metabolism, Clinical Research Institute of Montreal, Quebec, Canada

Abstract

Although the complete amino acid sequence of human apolipoprotein (apo) B100 is known (4536 amino acids), the structure of apo B48 has not been defined. The objective of our study was to define the structure of apo B48 and its relationship to apo B100. Antibodies were produced against 22 synthetic peptides corresponding to sequences in human apo B100. The levels of immunoreactivity of the antipeptides to apo B100 and apo B48 were used to define the structural relationship between these two species of apo B. Six antibodies from sequences in the amino-terminal half of apo B100, including antipeptide 2110–2129, bound to both apo B100 and apo B48. 15 other apo B-specific antipeptides from sequences carboxyl-terminal to residue 2152 bound to apo B100, but not to apo B48. Immunoblots of cyanogen bromide digests of apo B100 and apo B48 with antipeptides 2068–2091 and 2110–2129 detected a 16-kD fragment (residues 2016–2151) in the apo B100 digest and a fragment of identical size in the apo B48 digest. Because apo B48 appears to contain the apo B100 cyanogen bromide fragment 2016–2151 and because an antiserum specific for the peptide 2152–2168 does not bind to apo B48, we conclude that apo B48 represents the amino-terminal 47% of apo B100 and that the carboxyl terminus of apo B48 is in the vicinity of residue 2151 of apo B100.

Introduction

Apolipoprotein (apo) B is a major protein constituent of LDL, VLDL, and chylomicrons. There are two species of apo B in plasma lipoproteins, designated apo B100 and apo B48 (1). Apolipoprotein B100 is found in LDL and VLDL and is synthesized primarily by the liver. Apolipoprotein B48 is found in chylomicrons and chylomicron remnants and, in humans, is synthesized exclusively in the intestine (1). Apolipoprotein B100 is an extremely large glycoprotein, consisting of 4536 amino acids (2–5). The precise structure of apo B48 is unknown, but, upon SDS-PAGE, appears to be about half the size of apo B100 (6).

Most genetic and biochemical evidence indicates that apo B100 and apo B48 are products of the same gene. In patients with abetalipoproteinemia, both forms of apo B are absent (7). Recently, Young et al. (8) have demonstrated the parallel expression in both apo B48 and apo B100 of a commonly occurring genetic polymorphism detected by an apo B-specific monoclonal antibody. Even so, the exact structural relationship between apo B48 and apo B100 and the molecular mechanisms responsible for their formation are unknown.

Although immunological studies have also advanced our knowledge, they have not fully elucidated either of these questions. Certain antibodies to apo B100 also bind to apo B48, whereas virtually all antibodies to apo B48 recognize apo B100. Studies with apo B-specific monoclonal antibodies have shown that apo B48 and apo B100 share certain epitopes (9). Marcel et al. (10) characterized the binding of seven apo B-specific monoclonal antibodies to apo B48 and to the apo B26 and apo B74 fragments of apo B100. These studies suggested that apo B48 is contained in the sequence of apo B100 and that the apo B26 fragment, the amino-terminal portion of apo B100, is contained in the sequence of apo B48 (10, 11). Protter et al. (12) demonstrated that an antiserum to a synthetic peptide representing a sequence near the amino terminus of apo B100 (residues 17–30) bound to both apo B26 and apo B48. These data suggested that apo B48 may be an amino-terminal fragment of apo B100. However, Hospattankar et al. (13) synthesized a peptide (MDB-18) corresponding to a sequence from the carboxyl-terminal portion of apo B100 (residues 3926–3940) and found that an antiserum to this peptide bound both apo B100 and apo B48. These immunochemical data suggested that apo B48 must contain at least some amino acid sequences from the carboxyl-terminal one-half of apo B100, raising the possibility that apo B48 is formed by a gene-splicing mechanism that would result in the inclusion of some sequences from the carboxyl-terminal region of apo B100.

The current study was undertaken to examine the structural relationship of apo B48 to apo B100. Antisera raised against 22 synthetic apo B peptides were used as immunological probes. The results indicate that apo B48 represents only the amino-terminal 47% of apo B100, and that the carboxyl terminus of apo B48 is in the vicinity of amino acid residue 2151 of apo B100.

