Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 32, Issue of November 15, pp. 23747–23750, 1993

Expression of Human Apolipoprotein B100 in Transgenic Mice

EDITING OF HUMAN APOLIPOPROTEIN B100 mRNA*

(Received for publication, September 2, 1993, and in revised form, September 17, 1993)

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Apolipoprotein B (apoB) is a large glycoprotein that circulates in plasma as a major constituent of numerous lipoproteins. ApoB exists in two forms: apoB48 and apoB100. ApoB48 is identical in sequence to the N-terminal region of apoB100 and is generated by sequence-specific mRNA editing of the apoB100 transcript. Here, we describe the development of a line of mice expressing a human apoB transgene driven by promoter/enhancer sequences from the transthyretin gene. In these mice, immunodetectable human apoB100 is synthesized by the liver, kidney, and brain. Human apoB100 is found in low concentration (~0.1 mg/dl) in the plasma of the transgenic mice and circulates in the low density lipoprotein fraction. The hepatic human apoB100 transcripts undergo mRNA editing at only slightly lower efficiency than the endogenous mouse apoB100 message. Therefore, there is no absolute species specificity to the apoB100 mRNA editing process.

Apolipoprotein B (apoB)[1] is a major integral component of numerous lipoproteins and is essential for both the synthesis of triglyceride-rich lipoproteins and the catabolism of cholesterol ester-rich particles. ApoB exists in two forms: apoB100 and apoB48. In humans, apoB100 is synthesized almost exclusively in the liver and is required for the formation and secretion of triglyceride-rich very low density lipoproteins (1). ApoB100 is the only apolipoprotein in low density lipoprotein (LDL), the major vehicle for cholesterol-ester transport in humans, and sequences within its C-terminal region mediate clearance of LDL in tissues by the LDL receptor (2). The other form of apoB, apoB48, is made in human small intestine and is necessary for the packaging of dietary lipids into chylomicrons. ApoB48 is identical to the N-terminal ~48% of the apoB100 protein and so does not contain the C-terminal sequences required for LDL-receptor binding (3).

Both apoB100 and apoB48 are products of the same gene (3, 4). In the intestine, a post-transcriptional modification of apoB100 mRNA results in a substitution of uridine for cytosine at nucleotide 6666. The likely mechanism responsible for this event is a site-specific cytosine deamination of the apoB100 mRNA (3–6). Evidence suggests that multiple proteins participate in this RNA editing event, and recently a 229-residue protein that is part of this complex has been purified and cloned (7–9).

There are important species differences in the proportion of apoB100 RNA that is edited in liver and intestines. Based on the relative amounts of apoB100 and apoB48 in human liver and intestine, it has been estimated that less than 1% of apoB100 mRNA is edited in the liver and >95% is edited in the intestine (10). In contrast, in both rats and mice, a variable percentage of the apoB100 mRNA is edited in the liver depending on the hormonal, nutritional, and developmental status of the animal (11–16). It has been suggested that there is not necessarily species specificity to the mRNA editing process *in vivo* (1, 17).

In this paper we describe the development of a line of transgenic mice expressing a human apoB100 minigene driven by the mouse transthyretin promoter. Despite low levels of apoB100 expression, these mice edit the human apoB100 mRNA with only a slightly decreased efficiency when compared to the mouse apoB100 transcript.

MATERIALS AND METHODS

Development of Human ApoB100 Transgenic Mice—A human apoB100 expression construct (pTTR-B100 (Leu-Leu), where TTR is transthyretin) was made using enhancer/promoter sequences from the mouse transthyretin gene (18, 19), exons 1–26 of the human apoB100 cDNA, and the last three exons and introns from the human apoB gene, including 2.3 kb of the 3′-untranslated region. A pGEM3 vector containing a 290-base pair fragment from the distal mouse transthyretin enhancer (−2.15 to −1.86 kb) fused to the promoter element (−1 to −202) was kindly provided by Dr. R. H. Costa, University of Chicago, Chicago, IL. An *Eco*RI(blunt-ended)/*Pst*I fragment from pGEM3 was inserted into pBSK3II (pBlueScript II SKS, Stratagene), which had been digested with *Sma*I and *Pst*I to generate pBSKSII-TTR2. An *Eco*RI fragment extending from nucleotide 17 to 6507 and encoding the N-terminal 46% of apoB100 from the apoB53 cDNA (20) was subcloned into the *Eco*RI site in the polylinker of pBSK SII-TTR2 to generate pBSKSII-TTR-B46.

