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Biochemistry

Expression of apolipoprotein B mRNAs encoding higher- and lower-molecular weight isoproteins in rat liver and intestine

(RNA/editing)

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ABSTRACT **Two B apolipoproteins (apo) are present in human plasma, designated apoB-100 and apoB-48, and represent translational products from mature apoB mRNAs that differ by a single base. Either the glutamine codon encoded by the single-copy apoB gene at nucleotide 6666 is transcribed and translated to produce apoB-100 or an RNA-editing mechanism substitutes a uracil for cytosine, altering this glutamine codon (CAA) to a stop codon (UAA), prematurely terminating translation to produce apoB-48. In the present report, editing of rat apoB transcripts was evaluated by amplification of RNA with the polymerase chain reaction by use of primers based on the apoB cDNA cloned from a rat liver cDNA library. The combined results of this study show that (*i*) a single copy of the apoB gene exists in the rat; (*ii*) the rat apoB gene encodes only the glutamine codon for the synthesis of apoB of higher molecular weight (apoB$_H$); (*iii*) rat apoB transcripts undergo RNA editing; (*iv*) apoB$_H$ and apoB of lower molecular weight (apoB$_L$) in the rat represent structural equivalents of apoB-100 and apoB-48 in humans, respectively; (*v*) RNA editing occurs in both the liver and intestine of the rat; (*vi*) rat hepatic apoB RNA is more extensively edited than is human hepatic apoB RNA, which is consistent with the marked increase in apoB$_L$ secretion by the rat liver when compared with human; and (*vii*) the definitive identification of apoB$_H$ mRNA as well as apoB$_L$ mRNA in the rat intestine provides a mechanism for the biosynthesis of both apoB$_H$ and apoB$_L$ by the rat intestine.**

The apolipoproteins comprise a group of proteins essential for the transport and metabolism of lipid particles. In humans apoB-100 (M_r 512,000) is a major apolipoprotein of very low density lipoproteins and low density lipoproteins, serving as a classical ligand for uptake by the low density lipoprotein receptor (1, 2). ApoB-48 (M_r 250,000) serves as the major B apolipoprotein in chylomicrons. Initial protein studies of human apoB by Marcel *et al.* (3) with monoclonal antibodies indicated that the B-48 apolipoprotein was colinear with the amino-terminal portion of apoB-100. Analysis of the human apoB gene indicated that it exists as a single-copy gene on chromosome 2 (4, 5). In human, studies by Powell *et al.* (6), Chen *et al.* (7), and our laboratory (8) established the mechanism of production of both apoB-48 and apoB-100 mRNAs from a single gene to be an RNA-editing process that substitutes a uracil for cytosine at nucleotide 6666. This substitution terminates translation of the apoB-48 isoprotein at amino acid 2152, replacing the CAA glutamine codon encoded by the gene with an in-frame UAA stop codon. In addition, we have recently reported the isolation of cDNA clones from human liver and intestinal libraries containing sequences encoding both apoB-100 and apoB-48 (9).

In the rat, two plasma apoB isoproteins are also present and have been designated higher-molecular weight apolipoprotein (apoB$_H$) and lower-molecular weight apolipoprotein (apoB$_L$) (10–12). Both apoB$_H$ and apoB$_L$ have been reported to be secreted by the rat liver (10–12). Reports of the apoB isoproteins secreted by the rat intestine have been inconsistent. Lee *et al.* (13) detected primarily apoB$_H$ in rat chyle, whereas apoB$_L$ was the primary intestinal apoB isoprotein reported by Van't Hooft *et al.* (11).

In this report we present data to establish that the rat apoB$_H$ and apoB$_L$ are structural equivalents of apoB-100 and apoB-48 in humans and result from an RNA-editing mechanism present in both liver and intestine.

METHODS

Isolation and Sequencing of the Rat ApoB Gln/Stop Codon Flanking Region. An oligonucleotide probe (5'-ATACTGATCAAATTATATCACATATGTCTG-3') complementary to the known rabbit apoB stop codon region (6) was ^{32}P-end-labeled and used to screen a rat liver λgt11 cDNA library (Clontech) as described (14). Six positive clones from 500,000 plaques were obtained, subcloned into M13 and pGEM4 (Promega), and sequenced using the dideoxynucleotide chain-termination method (15). A single clone, containing a 1.3-kilobase (kb) *Eco*RI insert that encompassed the desired apoB region was used for further analysis.

