Regulation of Intestinal Apolipoprotein B mRNA Editing Levels by a Zinc-Deficient Diet and cDNA Cloning of Editing Protein in Hamsters[1,2]

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ABSTRACT This study was conducted to investigate the influence of dietary zinc on intestinal apoB mRNA editing in hamsters. Apolipoprotein B-48 (apoB-48) is synthesized from the same gene as apoB-100 by a post-transcriptional, site-specific cytidine deamination, a process known as apoB mRNA editing. A cDNA encoding the hamster apoB mRNA editing enzyme was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) and the deduced amino acid sequence was found to possess high amino acid sequence identity to apoB mRNA editing enzymes from several other species. Editing activity was detected in the small intestine and colon but, like humans, none was detected in the liver. Analysis by RT-PCR indicated that the small intestine possessed the highest expression of editing enzyme mRNA abundance, whereas both liver and small intestine expressed relatively high levels of apoB mRNA. The influence of dietary zinc on intestinal apoB mRNA editing levels was examined in Golden Syrian hamsters (7 wk old) assigned to one of the following three dietary treatments: Zn-adequate (ZA, 30 mg Zn/kg diet), Zn-deficient (ZD, <0.5 mg Zn/kg diet), or Zn-replenished (ZDA, ZD hamsters receiving ZA diet for last 2 d) for 7 wk. Hamsters consuming the ZD diet had modestly but significantly lower intestinal editing activity than ZA hamsters. Intestinal editing activity in the ZDA group was not different from that of ZA hamsters. Data derived from these studies contribute to the understanding of lipoprotein metabolism in hamsters, a suitable model for the study of atherosclerosis. J. Nutr. 130: 2166–2173, 2000.

KEY WORDS: • *hamsters* • *dietary zinc* • *cardiovascular disease* • *cholesterol* • *low density lipoprotein*

Apolipoprotein B (apoB)[4] is an obligatory structural and functional component of triglyceride-rich VLDL and chylomicrons. ApoB is also an essential protein of LDL particles, the main transporter of plasma cholesterol in humans. There are two major isoforms of apoB, apoB-100 and apoB-48, both of which are synthesized from the same gene by a post-transcriptional mechanism (Chen et al. 1987, Powell et al. 1987) known as apoB mRNA editing. A cytidine to uridine conversion in the apoB transcript results in changing a glutamine CAA codon to UAA, an in-frame translational stop codon. This editing results in a truncated protein (apoB-48) that is approximately half the size of apoB-100. ApoB-48 is identical to the amino-terminal half of apoB-100 but lacks the COOH-half of apoB-100. Consequently, apoB-48 lacks the LDL receptor-binding domain required for uptake via LDL receptor–mediated endocytosis. Instead, apoB-48–containing lipoproteins are cleared faster than apoB-100–containing lipoproteins by a distinct pathway. ApoB-48 also does not possess the region present in apoB-100 that can combine with apo(a) to form the atherogenic lipoprotein (a) molecule (Scanu and Fless 1990). Because humans do not have the ability to edit apoB mRNA in the liver, human VLDL and LDL particles contain apoB-100. This lack of hepatic editing activity is thought to contribute to our susceptibility to develop atherosclerosis. Consequently, numerous laboratories are developing ways to induce hepatic editing using techniques such as gene therapy.

The editing reaction occurs in the nucleus (Lau et al. 1991) and is performed by a complex or "editosome" (Harris et al. 1993) comprised of a 27-kDa enzyme termed *apoB* mRNA *e*diting *c*atalytic subunit *1* (apobec-1) and other complementary protein factor(s) (Lau et al. 1990, Navaratnam et al. 1993b). Apobec-1 exists as a homodimer (Lau et al. 1994) and is an RNA-specific cytidine deaminase that exhibits considerable sequence homology with other cytidine deaminases (Navaratnam et al. 1995). A zinc-coordinating region that resembles that of other cytidine deaminases is present in apobec-1, and apoB mRNA editing has been shown to be zinc dependent in vitro (Barnes and Smith 1993, Navaratnam et al. 1993a). Editosome assembly is facilitated by an 11-nucleotide "mooring" sequence that begins five nucleotides downstream of the editing site in the apoB transcript (Smith et al. 1991); this

[1] Supported by U.S. Department of Agriculture Human Nutrition Competitive Grants 96–35200-3248 and 95–37200-1672 and funds from the University of Arizona Agricultural Experiment Station.