To synthesize a full-length apoB100 construct, a *Spe*I-*Cla*I fragment encompassing the TTR enhancer/promoter elements plus the coding sequences for apoB42 (from pBSKII-TTR-B46) was inserted into the expression vector pB100 (Leu-Leu).[2] This human apoB100 expression

* This work was supported in part by National Institutes of Health Grants HL-20948, HL-47619, and HL-47660 and by the Perot Family Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a grant from the Italian Ministry of Scientific and Technological Research.

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[1] The abbreviations used are: apoB, apolipoprotein B; LDL, low density lipoprotein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; kb, kilobase(s).

[2] Z. Yao, unpublished observation.

construct had been modified by site-directed mutagenesis to change codon 2153 from CAA (Gln) to CTA (Leu). Neither the edited (UUA) nor unedited (CUA) transcript terminates translation at this position, so no apoB48 is made (21). A 18.3-kb *Spe*I-*Kpn*I fragment was purified from pTTR-B100 (Leu-Leu) injected into fertilized C57Bl/6 × SJL F_2 hybrid mice eggs. Mice containing the transgene were identified by dot blot hybridization of genomic DNA extracted from tail homogenate. One male mouse (578-7) expressed the transgene, and a line was established by breeding with C57Bl/6 × SJL F_1 females.

Immunoblot Analysis of ApoB100 Synthesis in Transgenic Mice—Mice were anesthetized using an intraperitoneal injection of 90 mg/kg sodium pentobarbital and perfused with phosphate-buffered saline (PBS). Tissues were collected and cell homogenates were made as previously described (22). A total of 12 mg of protein from each tissue were subjected to immunoprecipitation using 30 μg of a rabbit anti-human apoB100 polyclonal antibody. The immune complexes were recovered using 100 μl of Sepharose G beads, and washes were performed as described (23). The immunoprecipitates were redissolved in 20 μl of PBS and 20 μl of loading buffer (20% (v/v) glycerol, 4.6% (w/v) SDS, 125 mM Tris-Cl, pH 6.8, 0.15% (w/v) bromphenol blue, 5% (v/v) β-mercaptoethanol). After boiling for 5 min, a 20-μl aliquot was loaded onto a 5% SDS-polyacrylamide gel and electrophoresis was performed for 1 h and 30 min at 150 V, 4 °C. The proteins were transferred and immunoblotted as described (22) using a monoclonal antibody against the N-terminal region of human apoB100 (MB3) (24, 25) at a dilution of 1:1000 followed by incubation with a sheep anti-mouse horseradish peroxidase-conjugated antibody (Amersham Corp.). The filter was developed using the ECL Western blotting detection kit (Amersham Corp.) and exposed to XAR-5 film (Kodak).

Quantification of Plasma Human ApoB100 Levels in Mouse Plasma—The human apoB100 concentrations in the mouse plasma were determined using a solid phase radioimmunoassay. Ninety-six-well flexible polyvinyl chloride microliter plates were coated for 3 h with PBS containing 2 μg/ml MB47, an immunopurified human apoB100-specific antibody (24), which binds apoB100 near amino acid residue 3500. Subsequently, the microliter plates were incubated with PBS containing 20 mg/ml radioimmunoassay grade bovine serum albumin (BSA) (Sigma) for 45 min. A total of 1–2 μl of mouse plasma was added to each well and incubated overnight at 4 °C. The plates were then washed six times with PBS containing 0.1% (w/v) BSA and 0.05% (v/v) Tween 20. Then, 50 μl of ^{125}I-labeled human apoB-specific monoclonal antibody, C1.4 (~10,000 cpm/ng), was added to each well (400,000 cpm/well). Antibody C1.4 (generously provided by Dr. Elaine Krul, Washington University, St. Louis, MO) binds apoB100 near amino acid 500 (25). The plates were then incubated with the ^{125}I-C1.4 for 6 h and washed as previously described, and radioactivity was quantitated. The standard curve was generated using purified human LDL (26) diluted in a PBS solution that contained 20 mg/ml BSA, 0.05% (v/v) Tween 20, and 0.04% (w/v) NaN_3. The standard curve ranged from 0.3 ng to 2.048 μg per well.

