Elimination of Apolipoprotein B48 Formation in Rat Hepatoma Cell Lines Transfected with Mutant Human Apolipoprotein B cDNA Constructs

(Received for publication, August 8, 1991)

Zemin Yao‡, Brian D. Blackhart†, David F. Johnson, Stacy M. Taylor, Kurt W. Haubold, and Brian J. McCarthy

From the Gladstone Foundation Laboratories for Cardiovascular Disease, Cardiovascular Research Institute, University of California, San Francisco, California 94140-0608

Rat hepatoma McA-RH7777 cell lines transfected with full-length human apolipoprotein (apo) B constructs produce mostly human apoB48 and only small amounts of apoB100, as a result of mRNA editing at codon 2153 (C to U conversion at nucleotide 6666). To abolish the formation of apoB48 and increase the yield of apoB100 and other forms of apoB longer than apoB48, site-specific mutations were introduced at or near the base of apoB mRNA editing. Among four mutations examined, only that in which codon 2153 was converted from CAA (Gln) to CTA (Leu) effectively precluded the formation of apoB48. In this mutant, a stop codon would not be generated even if the C to U conversion occurred. The three other mutations were introduced to disrupt the proposed stem-loop structure encompassing the editing site. Changes made in the third positions of five codons on the 5' side of the edited base or of four codons 3' of the edited base failed to eliminate the production of a protein with the approximative size of apoB48. A construct in which codon 2153 was changed from CAA to CAT (Asp) also failed to eliminate the production of a protein the size of apoB48. Analysis of the region between nucleotides 6200 and 6900 of the cDNA did not detect any prevalent alternate editing sites. Immunoblot analysis using polyclonal antibodies raised against synthetic peptides of human apoB100 indicated that the carboxyl terminus of the apoB48-like proteins probably resides between amino acid residues 2046 and 2129 of apoB100. These results provide some insight into the mechanism of apoB mRNA editing and will facilitate further studies on apoB-containing lipoproteins.

Apolipoprotein (apo)

1 B100 and apoB48, both of which are present in the plasma of mammals, are encoded by the same apoB gene (1, 2). Apolipoprotein B48, an amino-terminal portion representing approximately 48% of the full-length apoB100, is generated by a unique mRNA editing mechanism in which an in-frame stop codon is created in the middle of the apoB mRNA by conversion of cytosine at nucleotide 6666 to uracil (1, 2). In humans, apoB mRNA editing is confined to the intestine, where apoB48 is the predominant product of the apoB gene (1, 2). In rodents, however, the apoB mRNA editing mechanism operates in both the intestine and the liver and the rodent liver produces apoB48 (3, 4).

Although the biochemical nature of the apoB mRNA editing mechanism has not yet been fully characterized, there is little doubt that the conversion is indeed from a cytosine to a uracil (5, 6), and the most likely mechanism responsible for this conversion is mediated by a nucleotide-specific cytosine deaminase. The minimum sequence that is sufficient for efficient C to U conversion is as short as 26 nucleotides in rat hepatoma McA-RH7777 cells transfected with human apoB constructs (7). Furthermore, a 26-base sequence surrounding the edited cytosine is fully conserved among humans, rabbits, rats, and mice (7). It has been suggested that this 26-base segment forms a stem-loop structure in which C-6666 is within the loop (8). The sequence specificity of the editing mechanism has been examined using site-specific single- or multiple-base mutants within a nine-base region flanking C-6666 (9). Among 22 different mutant apoB constructs, 20 were edited, implying that the C to U editing mechanism is relatively insensitive to a variety of nucleotide changes. For example, changing the adenine at position 6667 to a guanine, cytosine, or thymidine had little or no effect on the efficiency of C to U conversion at position 6666 (9).

We have been using rat hepatoma McA-RH7777 cells transfected with constructs encoding full-length or truncated forms of human apoB100 as a means to understand structure-function relationships within apoB (10, 11). Attempts to obtain high yields of full-length apoB100 have been frustrated by the fact that most of the human apoB mRNA is edited, so that far more apoB48 than apoB100 is produced in these cells.