Methods

Lipoprotein isolation. Human LDL ($d = 1.02$ – 1.05 g/ml) were isolated from the plasma of normal fasted donors by sequential ultracentrifugation. Human $d < 1.006$ g/ml (VLDL) fractions were isolated from

Address reprint requests to Dr. Innerarity.

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the plasma of an atypical type III hyperlipoproteinemic subject by ultracentrifugation (14). The apo B48-containing lipoproteins in the $d < 1.006$ g/ml density fraction were separated from the apo B100-containing lipoproteins by using a monoclonal antibody immunoaffinity procedure (15).

Preparation of apolipoprotein B48 and the thrombolytic fragments of apolipoprotein B100. The LDL and the apo B48-containing lipoproteins were delipidated with chloroform-methanol (2:1, vol/vol). Apolipoprotein B48 was isolated by preparative SDS-PAGE according to the method of Stephens (16), and electroeluted from the gels as described by Knott et al. (17). Thrombin-treated LDL were prepared by incubation for 24 h at 23°C using a thrombin to LDL protein ratio of 1:100 (wt/wt) (17).

Cyanogen bromide digestion of apolipoprotein B48 and apolipoprotein B100. Cyanogen bromide (CNBr; Pierce Chemical Co., Rockford, IL) digestions of the purified apo B48 and delipidated LDL (apo B100) were performed by solubilizing the protein in 70% formic acid, adding CNBr equal to 50 times the weight of the apolipoprotein, and gently mixing the digest for 16 h at room temperature. A second addition (50-fold weight excess) of CNBr was made and the mixture digested for another 2 h. The digest was then diluted 10-fold with deionized water and lyophilized.

Peptide synthesis and purification. Peptides of 14 to 40 amino acids in length, whose sequences were derived from the cDNA sequence of apo B100 and to which an amino-terminal cysteine residue was usually added, were synthesized by the Biomolecular Resource Center of the University of California, San Francisco, Johnson and Johnson, Inc. (San Diego, CA), Biosearch, Inc. (San Rafael, CA), or Applied Biosystems, Inc. (Foster City, CA). The peptide sequences were 12–27, 259–279, 890–908, 2008–2024, 2068–2091, 2110–2129, 2152–2168, 2177–2196, 2236–2253, 2272–2292, 2301–2325, 2404–2425, 2481–2499, 3120–3159, 3352–3371, 3492–3511, 3668–3687, 3926–3940, 3934–3956, 3958–3971, 4004–4021, and 4520–4536 (see Fig. 1). The peptides were analyzed by reversed-phase high-pressure liquid chromatography using an analytical column (Vydac C18; Separations Group, Hesperia, CA). The peptides eluted as a major peak constituting 60 to 90% of the total UV-absorbing material. When necessary, peptides were repurified using a thiopropyl-Sepharose 6B column. All of the peptides showed the expected amino acid compositions, and several of the peptides were sequenced to confirm their identity and purity.

Peptide conjugation and immunization procedures. Synthetic peptides were conjugated to large protein carrier molecules by one of two procedures. Peptides containing an amino-terminal cysteine residue were conjugated to keyhole limpet hemocyanin using the heterobifunctional reagent *n*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (11). Peptides synthesized without a terminal cysteine were conjugated to RIA-grade bovine serum albumin (Sigma Chemical Co., St. Louis, MO) with glutaraldehyde (18). Antisera against synthetic

peptides, individual thrombolytic fragments of apo B100, and purified apo B48 were prepared as described (11).

Gel electrophoresis and immunoblotting. Reduced beta-migrating VLDL (β -VLDL) and thrombolytic fragments of LDL were analyzed by SDS-PAGE on 5% gels, whereas CNBr fragments of apo B48 and apo B100 were separated on SDS-PAGE gradient gels of 10–15% or 15–20%. Immunoblots of apolipoproteins were performed as described (19). Preimmune sera were used as controls in parallel with the antisera for immunoblots, and specific reactivity was demonstrated in all cases but one (antipeptide 3926–3940). Autoradiographs were obtained by exposing the dried blots to Kodak XAR-2 film with Cronex enhancing screens. Solid-phase RIAs were performed as described (8).