Analysis of Distribution of Human ApoB100 Distribution in Transgenic Mice Plasma—A total of 100 μl of plasma from a human, an apoB100 transgenic mouse, and a control littermate was adjusted to a density of 1.215 g/ml with KBr. Ultracentrifugation was performed for 11 h at 4 °C using a Beckman TLA-100 rotor at 436,000 × *g*. Aliquots of 10 μl from the top and bottom fractions were added to 10 μl of loading buffer (see above), boiled for 5 min, and loaded onto a 5% SDS-polyacrylamide gel. The human sample was diluted 1:100 in PBS prior to electrophoresis. Electrophoresis, transfer, and immunoblotting were performed exactly as described above.

Plasma from three human apoB100 transgenic mice (500 μl) was adjusted to a density of 1.215 g/ml with KBr and subjected to ultracentrifugation at 240,000 × *g* for 44 h at 4 °C using a Beckman Ti-50.3 rotor. The lipoprotein fraction was adjusted to a volume of 3 ml using 0.15 M NaCl, 0.01% (w/v) Na_2 EDTA, 0.02% (w/v) NaN_3 at pH 7.2. A 2-ml aliquot was subjected to gel filtration on a Superose 6B column using a fast protein liquid chromatography (FPLC) apparatus (27). Fractions of 2 ml were collected, and 200 μl from each fraction was used to measure the cholesterol content with an enzymatic calorimetric assay (Boehringer Mannheim). Every three fractions were pooled and subjected to trichloroacetic acid precipitation as described (27). The pellet was solubilized in the loading buffer (see above), boiled for 5 min, and loaded onto a 5% polyacrylamide gel containing SDS. After electrophoresis, the proteins were transferred and immunoblotting was performed using MB3, as described above.

Analysis of Transgenic Liver RNA—Total liver and small intestinal RNA from three transgenic and three control mice were isolated (28) and diluted to 1.0 μg/μl. Two μg of each RNA sample was amplified with either mouse- or human-specific primers and 2 units of Retrotherm™ RT polymerase (Epicentre Technologies, Madison, WI) using buffers supplied by the manufacturer. Two oppositely oriented human-specific oligonucleotides, M49 (5′-CTG AAT TCA TTC AAT TGG GAG AGA CAA GTT TCA A-3′) and M50 (5′-CGG ATA TGA TAG TGC TCA TCA AGA C-3′), were employed to amplify a 278-base pair fragment extending from nucleotide 6506 to 6784 of human apoB100. The corresponding region of the mouse apoB100 RNA was amplified using mouse-specific oligonucleotides, M49R (5′-CGG AAT TCA TCT GAC TGG GAG AGA CAA GTA CGT G 3′) and M50mus (5′-CGG ATA TGA TAC TGT TCA TCA AGA A-3′). After amplification, the reaction products were extracted with phenol and chloroform (1:1), passed over a Chromaspin-100 column (Clontech Laboratories, Inc., Palo Alto, CA) to remove free nucleotides, and precipitated with three volumes of ethanol. The PCR products were resuspended in TE (10 mM Tris, 1 mM Na_2 EDTA, pH 8) and subjected to primer extension analysis as described (5). The extent of editing was determined by scanning the primer extension gels with an Ambis-100 radioactive imaging system.

RESULTS AND DISCUSSION

A human (apoB100) fragment driven by the transthyretin enhancer/promoter element (Fig. 1) was microinjected into 725 fertilized C57Bl/6 × SJL F_2 hybrid eggs. The eggs were transferred into pseudopregnant females, and 34% (29 mice) of the 86 offspring contained the human apoB100 transgene, as assessed by dot blot hybridization of total nucleic acids. A 10-μl aliquot of plasma from each transgenic mouse was subjected to immunoblotting, and a single male mouse had detectable amounts of human apoB100 (data not shown). The copy number of the transgene in the offspring of this founder was estimated to be 20 by quantitative genomic blotting.