There are, in principle, several approaches to overcome this difficulty. The most obvious one, to use cells in which the apoB mRNA editing mechanism is absent, has not proved feasible. While it is apparent that among many cell lines of hepatic and intestinal origin tested, McA-RH7777 cells exhibit the highest level of editing activity, other cell lines also display this activity (5). In fact, the activity is not restricted to cell lines originating from tissues that actually synthesize apoB (6). To date, we have failed to identify any non-human cell line, including McA-RH7777, that can be efficiently transfected and will secrete apoB but does not edit the human apoB mRNA. An alternate approach to eliminate editing
might involve the use of a decoy substrate to absorb most of the editing activity. However, when excess amounts of a small construct (pHER-354) containing the region of the apoB gene surrounding the edited site (5, 12) were cotransfected with the full-length apoB100 construct, no improvement in the yield of apoB100 was achieved.2

For these reasons, we embarked upon a third strategy: we performed site-specific mutagenesis at or near the site of apoB mRNA editing to abolish the formation of apoB48 and increase the yield of apoB100 or other forms of apoB longer than apoB48. Among four mutations examined, only that in which codon CAA (Gln) at position 2153 was changed to CTA (Leu) effectively eliminated the formation of human apoB48. Unexpectedly, expression of constructs containing other mutations in this region continued to yield significant amounts of a protein of approximately the size of apoB48.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco's modified Eagle's medium (DMEM), fetal bovine or horse serum, and G418 were all obtained from Gibco.

Restriction enzymes and DNA modification enzymes were obtained either from Pharmacia or from New England Biolabs, Inc. (Beverly, MA). Protein inhibitors leupeptin, aprotinin, pepstatin, soybean trypsin inhibitor, benzamidine, and phenylmethylsulfonyl fluoride were all purchased from Sigma. Reagents for polyacrylamide gel electrophoresis and the immunoblot assay were obtained from Bio-Rad. Monoclonal antibody 1D1 was a gift from Drs. R. W. Miller and Y. L. Marcel (Laboratory of Liprotein Metabolism, Institut de Recherches Cliniques de Montreal, Montreal, Canada). Polyclonal antibodies raised against human apoB100 peptides 2008 (residues 2008–2024, 2008 residues 2068–2091, 2110 residues 2110–2129), 2140 residues 2140–2151, and 2301 residues 2301–2325) were gifts of Drs. T. L. Ingersent and S. G. Young of the Gladstone Foundation Laboratories. None of these polyclonal antibodies cross-reacts with rat apoB48 secreted by McA-RH7777 cells. Sheep anti-human apoB antiseraum was obtained from Boehringer Mannheim. The sheep antiseraum not only quantitatively precipitates human apoB proteins, but also recognizes rat apoB100 and apoB48. Protein A-Sepharose CL-4B beads were obtained from Pharmacia-LKB Biotechnology Inc. TranS-S-label (1100 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA), and 35S-labeled anti-mouse or anti-rabbit immunoglobulin G antibodies were from Amersham Corp. Enlightening was obtained from Du Pont-New England Nuclear.

**Preparation of Expression Plasmids**—The parental expression plasmids were validated by double-stranded DNA sequencing using a kit from Amersham Corp. The regions encompassing mRNA editing, an EcoRI fragment that encompassed the apoB RNA region were cloned back into the parental pB53, which had been digested with Clal (nucleotide 5849) and HpaI (nucleotide 6507). The resultant plasmids (pB60) contained the reading frames for the amino-terminal 60% of apoB100 and a duplicate of the sequences encoding the amino-terminal 53% of human apoB as described previously (11). To generate mutations in the region of the apoB48 mRNA editing, an EcoRI fragment that encompassed the apoB RNA editing site extending from nucleotides 6507 to 8257 of the apoB cDNA was cloned into pB53. Four different mutations (“QD,” “ED5,” “ED3,” and “Leu-Leu”) were introduced at or near the edited base C-6666 (Fig. 1), by methods described by Kunkel et al. (13). The details on the mutated nucleotides will be described under “Results.” The EcoRI fragments containing the introduced mutations were ligated into the EcoRI site at nucleotide 6507 of apoB cDNA in the parental pB53. The resultant plasmids (pB60) contained the reading frames for the amino-terminal 60% of apoB100 and a duplicate sequence from EcoRI (nucleotide 6507) to HindIII (nucleotide 7336) of apoB cDNA. To eliminate this sequence duplication, pB60 plasmids were digested with Clal (nucleotide 5849) and HpaI (nucleotide 7377), and the fragments containing the mutated apoB48 editing region were cloned back into the parental pB53, which had been cleaved with Clal and SmaI. (This SmaI site is located in the polylinker of pCMV5.) The mutant nucleotide sequences in the resulting apoB expression plasmids were validated by double-stranded DNA sequencing with Sequenase according to the manufacturer’s instructions (United States Biochemical Corp., Cleveland, OH). All DNA samples used for transfection or sequencing were purified by centrifugation twice in a CsCl gradient.