[2] The nucleotide sequences reported in this paper have been submitted to GenBank with accession numbers AF176576 (apoB) and AF176577 (apobec-1).

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[4] Abbreviations used: apoB, apolipoprotein B; apobec-1, apoB mRNA editing catalytic subunit 1; DEPC, diethyl pyrocarbonate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; GCG, Genetics Computer Group; MT, metallothionein; RACE, Rapid Amplication of cDNA Ends; RPA, ribonuclease protection assay; RT-PCR. reverse transcriptase-polymerase chain reaction; ZA, zinc-adequate; ZD, zinc-deficient; ZDA, zinc-replenished.

Manuscript received 16 December 1999. Initial review completed 8 February 2000. Revision accepted 12 May 2000.

region has been shown to be critical for editing efficiency (Shah et al. 1991). Although the complementary factors are absolutely essential for editing (Anant et al. 1995, Teng et al. 1993), they are present in a wide variety of tissues including some that do not edit apoB mRNA (Teng and Davidson 1992). Because of the ubiquitous nature of the complementary factors, it often appears to be the tissue-specific expression of apobec-1 that determines the ability or inability of a tissue to edit. Although intestinal editing occurs at high levels in nearly all mammals examined, hepatic editing is much less common and occurs only in certain species. Greeve et al. (1993) compared hepatic editing in 12 different species and showed that mice, rats, dogs and horses exhibit hepatic editing. Humans, pigs, cows, monkeys, sheep and hamsters, as we have reported here, do not possess detectable hepatic editing. This finding may explain in part why hamster lipoprotein metabolism is more similar to humans than that of most other rodents. For example, a relatively high portion of plasma cholesterol is LDL cholesterol in both humans and hamsters. Both species respond similarly to atherogenic diets (Spady and Dietschy 1988), and hamsters also develop atherosclerotic lesions that resemble those found in humans (Nistor et al. 1987). These characteristics make the hamster an intriguing model with which to study lipoprotein metabolism and prompted us to examine apoB mRNA editing in hamsters.

Previous work in other laboratories has shown that hamster hepatocytes secrete apoB-100 but not apoB-48 (Liu et al. 1991). These studies did not define the mechanism for the lack of apoB-48 synthesis. We hypothesized that hamsters do not synthesize apoB-48 in the liver because they do not edit apoB mRNA in the liver due to a lack of expression of apobec-1, the catalytic component of the editing reaction. Because the editing reaction is zinc dependent in vitro and zinc-deficient rats exhibit impaired lipid absorption, we hypothesized that dietary zinc status may affect intestinal editing activity in hamsters. Similarities between hamster and human lipoprotein metabolism and the prevalence of zinc deficiency in certain human subpopulations (Sandstead 1995) prompted us to examine whether zinc deficiency in hamsters affected intestinal editing levels.

MATERIALS AND METHODS

Tissue collection and RNA isolation. For hepatic RNA isolation, fresh liver sample was immediately combined with TRIzol reagent (Life Technologies, Grand Island, NY) and homogenized. Subsequent steps were performed according to the manufacturer's protocol. For intestinal RNA isolation, the small intestine was flushed with ice-cold diethyl pyrocarbonate (DEPC)-treated PBS and the mucosa was obtained by scraping. The intestinal mucosa was immediately combined with TRIzol for homogenization and isolation of intestinal RNA. Other tissue samples were snap-frozen in liquid nitrogen immediately after their excision and stored at −80°C for subsequent RNA isolation. RNA samples were digested with RQ1 DNase in a 60-μL reaction containing ~5 μg RNA, 5 μL RQ1 DNase (1 U/μL; Promega, Madison, WI), 1 μL RNasin (40 U/μL; Promega), 6 μL of 100 mmol/L dithiothreitol (DTT), 6 μL of 10X RQ1 buffer and 37 μL DEPC-H_2O. Samples were digested for 90 min at 37°C. RNA was then extracted with phenol/chloroform, precipitated with ethanol and pellets were resolubilized in 10 μL DEPC-H_2O.