To determine the tissue distribution of expression of human apoB100, tissue homogenates were subjected to immunoblotting using MB3, a human-specific anti-apoB100 mouse monoclonal antibody that binds near apoB100 amino acid residue 1000 (29). ApoB100 protein expression was not detectable using standard immunoblotting techniques. Therefore, immunoprecipitation studies were performed on tissue extracts from animals, which had been perfused extensively with PBS prior to organ removal. Immunoreactive material of the expected size was identified in liver and brain and in trace amounts in the kidney (Fig. 2). Previously, a similar promoter/enhancer construct from the TTR gene was shown to result in liver-specific expression (18). However, mice with high copy numbers of this promoter element also expressed TTR in brain and kidney (30).

The antibody employed for immunoprecipitation detection, MB3, also recognizes apoB48 (29). No human apoB48 protein was identified in any of the tissue samples. This was the expected result, since the construct used to make the transgenic mice was engineered to preclude the formation of apoB48. The construct contained a substitution of a thymidine for adenine in the second position of codon 2153 from CAA (Gln) to CTA (Leu). Message editing would not effect the amino acid encoded, since the edited transcript (UUA) also encodes a leucine residue. Importantly, this base pair substitution has been previously

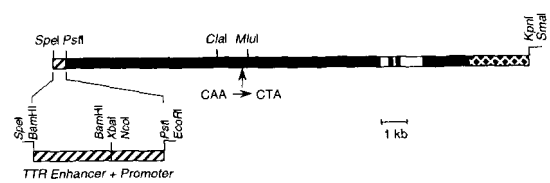

FIG. 1. **Human apoB100 expression construct.** The expression vector (pTTR-B100 (Leu-Leu)) contains enhancer-promoter elements from the mouse transthyretin gene (*striped box*), the coding region of the human apoB100 cDNA (*solid boxes*), the last three introns of the apoB100 gene (*white boxes*), and 2.3 kb of the 3′-untranslated region (*cross-hatched boxes*). Site-directed mutagenesis was performed at the site at which the apoB100 mRNA is edited (CAA → CTA) to prevent apoB48 synthesis.

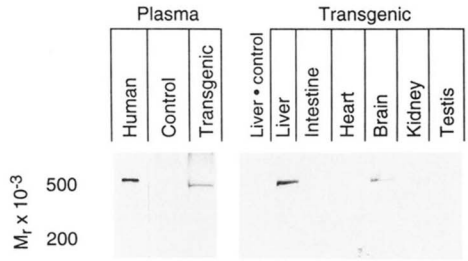

FIG. 2. **Tissue distribution of human apoB100 protein expression.** Twelve mg of total protein from tissue homogenate were subjected to immunoprecipitation using a rabbit anti-human apoB100 polyclonal antibody as described under "Materials and Methods." The precipitate was size-fractionated on a 5% SDS-polyacrylamide gel under reducing conditions, transferred to nitrocellulose, and incubated with a mouse anti-human apoB100 antibody (MB3) and then with a sheep anti-mouse IgG that was conjugated to horseradish peroxidase (*HRPO*). The membrane was exposed to XAR-5 film for 30 s (plasma samples) and 3 min (tissue samples) prior to developing.

demonstrated *in vitro* not to interfere significantly with apoB100 mRNA editing (21).

The plasma levels of apoB100 were quantitated using a sensitive sandwich radioimmunoassay. The concentrations of apoB100 in the F_1 apoB100 mice ranged from 0.035 to 0.10 mg/dl with a mean level of 0.07 mg/dl. It is likely that the low level of human apoB100 expression is due to the fact that the construct does not contain all of the gene-specific sequences necessary for high levels of apoB100 expression. Though all the *cis*-acting sequences necessary for apoB100 transcription have not yet been characterized, an enhancer element has been identified in intron 2 of the human apoB100 gene and these sequences are not included in the human apoB100 transgene (31, 32).

The total cholesterol (mg/dl) (74.2 ± 10.5 (transgenic) *versus* 79.6 ± 2.8 (control)) and triglyceride levels (mg/dl) (87.5 ± 4.7 (trangenic) *versus* 129.8 ± 15.2 (control) were similar in five apoB100 transgenic mice and their littermate controls. To determine if the apoB100 expressed in the plasma was associated with lipoproteins, aliquots of plasma from human, a control, and a transgenic mouse were subjected to ultracentrifugation at a density of 1.215 g/ml. Aliquots from the top (d < 1.215 g/ml) and bottom (d > 1.215 g/ml) fractions were size-fractionated on a 5% reduced SDS-polyacrylamide gel and immunoblotted with a human apoB-specific monoclonal antibody (MB3) (Fig. 3*A*, *top panel*). All the immunoreactive material was present in the top fraction of the mouse sample, so the human apoB100 circulates as part of a lipoprotein in the mouse.