Hybridization Analysis of Rat Genomic DNA. High-molecular weight genomic DNA, prepared from rat liver (14), was digested with restriction enzymes and size-fractionated on a 0.6% agarose gel, and the gel was analyzed as described (16). Hybridization was performed using the 1.3-kb apoB cDNA sequence obtained from screening the rat liver library. The rat apoB cDNA was ^{32}P-labeled as described (17), and the dried gel hybridizations were performed for 16 hr at 60°C in 6× SSPE (20× SSPE = 3 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA), salmon sperm DNA at 100 μg/ml, 0.1% $NaDodSO_4$, and probe at 1×10^6 cpm/ml, followed by three room temperature washes in 6× SSPE for 10 min and one 4-hr wash in 6× SSPE. A final wash was performed at 60°C for 1 hr in 6× SSPE. The washed gel was exposed to Kodak XAR-5 film for 24 hr at −70°C with two intensifying screens.

Rat genomic DNA was amplified using the polymerase chain reaction (PCR) as described (18) by use of primers based on the rat apoB cDNA sequence as follows: SO 172 (5'-TAGAGGATCCCTGAGCAGGCTTCCTCAGCAG-3') and SO 173 (5'-TTTAAAGCTTCAATGATTCTATCAATAATCTG-3'). Primer SO 172 is a sense primer containing a

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Abbreviations: apo, apolipoprotein; apoB$_H$, apoB of higher molecular weight; apoB$_L$, apoB of lower molecular weight; PCR, polymerase chain reaction.

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*Bam*HI site. Primer SO 173 is an antisense primer containing a *Hin*dIII site.

Analysis of Rat Liver and Intestine mRNA. Total RNAs were isolated from ad libidum-fed (lab chow) 6-month-old adult male Sprague–Dawley rats maintained in a light cycled environment (sacrificed at 10 a.m.) using the guanidine thiocyanate/cesium chloride method (19). Ten micrograms of total RNA from rat liver or intestine was hybridized to 1.0 μg of PCR primer SO 173 at 70°C for 10 min in the presence of 80 units of RNasin (Promega), 150 μM dNTPs, 15 μl of 5× reverse transcription buffer (1× transcription buffer = 100 mM Tris·HCl, pH 8.3 at 42°C/10 mM $MgCl_2$/140 mM KCl/20 mM 2-mercaptoethanol) and H_2O in a final volume of 75 μl. The mixture was allowed to cool to 42°C; 60 units of avian myeloblastosis virus reverse transcriptase was added (Promega), and extension was performed for 1 hr at 42°C. Samples were phenol/chloroform extracted and ethanol precipitated, and the resultant first-strand cDNA was amplified with *Taq* (*Thermus aquaticus*) DNA polymerase and a Perkin–Elmer/Cetus DNA thermal cycler, using primers SO 172 and SO 173.

PCR products obtained from RNA amplification were treated with RNase A, phenol/chloroform extracted, ethanol precipitated, and resuspended in 10 mm Tris·HCl, pH 8.0/1 mM EDTA. Double-stranded cDNAs were then digested with appropriate restriction enzymes and electrophoresed through a 2% Nusieve (FMC)/1% agarose gel in Tris borate EDTA (pH 8.3). Fragments were isolated on DEAE paper (Schleicher & Schuell), ligated into appropriate sites of M13mp19 and transformed, and recombinant plaques were randomly selected and sequenced using the dideoxynucleotide chain-termination method.

RESULTS

Characterization of the Rat ApoB cDNA Sequence Encoding the Gln/Stop Codon Region. Initial studies were undertaken to establish the rat apoB cDNA sequence homologous to the previously established site of RNA editing in the human and rabbit (6–9). The nucleotide sequence of the Gln/stop codon flanking region of an M13 clone, containing a 1.3-kb *Eco*RI insert, was initially determined using M13 sequencing primers. This clone contained the sequence of the rat apoB cDNA immediately upstream and downstream from the predicted Gln/stop codon region, with the codon of interest located 300 base pairs (bp) from the M13 primer site. For unequivocal sequencing of the Gln/stop codon site, a rat sequence-specific primer was synthesized (SO 166 5′-AAAACTATAGAATTACAGAT-3′), hybridizing 97 bases upstream from the nucleotide of interest. By this approach, the sequence of the rat liver apoB cDNA clone was definitively established and contained the edited stop codon (TAA), shown in Fig. 1.