**Cells**—Transfection— McA-RH7777 cells were maintained in 10% fetal bovine serum and 10% horse serum as described previously (10). The cells (approximately 20% confluent in T75 flasks) were cotransfected with human apoB expression plasmids (5–10 pg of DNA) and pSV2neo plasmids (0.3 pg of DNA); stable transformants were selected and maintained in the culture medium, which was supplemented with G418 (400 mg/ml) the day after the transfection. The transfected clonal stable transformants were screened for their ability to synthesize and secrete apoB proteins as described below, and individual cell lines producing the desired apoB proteins were selected. Total RNA was isolated from each of the stable cell lines (14), 5 µg of RNA was treated with 20 units of RNase-free DNase (Stratagene) at 37°C for 2 h, and the DNase-treated RNA was used as a template for cDNA synthesis using a kit from Amersham Corp. The regions encompassing the edited site were amplified by the polymerase chain reaction (PCR) using Taq DNA polymerase (Perkin-Elmer Cetus). Pairs of oligonucleotide primers used in the PCR were designed specifically for the human apoB gene sequence. For example, an oligomer (GkB48II) containing 24 nucleotides from position 6224 to 6247 was prepared in which nucleotide 6246 was changed from T to C in order to create an XbaI site (ATCTATGATCCCTAGATTAGA), and another oligomer (48ScaI) encompassing nucleotides 6850–6879 was designed in which nucleotide 6868 (C) and 6871 (G) were changed into T and A, respectively, to generate a second XbaI site (TTTGAATCTAGA-ATGCGACTACTATTTCCAC). The resulting DNA fragments were either used for primer extension experiments to determine the effectiveness of apoB mRNA editing as described previously (12), or inserted into cloning vectors for sequence analysis. The nucleotide sequences were determined using standard DNA sequencing procedures. Primers used in the primer extension assays were: (i) M51, a 24-mer complementary to wild-type apoB cDNA from nucleotide 6671 to 6794 that detects the C to U change at nucleotide 6666 (5); (ii) M51b, a 24-mer that is similar to M51 except complementary to pB53 ED5; and (iii) B47PE, a 24-mer that is complementary to nucleotides 6535–6558 and is used to detect the C to U change at nucleotide 6528.

**Characterization of Secreted Apolipoprotein B-containing Lipoproteins**—Stably transfected cells were grown to 50–60% confluence in 10% serum-free medium. Cells were detached from the Primaria dishes with 8 ml of the same medium (without G418) for 24 h. In some experiments, the culture medium was supplemented with protease inhibitors leupeptin (10 µM) and aprotinin (100 units/ml). Conditioned medium from two flasks (16 ml) was collected on ice, supplemented with EDTA (0.5 mM) and phenylmethylsulfonyl fluoride (0.015% v/v), centrifuged at 10,000 rpm for 4°C for 15 min to remove cell debris, and subjected to ultracentrifugal fractionation in a salt gradient (11, 15). After centrifugation, 20 fractions (approximately 2 ml/fraction) were collected from the bottom of the centrifuge tubes, and lipoproteins in each of the fractions were concentrated with Cab-O-Sil (8 mg/sample). Lipoproteins (40 µg of protein) were recovered from the Cab-O-Sil with 100 µl of sample buffer containing 6 M urea, 2% (w/v) sodium dodecyl sulfate (SDS), and 0.1% (w/v) dithiothreitol (11) and stored at −80°C before electrophoresis. In some experiments, additional protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 µM pepstatin, 20 µg/ml soybean trypsin inhibitor, 10 µM leupeptin, and 10 µM aprotinin) were added to the samples prior to storage. Aliquots of the samples were subjected to electrophoresis on 5% polyacrylamide gels in the presence of 0.1% SDS. Proteins were transferred to nitrocellulose membranes and immunoblotted with monoclonal antibody 1D1, whose epitope is near the amino terminus of human apoB100 (16). In some experiments, conditioned serum-free medium was supplemented with KBr to d = 1.21 g/ml and subjected to flotation in a Ti-60 rotor at 50,000 rpm and 4°C for 48 h. After centrifugation, lipoproteins (d < 1.21 g/ml) were collected from the top of the tubes, sequences extending from the wild-type apoB cDNA were determined using standard DNA sequencing procedures, and the results were compared with that obtained from the culture medium. The results were compared with that obtained from the culture medium.

**Pulse-chase Studies of Human Apolipoprotein B Proteins**—The early events during the biosynthesis of wild-type or mutant human apoB proteins were examined by pulse-chase experiments. Stably transfected cells or nontransfected cells (at 70–75% confluence in T75 flasks) were split into seven 60-mm Primaria dishes (Beckton Dickinson Labware, Lincoln Park, NJ) with 4 ml of medium (without G418) per dish and incubated at 37°C for 16 h. The enhanced adhesion of cells to the Primaria dishes completely prevents detachment of cells during the chase period. The cells were washed with serum-free and methionine-free DMEM twice and pulse-labeled with TranS-S-label (400 µCi/dish) in 0.8 ml of the same medium at 37°C for 5 min. After the pulse, the labeling medium

2Z. Yao, B. D. Blackhart, S. M. Taylor, and B. J. McCarthy, unpublished observations.
was aspirated and the cells were incubated with 1 ml of serum-free DMEM containing 200 μM methionine for 0, 2.5, 5, 10, 20, and 30 min at 37 °C. At each time point, medium was collected and cells were lysed with 0.5 ml of radiolabeled buffer containing 1% SDS as described previously (15). Both cellular and medium samples were adjusted to 0.1% SDS, stained with Coomassie Brilliant Blue R, soaked in Enlightning, and subjected to fluorography as described previously (15). The intensity of the radioactively labeled apoB protein bands was determined by scanning the fluorograms with a densitometer (Hoefer Scientific Instruments, San Francisco, CA).