Results and Discussion

To determine if the amino-terminal sequences of apo B48 and apo B100 were similar or identical, 700 pmol of apo B48 isolated from a single subject was subjected to amino-terminal amino acid sequencing. The sequence obtained, Glu-Glu-Glu-Met-Leu-Glu-Asn-Val-Ser-Leu-Val-(Cys)-Pro-, is identical to the amino-terminal amino acid sequence of apo B100 (2–5). Antisera to specific regions of apo B100 were developed and used to probe further the relationship of apo B48 to apo B100. Specific polyclonal antibodies were also made against apo B48 and each of the thrombolytic fragments of apo B100 (11). As shown schematically in Fig. 1, the thrombolytic fragments of apo B100 are T4 (residues 1–1297), T3 (residues 1298–3249), T2 (residues 3250–4536), and T1 (T4 plus T3, not shown) (11). Binding of each of the polyclonal antisera to apo B48 and apo B100 thrombolytic fragments was tested using immunoblots. Anti-T4 and anti-T3 bound to apo B48, whereas anti-T2 did not (Fig. 2). Anti-apo B48 bound to T1 and T4 strongly, to T3 weakly, but not to T2 (Fig. 2). These results suggested that apo B48 contains the amino-terminal portion of apo B100.

Nevertheless, the possibility that apo B48 contains carboxyl-terminal sequences of apo B100 was considered. Therefore, 22 peptides were synthesized according to the amino acid sequences derived from the nucleotide sequence of the apo B100 cDNA (2), and antibodies were prepared. The peptides included sequences derived from all four exons in T3 and T2; most were concentrated in the region where apo B48 would be estimated to end if it constituted the amino-terminal 48% of apo B100 (Fig. 1). All peptide-specific antibodies, except antipeptide 3926–3940, bound to apo B100 by immunoblots and

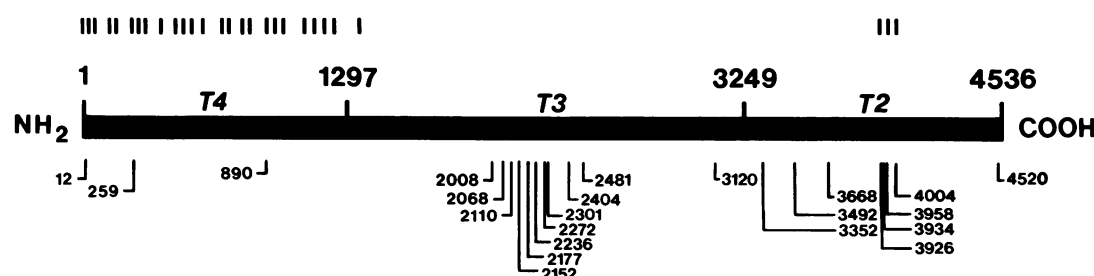


Figure 1. Location of the synthetic peptides corresponding to sequences of apo B100. The long horizontal bar represents the structure of apo B100. The number below the bar designates the amino-terminal residue of the synthetic peptide. Peptide antibodies whose numbers are found to the left of their respective lines bound to human apo B48 and apo B100, and those whose numbers are found to the right of their lines bound only to apo B100. The exception is

antibody 3926, which bound to neither. Thrombolytic fragments T4, T3, and T2 are also shown. The small bars above the apo B100 structure represent the intron-exon junctions in the gene (20), and the space between these bars indicates the relative size of the exon. Exon 26 is 572 base pairs in length and codes for most of T3 and a portion of T2.