Rat Genomic DNA Analysis. The 1.3-kb apoB cDNA was used to evaluate the apoB gene in the rat. Genomic DNA was isolated from rat liver, and the DNA was digested with restriction enzymes, electrophoresed, and probed with the rat apoB cDNA, as shown in Fig. 2. A single band was observed with all digests. These results are consistent with a single copy of the apoB gene in the rat, similar to the previously established copy number of the apoB gene in the human (4, 5) and rabbit (6).

The nucleotide sequence of the rat apoB gene in the region homologous to the edited glutamine codon of human apoB was evaluated using the PCR with primers SO 172 and SO 173. The PCR product analyzed by agarose gel electrophoresis was 297 bp, the predicted size of an uninterrupted exonic sequence (Fig. 3). This DNA fragment was subcloned into pGEM3Z, and the nucleotide sequence was determined by the dideoxynucleotide chain-termination method. Only the CAA codon encoding glutamine was present; no TAA stop codon could be detected in rat genomic DNA (data not shown). These results are consistent with the results obtained with the human apoB gene (6–9).

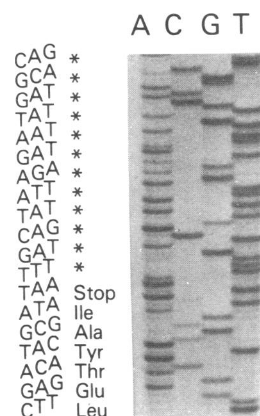

FIG. 1. Nucleotide and predicted amino acid sequence of the rat $apoB_L$. A portion of the nucleotide sequence of the rat apoB cDNA, obtained from screening a rat liver library and cloned into pGEM4, is shown. The predicted amino acids for the encoded $apoB_L$ are aligned with the edited termination codon (TAA) noted.

Analysis of Rat Liver and Intestinal ApoB mRNA. Studies were initiated to determine the tissue distribution and quantity of RNA editing occurring in rat apoB transcripts, adding to the observations of apoB gene expression reported (20, 21). Total RNAs were isolated from liver and intestine, and the RNA was amplified by the PCR using primers flanking the region of the Gln/stop codon. Specifically, first-strand cDNAs were synthesized with reverse transcriptase after hybridization of RNAs to SO 173, an antisense 32-mer hybridizing 91 bases downstream from the Gln/stop codon. Amplification of the first-strand cDNAs, as well as rat genomic DNA, was performed with *Taq* DNA polymerase, and primers SO 172, a sense 31-mer hybridizing 205 bases upstream from the Gln/stop codon, and SO 173. PCR products of the predicted size, 297 bp, were obtained upon amplification of rat genomic DNA and liver, as well as of intestinal RNA (Fig. 3).

To ascertain the ratio of glutamine to stop codons present in the liver and intestine in the ad libidum-fed rat, products of the PCR were subcloned into M13, and plaques were randomly selected and sequenced by use of a single-lane approach with only dideoxythymidine terminations. This

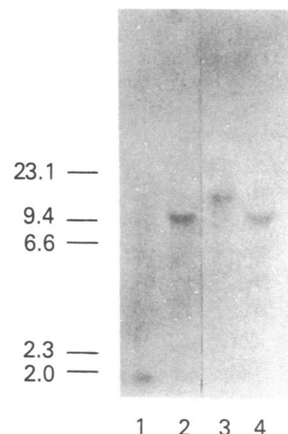

FIG. 2. Agarose gel hybridization analysis of rat genomic DNA. Ten micrograms of rat genomic DNA were digested with restriction enzymes for 18 hr (Lanes: 1, *Eco*RI; 2, *Bgl* II; 3, *Eco*RV; 4, *Hin*dIII) and electrophoresed through a 0.6% agarose gel; the gel was then hybridized with a ^{32}P-labeled 1.3-kb *Eco*RI rat apoB cDNA containing the edited Gln/stop codon region. Molecular sizes of *Hin*dIII digested λ DNA are shown on the left in kb.