Cloning of apoB cDNA sequence flanking the editing site. Human and rat apoB cDNA sequences flanking the editing site were obtained from GenBank (NIH, Bethesda, MD), and the conserved regions were analyzed by Genetics Computer Group (GCG) program (Madison, WI). A pair of primers, RP1–5′ and RP1–3′, were then designed on the basis of the rat sequence. DNase I-digested hamster hepatic or intestinal RNA (500 ng) were mixed with 2 μL of 5X first-strand synthesis buffer (Life Technologies), 50 pmol RP1–3′ primer and DEPC-water to 10 μL total volume. The sample was heated to 70°C for 10 min, then cooled to 37°C for 15 min, during which time 4 μL reverse transcription (RT) buffer, 2 μL of 10 mmol/L dNTPs and 1 μL of MMLV RT (Life Technologies) were added. Reverse transcription was performed at 42°C for 30 min, then stopped by heating to 95°C for 5 min and brief placement on ice. Thirty cycles of polymerase chain reaction (PCR) were then performed with 50 pmol RP1–5′ primer, and the cycling condition was set to 92°C for 1 min, 42°C for 2 min and 65°C for 2 min. The resultant PCR products were cloned, and the positive clones were verified by sequence analysis. Multiple clones were derived and sequenced from separate PCR reactions to reduce the possibility of PCR-introduced mutations. The editing site for hamster apoB was deduced from the sequence alignment of hamster, human, rat, pig and mouse cDNA flanking region of the editing site, and was further confirmed by the comparison of apoB sequences derived from both hamster liver and intestine. Oligo sequences were as follows: 5′-TCCTCAGCAGATTCATGATTATCT-3′ (RP1–5′; rat apoB cDNA nt 6482–6505), 5′-AGCATTTTTAGCTTTTCAATGATT-3′ (RP1–3′; rat apoB cDNA nt 6740–6763).

Cloning of the hamster apobec-1 cDNA. The hamster apobec-1 cDNA was obtained by the method of RT-PCR, as described previously (Wu et al. 1998) with some modifications. Apobec-1 mRNA sequences for humans, rats and mice were obtained from the GenBank database and analyzed for conserved regions. Three primers, Fa, Ra and Rb, were then designed on the basis of the human sequence. RT was performed with DNase I–digested hamster intestinal RNA, primer Ra and rTth (Perkin-Elmer, Norwalk, CT). The mixture was heated at 70°C for 10 min, annealed at 55°C for 10 min and the reverse transcription performed at 70°C for 10 min. For PCR, 8 μL of 10X chelating buffer, 50 pmol Fa primer, 6 μL of 25 mmol/L $MgCl_2$, and 64 μL distilled water were added. After 3 min at 94°C, PCR was performed for 30 cycles in a Perkin-Elmer thermocycler as follows: 94°C/1 min, 55°C/2 min and 72°C/2 min, with a 10-min final extension at 72°C. To reduce downstream selection and verification workload, the resultant PCR fraction was then reamplified with primers Fa and Rb, and this PCR resulted in a ~500-bp product. This fragment was cloned and found to share high homology to all known apobec-1 mRNA sequences; it was designated as the central part of hamster apobec-1 cDNA. On the basis of this sequence, two hamster primers, Fb and Rc, were designed for cloning the 5′ and 3′ ends of hamster apobec-1 cDNA using the method of RACE (Rapid Amplication of cDNA Ends).

For 5′ RACE, the RT reaction was performed as described above with the primer Rb; samples were prepared as previously described (Wu et al. 1998). To enhance the selectivity, the secondary PCR with primers dT18-RI and Rc was then performed. The resultant PCR product was cloned and verified by sequencing analysis. In 3′ RACE, the RT reaction was first performed with dT18-RI primer, using MMLV, and then amplified with dT18-RI and primer Fa. The resultant PCR was divided into four aliquots, and further amplified with the primers Fb and one of dT16GACG, dT16GACA, dT16GACT or dT16GACC. Positive bands were observed in the reactions from dT16GACA and dT16GACC. The PCR products were cloned and verified by sequencing analysis. Multiple clones were derived from separate PCR reactions to reduce the likelihood of sequence mutations during PCR. All positive clones derived from 5′ and 3′ RACE were analyzed with GenBank sequence homology search, and the one containing the longest insert was selected as hamster apobec-1 5′ or 3′-end cDNA.