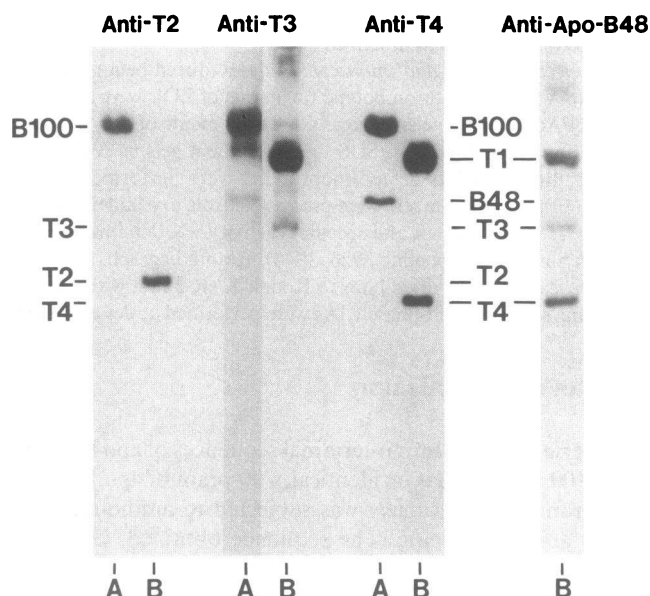


Figure 2. Immunoblots of SDS-gels of (A) human type III β -VLDL that possess both apo B100 and apo B48 and of (B) thrombolytic fragments of apo B100 from LDL. 15 μ g of protein was applied to each lane of 5% gels. T1 contains T4 and T3.

by solid-phase RIA (data not shown). The specificity of each peptide-specific antibody for apo B100 thrombolytic fragments was examined by immunoblots. In each case, the peptide-specific antibody bound to the thrombolytic fragments of apo B100 with the expected specificity. Six representative examples of peptide antibodies from the T2, T3, and T4 regions of apo B100 are shown in Fig. 3. The amino-terminal 12–27 and 259–279 antibodies reacted only with T1 and T4 thrombolytic fragments. The 2110–2129 peptide and 2404–2425 peptide antibodies located in the center of apo B100 were

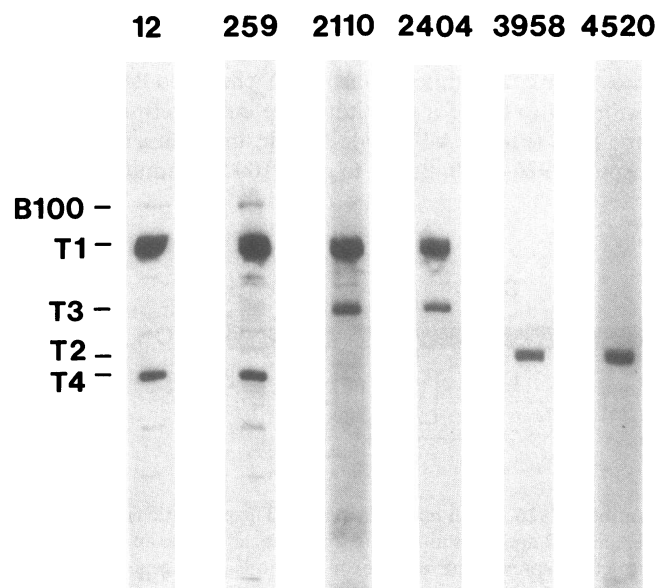


Figure 3. Immunoblots of thrombolytic fragments of LDL separated by SDS-polyacrylamide gel electrophoresis using apo B100 antipeptides. 15 μ g of protein was applied to each lane of 5% gels.

specific for T1 and T3, and the carboxyl-terminal 3958–3971 peptide and 4520–4536 peptide antibodies reacted only with T2.

The peptide-specific antibodies were then immunoblotted to apo B100 and apo B48. The results demonstrated that all antibodies amino-terminal to peptides up to and including synthetic peptide 2110–2129 reacted with both apo B48 and apo B100. Antibodies to synthetic peptide 2152–2168 and all other peptides carboxyl-terminal to this peptide (with the exception of antipeptide 3926–3940) bound to apo B100, but not to apo B48. The immunoblots of the six most informative peptide antibodies in this central region are shown in Fig. 4. Clearly, peptide antibody 2110–2129 reacted with apo B48, whereas peptide antibody 2152–2168 did not. Similar results were obtained with antipeptides 2110–2129 and 2152–2168 by solid-phase RIA (data not shown).