RESULTS

Generation of Stable Cell Lines That Express Wild-type and Mutant Apolipoprotein B53 Proteins—Our intention was to test constructs containing four mutations at or around the edited base of the apoB mRNA for their effects on the efficiency of editing and on the extent to which proteins larger than apoB48 are produced. Because the large size of the full-length apoB100 cDNA construct results in poor transfection efficiency, we chose to conduct these studies with a shorter construct that encodes apoB53. In cells transfected with wild-type pB53 plasmids, both human apoB53 and apoB48 are produced (11), indicating that the pB53 construct contains sufficient sequence information for recognition by the apoB mRNA editing mechanism. The properties of four site-specific mutants at or around the edited base C-6666 are shown in Fig. 1. In mutant QD, the codon CAA for amino acid Gln at position 2153 of apoBl00 was changed into GAT (Asp), thereby eliminating the C at position 6666. In mutants ED5' and ED3', respectively, the third positions of five codons 5' of the edited base and four codons 3' of the edited base were altered, while the edited base C-6666 was retained. In these latter two constructs, the mutations should disrupt the formation of the postulated stem-loop structure encompassing the apoB mRNA editing site without changing the encoded amino acid sequence. In the mutant Leu-Leu, the edited base C-6666 was retained but the base A at position 6667 was changed to T, and the resulting CTA codes for Leu. The Leu-Leu mutation prevents the formation of an in-frame stop codon even if the conversion of C to U has occurred at position 6666 because the edited codon UUA also codes for Leu.

Stable cell lines expressing the wild-type pB53 and each of the mutant constructs were generated in McA-RH7777 cells, and the apoB protein products secreted from the cells were detected by immunoblot analysis using monoclonal antibody 1D1 (Fig. 2). Among the four mutants examined, only the Leu-Leu mutation effectively precluded the formation of apoB48 (Fig. 2A, lanes 2–4). The nature of these apoB48-like proteins produced by each of the mutant pB53-transfected cell lines was established by using polyclonal antibody 1D1. This antibody was raised against the carboxyl-terminal 11 amino acids of apoB48; it reacts strongly with apoB48 but binds with low affinity to apoB100 and other truncated apoB forms larger than apoB48 (10, 11). In cells transfected with wild-type pB53, antibody 2140 specifically recognized the recombinant human apoB48 but did not react with apoB53 (Fig. 2B, lane 1); this result is consistent with earlier observations (11). The apoB48-like proteins produced by cells transfected with mutant ED5' also interacted with antibody 2140 (Fig. 2B, lane 3), suggesting that these proteins are probably related to wild-type human apoB53.
apoB48. The production of apoB48-like proteins by mutant ED5'-transfected cells implies that changes created at the third positions of five codons 5' of the edited base did not eliminate apoB mRNA editing. In contrast, proteins with the size of apoB48 that were produced in mutant QD- or ED3'-transfected cells were not recognized by antibody 2140 (Fig. 2B, lanes 2 and 4), indicating that the carboxyl-terminal sequences of these proteins differed from that of wild-type apoB48.

The Apolipoprotein B48-like Proteins Produced by the Mutant pB53-transfected Cells Do Not Result from Proteolysis—Consideration was given to the possibility that the apoB48-like proteins that were derived from the cells transfected with mutant pB53 constructs (ED3' and QD) resulted from protein degradation; proteolysis of the mutant human apoB53 proteins might occur either intracellularly or in the medium after secretion, or both. First we asked whether proteolysis occurred in the medium after secretion of the recombinant apoB-containing lipoproteins. The buoyant density of apoB-containing lipoproteins can be characterized by ultracentrifugation in a salt density gradient (11). It has been shown previously (11) and also confirmed in the present work (Fig. 3, panels 3-5), that the uinted recombinant apoB53 and apoB48 proteins were associated with particles of different buoyant density. The peak density for apoB53-containing lipoproteins is \( d = 1.06 \) g/ml, and for apoB48-containing particles is \( d = 1.10 \) (11). We reasoned that if proteolysis occurred in the medium, the density distribution of the resulting apoB48-like proteins should be coincident with that of apoB53, assuming that apoB48 does not exchange between lipoprotein particles (17). On the other hand, the assembly of lipoproteins has been shown to occur after the translation of apoB proteins (18, 19). Therefore, if proteolysis occurred intracellularly during or immediately after apoB53 translation, the resulting B48-like proteins would assemble lipoproteins with their own characteristic densities and thus be separable from apoB53-containing particles by density centrifugation.