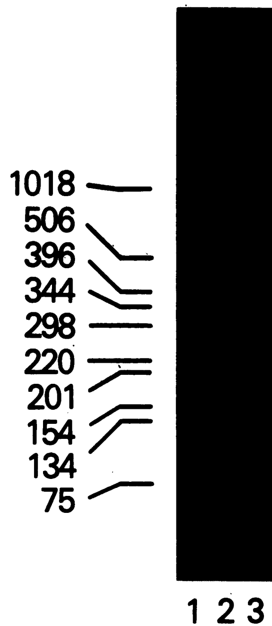

FIG. 3. Analysis of amplified rat apoB cDNAs. Amplified PCR products from rat genomic DNA (lane 1), rat liver RNA (lane 2), and rat intestinal RNA (lane 3) were electrophoresed through a 2% NuSieve/1% agarose gel and stained with ethidium bromide. Molecular sizes of a 1-kb ladder (Bethesda Research Laboratories) are shown on the left.

approach allowed the rapid discrimination of clones containing the glutamine and stop codons. The CAA codon encoding glutamine and the TAA stop codon were identified in both rat liver and intestinal cDNAs (Fig. 4). In total, 39 of 45 rat liver cDNA clones contained the stop codon, with 6 of the 45 clones encoding the glutamine codon (Fig. 5). Therefore the percentage of apoB mRNAs in the rat liver examined was 87% $apoB_L$ and 13% $apoB_H$. Similar sequence analysis of rat intestinal cDNA clones revealed 40 of 41 clones containing the stop codon, representing 98% $apoB_L$ and 2% $apoB_H$ in the original mRNAs.

Comparison of apoB Sequences from the Rat, Human, and Rabbit Species. The nucleotide and predicted amino acid sequences of the reported human (6–9) and rabbit apoB (6) and the rat apoB sequence determined in this study were aligned using the GENALIGN [Bionet National Computer Resource (1988, release 5.1)] and FASTA [National Institutes of Health molecular biology users group (1987, release 0.9)] programs. Fig. 6 illustrates the alignment of the rat, human, and rabbit apoB sequences in this region. The rat sequence has greater divergence from the human apoB sequence than does the rabbit apoB sequence, both at the nucleic acid and at the protein levels. There is 90% homology between the human and rabbit cDNAs and 81% homology between human and rabbit apoB isoproteins in the region shown. In contrast, there is 79% homology between rat and human apoB cDNAs and 79% homology between rat and rabbit cDNAs. The homology between the predicted amino acid sequences of the rat and human as well as the rat and rabbit were 65% and 64%, respectively.

For the regions illustrated in Fig. 6, the longest sequence conserved in all three species spans 23 nucleotides, containing 4 bases upstream through 18 bases downstream from the cytosine that is edited. No other completely conserved nucleotide sequence extends more than 15 bases in these three species in the regions analyzed.

DISCUSSION

Previous studies in the human and rabbit species have established that apoB-48 is synthesized from mRNA generated by an RNA-editing process that changes the CAA codon encoding glutamine in apoB-100 to a UAA premature stop codon, resulting in the termination of translation at residue 2152 in apoB-48 (6–8). Powell et al. (6) and Chen et al. (7) proposed that the RNA editing occurred only in the intestine of the species studied; however, our studies indicated that the edited premature stop codon, coding for the biosynthesis of apoB-48, was present in both the intestine as well as the liver (9). Studies reported in this manuscript establish that the RNA-editing mechanism is present in rat liver and intestinal mRNAs. Therefore, a similar RNA editing mechanism allows biosynthesis of two apoB isoproteins in the rat, $apoB_H$ and $apoB_L$, and in the human, apoB-100 and apoB-48, from single-copy apoB genes. Thus, rat $apoB_H$ and $apoB_L$ are structural equivalents of the apoB-100 and apoB-48 synthesized in humans.