Cyanogen bromide digests of apo B100 and apo B48 were prepared to verify the specificity of the key peptide antibodies. The results confirmed the observations made using undigested apo B48 and apo B100. As shown in Fig. 5, a number of related CNBr fragments were identified in both apo B48 and apo B100 by peptide antibodies 2068–2091 and 2110–2129. Based on its immunoreactivity and apparent molecular weight (16,000), the smallest CNBr fragment (Fig. 5, *arrows*) has been identified as the apo B100 CNBr fragment 2016–2151 (calculated $M_r = 16,300$). As shown in Fig. 5, the corresponding fragment from the apo B48 digest appears to be very similar, if not identical, in apparent molecular weight. The apparent identity of this fragment from the apo B48 and apo B100 digests and the fact that antipeptide 2152–2168 does not react with apo B48 suggest that the carboxyl terminus of apo B48 is in the vicinity of residue 2151. The carboxyl terminus of apo B48 could be located 5 to 10 residues on either side of 2151 and still be consistent with the data. Apolipoprotein B48, as defined by this study, has 47% of the molecular weight of apo B100, and the derived amino acid composition of the apo B48 ending with residue 2151 is in excellent agreement with the published amino acid composition of apo B48 (Table I).

Our data argue strongly against the possibility that apo B48 is formed by an alternative splicing mechanism that eliminates

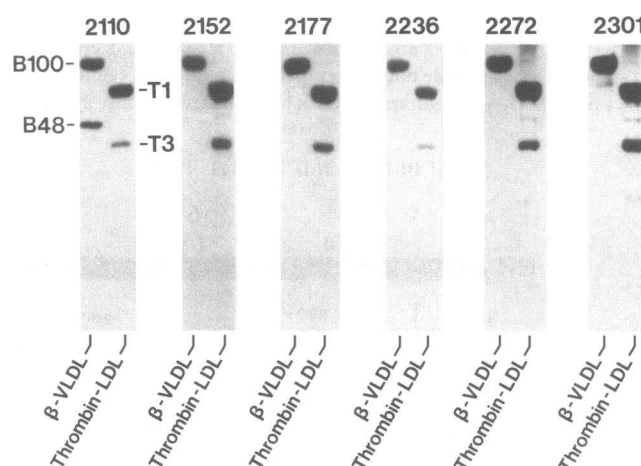


Figure 4. Immunoblots of β -VLDL, which possess both apo B100 and apo B48, and of thrombolytic fragments of apo B100 from LDL (thrombin-LDL) using antipeptides to apo B100. The numbers above the lanes represent the amino-terminal residue of the synthetic peptides. 15 μ g of protein was applied to each lane of 5% gels.

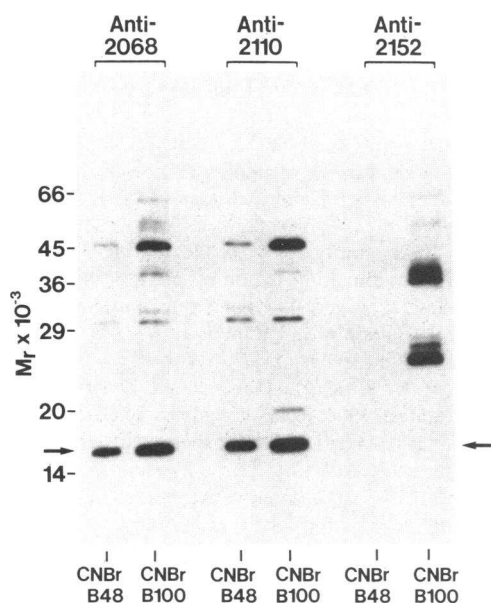


Figure 5. Immunoblots of CNBr digests of human apo B100 and apo B48 with anti-peptides 2068–2091, 2110–2129, and 2152–2168. The band denoted by the arrows has a $M_r = 16,000$. 15 μ g of protein was applied to each lane of 10–15% gels.

the very large 26th exon and results in the inclusion of both amino-terminal and carboxyl-terminal portions of apo B100. None of the antisera to carboxyl-terminal peptides of apo B100 bound to apo B48, and the sequences of these peptides were taken from each of the exons encoding the carboxyl-ter-

Table I. Amino Acid Composition of Apolipoprotein B48 and of Residues 1–2151 of Apolipoprotein B100

Amino acid	Apo B48*	Apo B100 1–2151†
Trp	14	13
Lys	166	165
His	52	50
Arg	82	77
Asp + Asn	229	228
Thr	146	145
Ser	193	192
Glu + Gln	236	235
Pro	83	81
Gly	120	117
Ala	141	140
Cys	14	18
Val	112	114
Met	45	47
Ile	100	106
Leu	259	260
Tyr	72	75
Phe	87	88
Total	2,151	2,151

* Calculated from apo B48 amino acid composition (21), assuming 2,151 residues.