Sequences for the 5′, central, and 3′ portions of apobec-1 cDNA were analyzed by GCG program to compile the full-length cDNA sequence. Primers used to obtain the hamster apobec-1 cDNA sequence were as follows: 5′-cggaattctttttttttttttttttt-3′ (dT18-RI), 5′-gtgaccccactctgaggagaagaat-3′ (Fa; corresponds to hamster apobec-1 cDNA nt 76–99), 5′-TTCCTGTCCTGGAGTCCCTG-3′ (Fb; hamster apobec-1 cDNA nt 305–324), 5′-tcttcttgaaatctttaaacagggtgg-3′ (Ra; corresponds to hamster apobec-1 cDNA nt 614–640), 5′-tacagcctcatccacagattt-3′ (Rb; corresponds to hamster apobec-1 cDNA nt 559–579), 5′-ATTTGAAGGTGGGTAGTT-

GAC-3′ (Rc; hamster apobec-1 cDNA nt 515–535). Upper-case letters denote hamster apobec-1 cDNA sequences.

ApoB mRNA editing assay. ApoB mRNA editing was determined by RT-PCR amplified primer extension assay, basically as described by Giannoni et al. (1994). This increases the sensitivity of the assay but does not change the ratio of apoB-48/apoB-100 mRNA. dNTPs (1 mmol/L), 4 μL of 5X first-strand synthesis buffer (Life Technologies), 1 μL RNasin (40 U/μL; Promega), 2 μL of 100 mmol/L DTT, and 50 pmol of downstream primer RP1–3′ were combined with 8 μL DNase-digested RNA (~750 ng) in a 19-μL reaction. Samples were heated to 70°C for 7 min, then annealed at 42°C for 5 min. SuperScript II RT (40 U; Life Technologies) was added and samples were incubated for 60 min at 37°C. After reverse transcription, mineral oil was added; samples were heated to 95°C for 5 min and then placed briefly on ice. For PCR, 80 μL of PCR buffer that contained 10 μL of 10X PCR buffer (Perkin-Elmer), 2 μL (50 pmol) primer RP1–5′, 10 μL dimethyl sulfoxide (DMSO), 10 μL of 25 mmol/L $MgCl_2$, 1 μL Taq polymerase (Perkin-Elmer) and 47 μL distilled H_2O were added to the 20-μL reaction followed by 30 cycles of 92°C/1 min, 40°C/2 min and 65°C/2 min. For the last cycle, extension was performed for 10 min. PCR with these primers resulted in the amplification of a 282-bp fragment flanking the editing site. PCR products were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA) after size verification by agarose gel electrophoresis. To check for DNA contamination, a control for each RNA sample was kept on ice during RT; in addition, PCR was performed using Taq DNA polymerase (Perkin-Elmer). Both methods verified the absence of DNA contamination. For primer extension analysis, the annealing primer HAMBEDIT (spanning hamster apoB cDNA nt 6674 to 6708) was 5′-end labeled with (γ-^{32}P)-ATP (Amersham), then purified with a QIAquick Nucleotide Removal Kit (Qiagen). Labeled primer, 2 μL of 250 mmol/L NaCl and 2 μL of 5X first-strand synthesis buffer (Life Technologies) were combined with 1 μL of diluted purified PCR product. Tubes were heated at 95°C for 10 min and after annealing at 70°C for 2 min, the mixture was cooled to 37°C over 2 min. Thereafter, 20 U of SuperScript II RT (Life Technologies), 1 μL of 100 mmol/L DTT, 1 μL of 5 mmol/L ddGTP and 0.5 μL of a 10 mmol/L mixture of dATP, dCTP and dTTP were added. Extension was performed at 37°C for 30 min. Samples were then placed on ice, and the products were ethanol-precipitated and separated on 6% polyacrylamide/8 mol/L urea gels at 55 W for 2 h. Gels were dried, subjected to autoradiography and analyzed by laser densitometry (Molecular Dynamics, Sunnyvale, CA). Primer extension of the unedited apoB-100 mRNA resulted in a product 43 bases in length, whereas the edited apoB-48 mRNA product was 54 bases in length. Primers used for the editing assay, 5′-AATCCTGTGGATCATAATTGTCTCTAATATACTGA-3′ (HAMBEDIT), RP1–3′ and RP1–5′, were described in the section on cloning of the apoB cDNA sequence flanking the editing site.