To determine the distribution of human apoB100 in the lipoproteins, FPLC was performed using plasma pooled from five transgenic and five control mice. The lipoprotein profiles were identical in the control and transgenic mouse plasma (control data not shown). In the transgenic mice, the apoB immunoreactive material was localized to fractions 12–18, which corresponds to the LDL peak (Fig. 3*B*).

Next we determined whether the full-length human apoB100 transcript underwent mRNA editing. Because the levels of human apoB100 mRNA in the livers of the transgenic mice were very low, the reverse transcriptase-polymerase chain reaction (RT-PCR) was used to amplify a 278-base pair fragment from the liver transgene apoB mRNA in the vicinity of the editing site (base pairs 6506–6784) from total hepatic mouse mRNA. The RT-PCR amplification of the liver mRNA from the transgenic mice, but not from the non-transgenic littermates, produced DNA fragments of the appropriate size (Fig. 4*A*). A primer-extension assay on the PCR amplification products of the human apoB100 mRNA isolated from transgenic livers revealed that the human apoB100 mRNA was edited (Fig. 4*B*). To

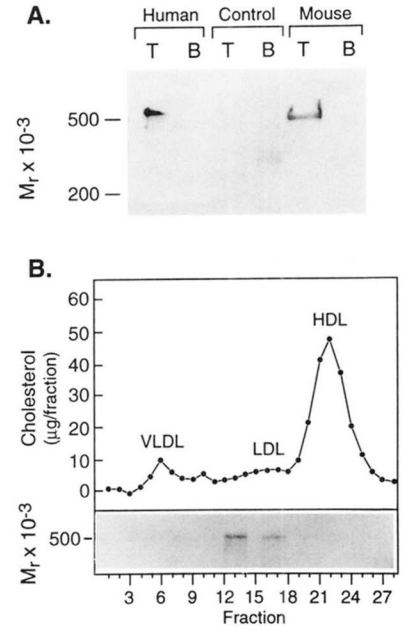

FIG. 3. **Distribution of human apoB100 in transgenic mice plasma.** A total of 100 μl of plasma from a human, control mouse, and transgenic mouse were subjected to ultracentrifugation at density 1.215 g/ml. Aliquots of the top (*T*) and bottom (*B*) fractions were loaded onto a 5% SDS-polyacrylamide gel under reducing conditions. After transfer to nitrocellulose, the proteins were immunoblotted using a mouse monoclonal anti-human apoB100 antibody (MB3) as described under "Materials and Methods." The membrane was exposed to XAR-5 film for 2 min (*panel A*). A total of 500 μl of plasma was pooled from five apoB100 transgenic mice and subjected to ultracentrifugation at d = 1.215 g/ml. A 2-ml aliquot of the top fraction (d < 1.215 g/ml) was subjected to gel filtration using a Superose 6B column and an FPLC apparatus. Two milliliter fractions were collected and the total cholesterol content was measured in each fraction. Every three fractions were pooled, and the proteins were precipitated using 15% trichloroacetic acid (v/v). The pellets were washed and resuspended in 40 μl of loading buffer, and 15 μl were size-fractionated onto a 5% polyacrylamide gel containing SDS under reducing conditions. Immunoblotting was performed using MB3, and the filter was exposed to film for 4 min prior to developing.

compare the efficiency of editing of the human and mouse apoB100 mRNA, mouse-specific oligonucleotides were employed to amplify the corresponding fragment from the mouse apoB100 mRNA. The human apoB100 mRNA transcript was edited with a somewhat lower efficiency than the endogenous mouse liver apoB100 transcript (Fig. 1*C*). The observed reduction in the efficiency of editing of the human apoB100 transcript cannot be attributed to the "Leu-Leu" mutation since a synthetic 354-nucleotide apoB RNA substrate that included this mutation was edited *in vitro* as effectively (7.8%) as the wild-type human sequence (6.0%) using a rabbit enterocyte editing extract (data not shown). Admittedly, the level of expression of human apoB in our transgenic mice is very low and this may impact on the efficiency of the editing activity. Higher levels of human apoB100 expression may result in a decrease in the proportion of human transcripts that undergo editing.