In cells transfected with mutant Leu-Leu, recombinant human apoB53 was the predominant product observed; the mutant apoB53 density distribution was essentially identical to that of wild-type apoB53 (Fig. 3, the first and second panels). The apoB48-like proteins produced by the cells transfected with the other mutants (QD, ED3', and ED5') distributed in fractions similar to those for wild-type apoB48 (Fig. 3, panels 3-5 from the top). The peak densities of these apoB48-like proteins derived from QD-, ED5', and, to a lesser degree, ED3'-transfected cells are clearly different from the peak densities of the corresponding apoB53 proteins secreted from the same cells (Fig. 3, panels 3-5). These results provide evidence that these apoB48-like proteins are associated with discrete particles and are secreted independently of the apoB53 proteins. Therefore, no significant proteolysis occurs after the secretion of apoB53-containing lipoproteins.

The density distribution of lipoproteins containing the mutant apoB53 secreted by Leu-Leu-transfected cells proves that one amino acid change in apoB53 (Glu to Leu at position 2153) had no measurable effect upon its ability to assemble lipids and form lipoprotein particles. However, in these experiments there was a trace amount of apoB48-like proteins detected in fractions 6-8 with antibody 1D1 (Fig. 3, panel 2), and the appearance of these proteins persisted even when the cells were cultured in medium supplemented with the protease inhibitors leupeptin and aprotinin (Fig. 3, panel 6, fractions 5 and 6). Inclusion of the protease inhibitors in the culture medium caused a density shift toward a higher density range of lipoproteins containing apoB53 or apoB48-like proteins (compare panel 2 with panel 6 in Fig. 3). The density shift of apoB53- or B48-containing lipoproteins secreted by mutant QD-, ED3', or ED5'-transfected cells was also seen when cells were cultured in medium supplemented with the protease inhibitors (Fig. 3, panels 3-5). The increased buoyant density of the lipoproteins probably reflects decreased lipid content. It is not clear whether the decreased lipid content results from interference by the protease inhibitors with lipid recruitment during lipoprotein assembly.

We next inquired whether the apoB48-like proteins resulted from intracellular degradation. The early events during apoB protein synthesis were analyzed by pulse (5 min) and chase (up to 30 min) studies. Secretion of the pulse-labeled apoB proteins during this time frame was shown to be negligible in all cell lines examined (data not shown). Fig. 4 illustrates experiments performed in cells transfected with mutants QD or Leu-Leu, using cells transfected with wild-type pB53 and non-transfected McA-RH7777 cells as controls. At the end of the 5-min pulse, discrete bands ranging from a molecular mass of 69 to 500 kDa (the size of apoB100) were immunoprecipitated in both the transfected cells and the controls (Fig. 4). These multiple apoB antiserum-reactive proteins are nascent apoB peptides, the discrete sizes of which are presumably attributable to translational pauses resulting from the secondary structure of the apoB mRNA. It is evident that in cells transfected with mutant Leu-Leu, apoB53 was the only protein product derived from the recombinant human apoB DNA (Fig. 4, panel 4), confirming that this mutation effectively abolished the formation of apoB48.

Based on this result, we concluded that the trace amounts of apoB48-like proteins present in the high density fractions derived from the cells transfected with mutant Leu-Leu (Fig. 3, panels 3 and 6) are the products of a low level of proteolysis. In contrast, in cells transfected with mutant QD both apoB53 and the apoB48-like proteins accumulated during the chase period, without any indication of a precursor-product relationship between the two proteins (Fig. 4, panel 3 of A and...
A similar result was obtained in control experiments with cells transfected with wild-type pB53 constructs (Fig. 4, panel 2 of A and B), providing additional support for the fact that the mutation QD did cause the formation of the apoB48-like proteins. An identical experiment performed in control non-transfected McA-RH7777 cells revealed a parallel pattern for the kinetics of synthesis of endogenous rat apoB100 and apoB48 (Fig. 4, panel 1 of A and B). No precursor-product relationship was expected in this case because apoB100 and B48 are translated from separate mRNAs (1,2).

Taken together, these results suggest that the proteolysis of apoB in these experiments is negligible; therefore, the formation of apoB48-like proteins from some of the mutant constructs (e.g. QD and ED3') cannot be attributed to apoB53 degradation either within the cells or in the medium.