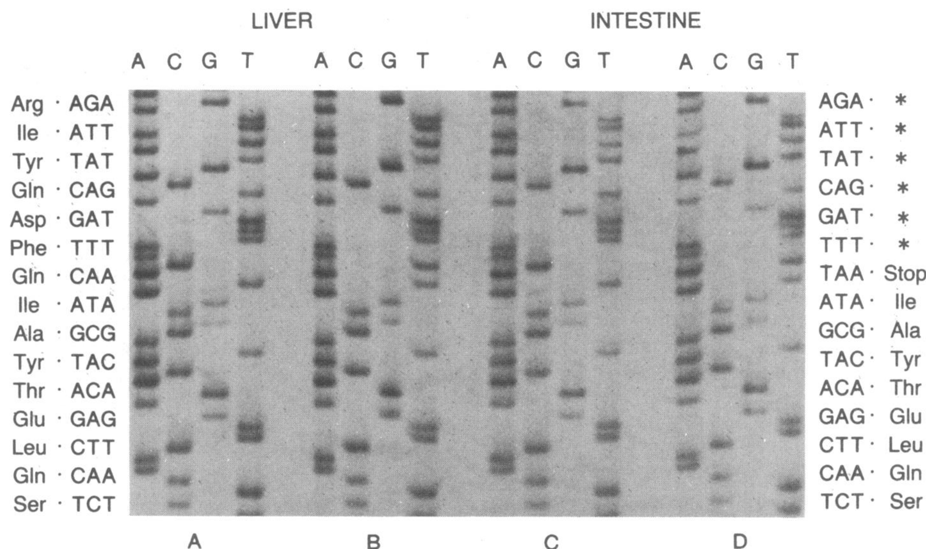

FIG. 4. Conventional sequence analysis of rat liver and intestinal apoB cDNA clones. Autoradiographs of 6% polyacrylamide sequencing gels containing sequencing reactions from cDNA clones from the rat liver (sets A and B) and intestine (sets C and D) are illustrated. Predicted amino acids for $apoB_H$ are noted on the left; predicted amino acids for $apoB_L$ are on the right. Sets A and C represent glutamine codon clones; sets B and D represent stop codon clones.

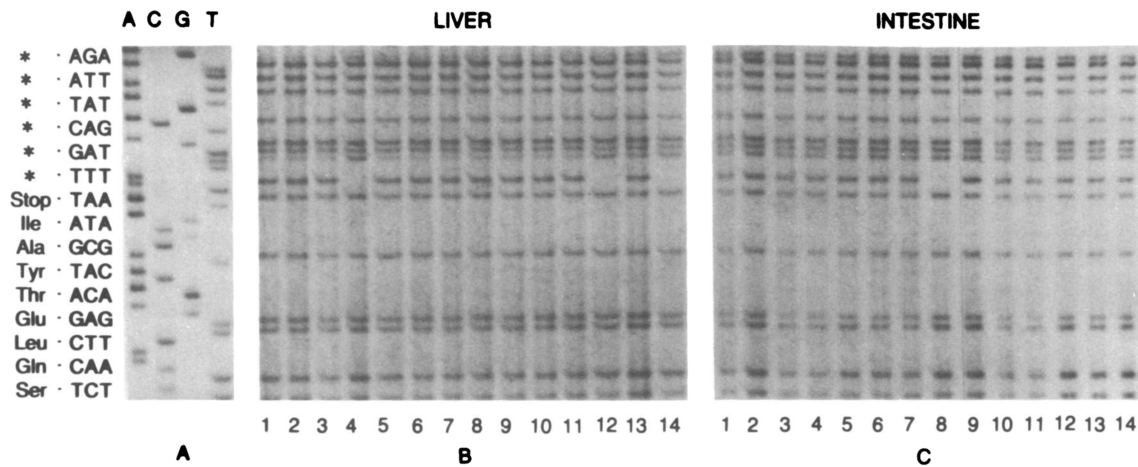

FIG. 5. Single-lane sequence analysis of randomly selected rat liver and intestinal apoB cDNA clones. (*A*) Conventional set of sequencing reactions from a known stop codon containing rat apoB cDNA clone. (*B*) Single-lane sequencing reactions with only dideoxythymidine terminations from 14 randomly selected rat liver apoB clones. (*C*) Similar reactions for randomly selected intestinal clones. Predicted amino acids for the rat $apoB_L$ encoded by this region are shown on the left.

The percentage of synthesis of $apoB_L$, as compared with $apoB_H$ from rat hepatocytes, is different when compared with rabbit and human hepatocytes. In these latter two species, apoB-100 is the predominant apoB isoprotein synthesized in the liver. In contrast, in the rat liver $apoB_L$ is the major apoB isoprotein secreted. The potential for the rat liver to modify apoB RNA transcripts to produce largely $apoB_L$ mRNAs is consistent with the apoB isoprotein data reported by Coleman *et al.* (12), who observed that rat hepatocytes secreted both $apoB_L$ and $apoB_H$, with the ratio of $apoB_L$ to $apoB_H$ increasing as the animal reached adulthood. The physiological reason for the markedly increased secretion of $apoB_L$ by the rat liver, when compared with the human and rabbit, is of great interest and will undoubtedly provide additional insights into lipoprotein metabolism in this species when studied further.