Our observation that the human liver apoB mRNA synthesized by transgenic mice is extensively edited is in contrast to data originally reported by Xiong *et al.* (17), which has subsequently been retracted (33). It appears that a multicomponent is necessary for apoB mRNA editing (6–8). The simplest complex would be one in which one subunit or protein recognizes and binds the apoB100 mRNA and another protein catalyzes the deamination of cytidine 6666. The site recognized by the mRNA-binding component has been identified as an 11-nucleotide sequence downstream from the editing site (34). This 11-nucleotide sequence is conserved perfectly in five species that

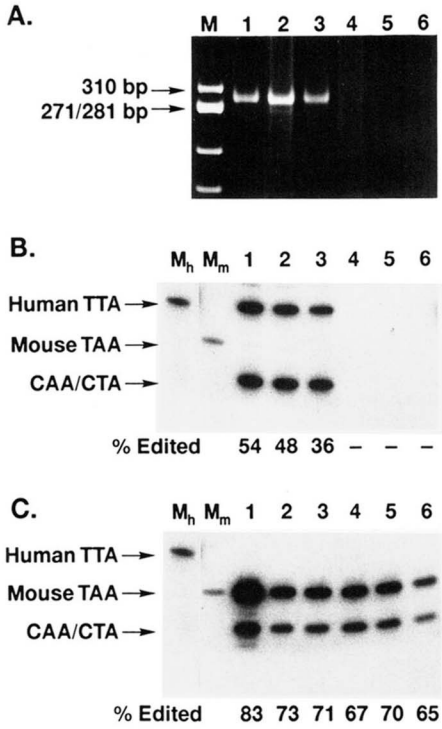

FIG. 4. **Primer extension analysis of transgenic liver RNA.** Total hepatic RNA from both transgenic and non-transgenic mice livers were amplified and analyzed by primer extension analysis. *Panel A*, the amplified products from 3 transgenic mice (*lanes 1–3*) and 3 control mice (*lanes 4–6*) were size-fractionated on a 5% polyacrylamide gel, and the gel was stained with ethidium bromide. *Panels B* and *C*, a primer extension assay was performed using RT-PCR-generated DNA fragments amplified employing either human-specific oligonucleotides (*panel B*, *lanes 1–6*) or mouse-specific oligonucleotides (*panel C*, *lanes 1–6*). As size controls, primer extensive products of synthetic RNA corresponding to edited human (*Mh*) or mouse (*Mn*) were used. Human-specific oligonucleotides (*Mh*) or mouse-specific oligonucleotides (*Mm*) were employed to amplify total transgenic mouse hepatic RNA. *Arrows* at *left* of figure designate the expected position of non-edited (CAA/CTA) and edited mouse (TAA) and human (TTA) extension products, respectively. The percentage of edited template was determined using the Ambis-100 imaging system and is given below each lane.

have been examined (35). Nevertheless, species-specific sequence differences of apoB100 substrates in flanking regions have resulted in moderate differences in editing efficiency *in vitro*. For example, previous studies have revealed that a 354-nucleotide rabbit apoB100 RNA substrate is edited 1.8 times more efficiently than a human apoB100 template of similar length by a rabbit enterocyte extract (36). As demonstrated in the present studies, apoB RNA editing *in vivo* also has limited species specificity. Endogenous mouse apoB100 mRNA was edited about 1.6 times more efficiently than the human apoB RNA. Thus, our results of slightly decreased editing efficiency of the human apoB transcript compared to the endogenous mouse transcript agree well with results from previous *in vitro* experiments (36). Therefore, even though our results, and those of previous investigations (36, 37) show that mRNA editing appears to be influenced by species-specific sequences flanking the binding and editing sites, the present results indicate that there is no absolute species specificity to the apoB mRNA editing process.

Acknowledgments—We thank Brian Blackhart and Brian McCarthy for contributions in the development of the human apoB100 expression construct. We thank Arlene Leon and Shanna D. Maika for technical assistance.

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