The Carboxyl Terminus of the Apolipoprotein B48-like Protein Derived from the Cells Transfected with Mutant QD Residues between Amino Acid Residues The carboxyl terminus of the resulting apoB48-like proteins, we reasoned, resides between amino acid residues 2068-2129. As shown in Fig. 5, the apoB48-like protein derived from mutant QD reacted strongly with monoclonal antibody 1D1 and polyclonal antibodies 2068, weakly with polyclonal antibody 2110, but failed to react with polyclonal antibodies 2140, 2140, or 2301 (see Fig. 5A, lane labeled pB60,QD). In contrast, the wild-type human apoB48 produced by the cells transfected with wild-type pB53 gave positive reactions with all of the antibodies tested except for polyclonal antibody 2301 (see Fig. 5A, lanes labeled with pB53). Apolipoprotein B53 and apoB60 produced by the corresponding cell lines were detected by antibody 2301 (Fig. 5A), with an epitope located downstream of the end of apoB48 between residues 2301 and 2325. These results suggest that the carboxyl terminus of the apoB48-like proteins probably resides between the epitopes of antibodies 2068 and 2110 (amino acid residues 2068-2129).

Are There Alternate Apolipoprotein B mRNA Editing Sites?—Results obtained for the peptide antibody mapping experiments (Fig. 5) prompted us to search for additional in-frame stop codon(s) in the mutant apoB transcripts that may be responsible for the formation of the apoB48-like proteins. First, we examined the effect of replacing the edited base C-6666 in the QD mutants. Total RNA was isolated from stable cell lines transfected with mutant pB53 or pB60 plasmids containing the QD mutation, and cDNA was prepared for direct DNA sequencing or primer extension analysis. Among approximately 20 clones of each that were sequenced, one C to U conversion at nucleotide 6528 was found in cDNA originating from cells transfected with the mutant pB60 QD (see Fig. 5B for the edited base). This C to U editing also caused a formation of an in-frame stop codon (CGA (Gln) to UAA (stop)) at codon 2107. In addition, another C to U conversion was found at nucleotide 6930 in cDNA from cells transfected with the mutant pB53 QD. In this case also, an
in-frame stop codon was generated (CAG (Gln) to UAG (stop)) at codon 2241 (sequencing data not shown). In the ED3' third positions of codons either 5' or 3' of C-6666 prevented amino acid sequences represent the corresponding antibody epitopes (21).

We then examined cDNA sequences derived from cells that contained the mutants ED5' and ED3'. In all of the five ED5' and four ED3' clones sequenced, we failed to detect any alternate C to U changes that would result in in-frame stop codons in the apoB messages (data not shown). Moreover, sequence analysis proved that base C-6666 was retained in all cDNA clones sequenced, indicating that changes made in the third positions of codons either 5' or 3' of C-6666 prevented most of the C to U conversions. Primer extension assays of cDNAs from pB53 ED5' revealed small amounts of edited sequence (Fig. 6A, lane 4), indicating the C to U conversion was diminished but not completely precluded by changing bases 5' of the edited base 6666. A 40-base fragment was produced in the primer extension assay using cDNAs from pB53 ED5' as templates because T-6659 was replaced by C in this mutant (see Fig. 1, sequence of ED5'). In contrast, primer extension assays of cDNAs derived from pB53 ED3' using oligomer M51b that is complementary to the sequence of mutant ED3' showed only the non-edited sequence (Fig. 6A, lane 5), suggesting that mutations introduced at the 3' side of the edited base abolish the C to U conversion. (Employing the primer M51b for mutant ED3' in the assay was

![Image](https://example.com/image.png)
necessarily because the primer M51 failed to anneal to the sequence 3' of C-6666 in this mutant.) Mutation Leu-Leu did not significantly affect the efficiency of C-6666 editing (Fig. 6A, lane 6 versus lane 2).

**DISCUSSION**

We have shown previously that full-length human apoB100 can be expressed from an apoB minigene construct in the rat hepatoma cell line McA-RH7777 (10). Unfortunately, the yield of apoB100-containing lipoproteins was quite low, largely because a large fraction of the apoB mRNA was edited, producing apoB48. The purpose of the present study was to develop an expression system in which the formation of apoB48 could be abolished, thus resulting in high yields of full-length apoB100. The lack of other well-characterized non-human cell lines that do not contain the apoB mRNA editing activity prompted us to circumvent the problem by mutating the apoB constructs so that production of apoB48 is significantly reduced or eliminated.