Definitive identification of $apoB_H$ mRNA in the rat intestine provides a mechanism for intestinal $apoB_H$ biosynthesis. However, characterization of apoB isoproteins synthesized and secreted by the rat intestine has been more controversial. Van't Hooft *et al.* (11) reported only $apoB_L$ secretion by the rat intestine, whereas Lee *et al.* (13) presented data describing predominantly $apoB_H$ in rat mesenteric chyle. The relative predominance of $apoB_L$ mRNA as compared with $apoB_H$ mRNA in the rat intestine reported in this study is consistent with the apolipoprotein data of Van't Hooft *et al.* (11). However, it should be noted that the dietary state of the animals in the reported studies [fasted for 24 hr in the Van't Hooft *et al.* studies (11), 3 hr after fat feeding in Lee *et al.* (13), and 3 hr after chow feeding in the present report] may influence the editing of the apoB transcripts and the apoB isoproteins secreted. Additional studies will be required to evaluate the effects of feeding as well as dietary constituents on intestinal apoB RNA modification and isoprotein secretion.

The precise single-nucleotide RNA-editing mechanism seen with the apoB mRNA is of particular interest. Fig. 6 contains a comparison of the nucleotide sequences of the apoB cDNAs in the region of the Gln/stop codon in the rat, human, and rabbit species. A specific, highly conserved, colinear sequence, such as the 23 bases flanking the Gln/stop codon described above, may interact with specific proteins or other factors. Secondary structure or nucleotide sequences removed from the site of editing may also be important in the mechanism involved in RNA processing. Further analysis of factors that may interact with apoB transcripts in the Gln/stop codon region is needed and should provide important information on this unusual RNA editing mechanism.

The combined results presented in this report establish that apoB RNA in the rat, like the human, undergoes editing in the

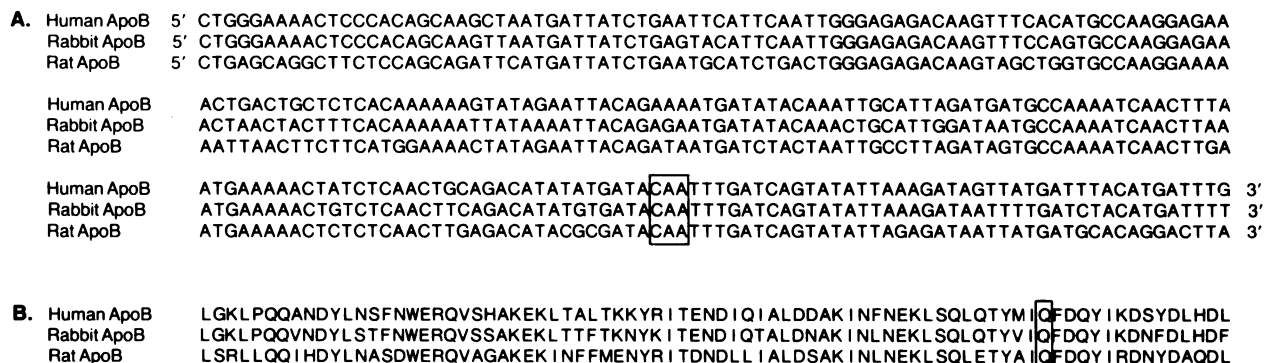

FIG. 6. Diagrammatic comparison of apoB sequences. (*A*) The nucleotide sequences flanking the edited glutamine codon in the human, rabbit, and rat apoB cDNAs are aligned. The glutamine codon that during RNA processing becomes a UAA stop codon is shown in the open box. Primers used to amplify rat genomic DNA and RNA hybridize 205 bases upstream (SO 172) and 91 bases downstream (SO 173) from the cytosine in the glutamine codon and result in a predicted amplified product size of 297 bp. (*B*) The predicted amino acid sequences of human, rabbit, and rat apoB proteins encoded by the cDNAs in *A* are aligned, with each sequence representing the large apoB isoprotein for the individual species (apoB-100 for human and rabbit and $apoB_H$ for the rat). The glutamine corresponding to the CAA codon, which is boxed in Fig. 6*A*, is boxed.

intestine as well as the liver. The mechanism for the biosynthesis of the previously designated $apoB_H$ and $apoB_L$ isoproteins was shown to be due to an RNA editing mechanism as has been reported for the human (6-9). These findings broaden the concept of RNA editing that produces premature stop codons to include the liver and intestine in multiple species. Characterization of hormonal, nutritional, and pharmacological modulators of this class of RNA editing may provide further insights into apolipoprotein synthesis in normal and disease states.

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