† From apo B100 cDNA-derived protein sequence (2).

minial half of apo B100. Three different antisera against peptide 3926–3940, which is identical to MDB-18 of Hospattankar et al. (13), bound strongly to the peptide 3926–3940, as measured by solid-phase RIA. Anti-peptide 3926–3940 bound to small tryptic fragments of apo B100 on solid-phase RIA, demonstrating that the antisera did recognize apo B100-specific sequences. However, none of our three MDB-18-equivalent antibodies bound to apo B100 or T2 on immunoblots or by solid-phase RIA. In contrast, an antiserum to a peptide with overlapping sequence (3934–3956) clearly bound to apo B100, but not to apo B48. It is known that some peptide antibodies do not react with well-ordered regions of proteins (22, 23); thus, the region in the vicinity of the 3926–3940 sequence of apo B100 may have a constrained conformation or may be sterically inaccessible to the peptide antibody.

Although this study clarifies the structure of apo B48, the molecular mechanism for the formation of apo B48 has been difficult to define. Strong evidence exists for a single gene coding for both apo B100 and apo B48 (7, 8, 24, 25), but other observations on the mechanism for synthesis of apo B48 are in dispute. The liver contains a single large message of 14 kb that encodes apo B100. A message of similar size exists in intestinal mucosal cells (17, 24–26). Until recently it was not known whether this mRNA was the same as that in the liver. Two laboratories previously have reported the existence of a smaller mRNA (~ 6–8 kb) in intestinal cells in addition to the larger mRNA, and they have suggested that this smaller message may code for apo B48 (5, 24). However, if apo B48 terminates near the middle of apo B100 (as indicated by the present study), this site occurs in the middle of the 7.5-kb exon 26 of the apo B gene (20), with the nearest upstream intron more than 2 kb away and the nearest downstream intron more than 5 kb away. Likewise, there is no obvious alternative splice site in the region of the exon where apo B48 terminates. Therefore, regardless of whether apo B48 might arise from a large intestinal mRNA that is different from the liver mRNA or whether it might arise from a smaller mRNA, the mechanism was not apparent until a very recent study by Powell et al. (27).

In their study, apo B cDNA clones from human and rabbit small intestine were compared with apo B cDNA clones from rabbit liver and the human hepatoma cell line HepG2. The human intestinal apo B cDNA sequence was identical to that for liver apo B100, except that after codon 2152 the nucleotide sequence was found to be TAA, which is an in-frame stop codon, instead of CAA, which was found in the human liver cDNA for apo B100. Identical results were observed when rabbit apo B intestine and liver cDNA clones were compared. However, genomic clones from the intestine revealed only one gene for apo B and, further, that the DNA sequence corresponding to codon 2153 was CAA, not TAA. Thus, a single C → U change is introduced into the apo B mRNA co- or posttranscriptionally to produce a stop codon. Moreover, from their study it can be predicted that the stop codon at 2153 would result in an apo B48 containing the amino-terminal 47% of apo B100 with its carboxyl terminus at residue 2152.

The study by Powell et al. (27) also revealed that both the larger (~ 14 kb) and smaller (~ 7 kb) mRNA for apo B occurred in the human and rabbit intestine and that both contained a UAA stop codon. The cDNA clones from the intestine of both rabbits and humans corresponding to the smaller mRNA were polyadenylated at different positions downstream from the termination codon. It could not be deter-

mined from their study if both the larger and smaller apo B mRNAs are actually translated into apo B48. That our conclusions about the structure of the apo B48 protein are in such close agreement with the structure predicted by the mRNA editing mechanism proposed by Powell et al. (27) indicates that at least one of the mRNAs is translated into apo B48.

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