Among four mutations that were created at or near the apoB mRNA editing site, only the one in which CAA at codon 2153 was changed into CTA effectively abolished the production of apoB48-sized proteins. Analysis of the products of cell lines transfected with this Leu-Leu mutant indicates that little, if any, apoB48-like protein is formed. In addition, the single amino acid substitution (Leu for Gln) at position 2153 does not seem to create any significant effect on the ability of apoB to assemble lipids or form lipoprotein particles. Taking advantage of codon degeneracy for Leu, we mutated the edited region such that an in-frame stop codon would not be formed even if C-6666 was converted into U. Primer extension analysis showed that the efficiency of the C to U conversion in the mutant Leu-Leu construct was similar to that of the wild type, a result consistent with data from Chan and his colleagues (9) showing that the in vitro editing efficiency for a mutant in which C-6667 changed into U is unaffected. The relative insensitivity of the editing process to at least some nucleotide changes implies that the secondary or tertiary structure of apoB mRNA is more crucial than primary structure for the deamination of C-6666. Evidence obtained in the current study (e.g. mutants ED5' and ED3') supports the view that the postulated stem-loop structure (8) encompassing the edited base may not be important. The editing efficiency seems to be far more sensitive to mutations introduced 3' rather than 5' of C-6666. The observation that sequences 5' of C-6666 are poorly conserved among mammalian species relative to sequences at the 3' end (7), together with the current data, suggests that information recognized by the editing mechanism may reside downstream of C-6666.

The other three mutants (QD, ED5', and ED3') still expressed significant amounts of apoB proteins of approximately the same size as apoB48. Further analysis of these proteins revealed that authentic apoB48 was made only by the ED5' mutant, presumably because of low levels of apoB mRNA editing in the mutant construct. The other two mutants, QD and ED3', produced a new protein(s) with a carboxyl terminus distinguishable from that of wild-type apoB48. The nature of these apoB48-like proteins remains elusive, although the present data suggest that they do not result from proteolysis.

The existence of two new alternate editing sites in cDNA preparations derived from the mutant QD-transfected cells (one in pB53 QD and one in pB60 QD) seemingly provides a possible explanation for the resulting apoB48-like proteins, especially that in pB60 QD-transfected cells, where the editing occurs at a position consistent with the carboxyl terminus of the protein as predicted by the peptide antibody mapping experiment. However, the frequency with which the alternate editing sites occur seems to be too low (one in 20 clones) to account for the amount of apoB48-like protein produced by the transfected cells, unless the modified mRNA is unusually stable or translated with unusual efficiency. Furthermore, because of the possibility of introducing errors during the Taq DNA polymerase chain reactions, care has to be exercised in the interpretation of the new editing sites.

Our results and several recent reports have suggested unexpected complexities in the mechanism responsible for apoB mRNA editing. Initially it appeared that the highly conserved base sequence surrounding the edited base acted as a recognition site for the enzymatic deamination of cytosine. However, as shown by site-specific mutagenesis, the editing is relatively insensitive to base substitutions in the immediate region flanking C-6666 (9). Furthermore, Navaratnam et al. (20) identified an alternative site of C to U editing in two out of 11 intestinal apoB cDNA clones. This new deamination site at nucleotide 6802 bears little sequence homology to the primary site and results in the substitution of Ile (AUA) for Thr (ACA) rather than a stop codon. We did not observe this alternate editing site at position 6802 (20) in McA-RH7777 cells transfected with any of the human apoB constructs. Introducing mutations into our apoB expression constructs also revealed unforeseen complications. For instance, in the QD mutants, simply eliminating the edited base C-6666 (by changing it to G) did not preclude the formation of proteins with the approximate size of apoB48 in either the pB53 or the pB60 constructs.

Continued formation of apoB48-like proteins in the cells transfected with QD, ED5', or ED3' mutants suggests the existence of an unknown mechanism within these cells that generates truncated apoB proteins even if the editing site is non-functional. The labeling kinetics of apoB48-like proteins and their ability to assemble lipoprotein particles with a distinct density distribution (Fig. 4) both suggest that these truncated apoB proteins are immediate translational products. This raises the question of whether the formation of apoB proteins with the approximate size of apoB48 is solely attributable to apoB mRNA editing. Could other kinds of mRNA modification, such as polyadenylation, result in interruption of apoB translation? It has been documented that there are two populations of apoB mRNA in human intestine, one of which is about half the size of the messenger encoding the full-length apoB100 (1, 2). Both kinds of intestinal apoB mRNA molecules are polyadenylated. Furthermore, sequence analysis of the apoB gene has revealed cryptic polyadenylation signal sequences located 3' of the apoB mRNA editing sites; these cryptic polyadenylation signals may be responsible for the polyadenylation of the short apoB mRNA editing sites; these cryiptic polyadenylation signals may be responsible for the polyadenylation of the short apoB mRNA editing sites; these cryptic polyadenylation signals may be responsible for the polyadenylation of the short apoB mRNA editing sites. Nonetheless, whether polyadenylation precedes the C to U conversion. Although increased mRNA stability is generally supposed to be its major function, polyadenylation in the middle of human apoB mRNA may interrupt apoB translation and cause premature termination. The relationship of cryptic polyadenylation signals to human apoB mRNA editing and the production of truncated forms of apoB merits further investigation.

More conclusive proof that the Leu-Leu mutation can prevent the formation of apoB48 remains to be established for constructs encoding the full-length apoB100. Nevertheless, we believe the constructs encoding the amino-terminal 53% of apoB already include all the information necessary for apoB mRNA editing. The results obtained from pB53 Leu-
Abolishing ApoB48 Formation

Leu-transfected cells are probably applicable to the full-length constructs or those producing truncated proteins larger than apoB48. Creating such modified apoB expression plasmids will facilitate studies of the metabolism of plasma apoB100- and apoB48-containing lipoproteins using expression systems in both cell culture and transgenic animals.

Acknowledgments—We wish to thank Drs. R. W. Milne and Y. L. Marcel for the monoclonal antibody 1D1; Dr. S. G. Young for human apoB peptide antibodies used in this study; and Dr. T. L. Innerarity for critical reading of the manuscript. We are also grateful to Tom Rolain and Charles Benedict for illustrations, Al Averbach for editorial assistance, and William Doolittle for manuscript preparation.

REFERENCES

1. Powell, L. M., Wallis, S. C., Pease, R. J., Edwards, Y. H., Knott, T. J., and Scott, J. (1987) Cell 50, 831–840
2. Chen, S. H., Yang, C. Y., Gu, Z. W., Lee, B. R., Weng, S. A., Silberman, S. R., Cai, S. J., Deshpere, J. P., Rosseneu, M., Gotto, A. M., Jr., Li, W. H., and Chan, L. (1989) Science 248, 363–366
3. Davidson, N. O., Powell, L. M., Wallis, S. C., and Scott, J. (1988) J. Biol. Chem. 263, 13482–13485
4. Tennyson, G. E., Sabatos, C. A., Higuchi, K., Meglin, N., and Brewer, H. B., Jr., (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 500–504
5. Boström, K., García, Z., Poksay, K. S., Johnson, D. F., Lusis, A. J., and Innerarity, T. L. (1990) J. Biol. Chem. 265, 22446–22452
6. Hodges, P. E., Navaratnam, N., Greeve, J. C., and Scott, J. (1991) Nucleic Acids Res. 19, 1197–1201
7. Davies, M. S., Wallis, S. C., Driscoll, D. M., Wynne, J. K., Williams, G. W., Powell, L. M., and Scott, J. (1989) J. Biol. Chem. 264, 13395–13398
8. Scott, J., Wallis, S. C., Davies, M. S., Wynne, J. K., Powell, L. M., and Driscoll, D. M. (1989) Gut Festschrif 30, 35–43
9. Chen, S. H., Li, X., Liao, W. S. L., Wu, J. H., and Chan, L. (1990) J. Biol. Chem. 265, 6811–6816
10. Blackhart, B. D., Yao, Z., and McCarthy, B. J. (1990) J. Biol. Chem. 265, 8358–8360
11. Yao, Z., Blackhart, B. D., Linton, M. F., Taylor, S. M., Young, S. G., and McCarthy, B. J. (1991) J. Biol. Chem. 266, 3300–3308
12. Boström, K., Lauer, S. J., Poksay, K. S., García, Z., Taylor, J. M., and Innerarity, T. L. (1989) J. Biol. Chem. 264, 15701–15706
13. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
14. Chirgwin, J. M., Przyhyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
15. Yao, Z. and Vance, D. E. (1988) J. Biol. Chem. 263, 2998–3004
16. Milne, R., Theolıs, R., Jr., Maurice, R., Pease, R. J., Weech, P. K., Rassart, E., Fruchart, J. C., Scott, J., and Marcel, Y. L. (1989) J. Biol. Chem. 264, 17954–17960
17. Kane, J. P. (1983) Annu. Rev. Physiol. 45, 637–650
18. Boren, J., Wettesten, M., Sjöberg, A., Thorlin, T., Bondjers, G., Wiklund, Ö., and Olufsson, S. O. (1990) J. Biol. Chem. 265, 10556–10564
19. Davis, R. A., Thrift, R. N., Wu, C. C., and Howell, K. E. (1990) J. Biol. Chem. 265, 10005–10011
20. Navaratnam, N., Patel, D., Shah, R. R., Greeve, J. C., Powell, L. M., Knott, T. J., and Scott, J. (1991) Nucleic Acids Res. 19, 1741–1744
21. Innerarity, T. L., Young, S. G., Poksay, K. S., Mahley, R. W., Smith, R. S., Milne, R. W., Marcel, Y. L., and Weisgraber, K. H. (1987) J. Clin. Invest. 80, 1794–1798