Tissue-specific distribution of apoB and apobec-1 mRNA. RT-PCR was used to amplify endogenous mRNA of both apobec-1 and apoB in the various tissues. Competitive RT-PCR was not utilized because a semiquantitative method was not required. For apoB, RT was performed as previously described in the last section. RT product (10 μL) was combined with 40 μL PCR mix in a 50-μL final volume containing 50 pmol RP1–5′, 5 μL of 10X PCR buffer (Perkin-Elmer), 2 μL of 25 mmol/L $MgCl_2$, 5 μL DMSO, 0.5 μL Taq polymerase and 1.5 μL diluted [α-^{32}P] dCTP (NEN Life Science Products, Boston, MA). The first cycle was performed as follows: 92°C/3 min, 40°C/2 min and 65°C/2 min. Twenty-nine cycles of 94°C/1 min, 55°C/2 min and 72°C/2 min were then performed, followed by a 10-min final extension.

For apobec-1, RT was performed essentially as described previously except that 50 pmol Rc primer was used in a 10-μL RT reaction. For subsequent PCR, 40 μL of PCR buffer containing 4 μL of 10X chelating buffer, 50 pmol Fb, 3 μL of 25 mmol/L $MgCl_2$ and 0.15 μL [α-^{32}P] dCTP were added. After 2 min at 94°C, 30 cycles were performed as follows: 94°C/30 s, 55°C/30 s and 72°C/2 min, with a 10 min final extension. Negative controls for all samples were included in which tubes were kept on ice during reverse transcription (RT−) to check for DNA contamination. RT-PCR products were electrophoresed on native 5% polyacrylamide gels. Migration of the products was compared with a 100-bp DNA ladder (Promega) 5′ end-labeled with [γ-^{32}P] ATP.

Experimental animals and diets. Male Golden Syrian hamsters (7 wk old; Harlan, Indianapolis, IN) were assigned randomly to one of the following three dietary treatment groups: zinc-adequate (ZA, n = 8), zinc-deficient (ZD, n = 8) or zinc-replenished (ZDA, n = 4). The replenished group was fed ZD diet until the last 2 d of treatment during which time they were fed the ZA diet. Dietary treatment lasted for 49 d during which time hamsters consumed their respective diet ad libitum. The basal diet was purchased from Dyets (Bethlehem, PA) and was formulated according to the AIN-93-M rodent diet recommendations (Reeves et al. 1993) except that zinc was omitted from the mineral mix. $ZnCO_3$ was added to the zinc-adequate diet at 30 mg Zn/kg diet, whereas the zinc-deficient diet contained <0.5 mg Zn/kg diet. All procedures were approved by the Animal Care and Use Committee of the University of Arizona.

Statistics. Values are means ± SD. Differences were considered significant at $P < 0.05$. The data were analyzed using one-way ANOVA and Duncan's new multiple range test (Jaccard and Becker 1990).

RESULTS

Cloned hamster apobec-1 cDNA shows high homology with those from other species. The hamster apobec-1 cDNA was cloned by RT-PCR and 5′, 3′-RACE. The complete nucleotide and the predicted amino acid sequence are shown in **Figure 1**. The nucleotide sequence is 829 bp in length and contains a consensus polyadenylation signal (unlike the rat in which the consensus signal is missing). The single open reading frame of 690 bp encodes a 229-amino acid protein with a calculated molecular mass of 27,574 Da. As indicated in Figure 1, the hamster, mouse and rat proteins each contain 229 residues, whereas those of humans and rabbits are each 236 residues in length. The highest similarity is with mouse apobec-1, sharing 83% amino acid sequence identity, followed by rat (81%), human (69%) and rabbit (64%).

A span of 18 nucleotides downstream of the editing site in apoB mRNA is conserved in hamsters. A 281-bp hamster apoB cDNA fragment was obtained by RT-PCR. The editing site is identified by sequence alignment with known apoB sequences, as shown in **Figure 2**. Only one nucleotide was found to be different when comparing the apoB sequences derived from hamster liver and intestine, at the editing site, indicating that the PCR method was accurate and did not introduce mutations. Alignment of the apoB mRNA sequences once again illustrates the importance of the sequence downstream of the editing site that has been shown to be critical for editing (Shah et al. 1991). Smith et al. (1991) proposed that nucleotides 6671–6681, 3′ to the editing site (nt 6666), constitute a "mooring sequence" in which the sequence functions to position the deaminase over the site to be edited. Indeed, a span of 18 nucleotides downstream of the editing site is conserved in each of the species shown in Figure 2.

Lack of hepatic apoB mRNA editing in hamsters. Using the sequence derived from the apoB mRNA adjacent to the editing site, we synthesized an annealing primer for the "poisoned" primer extension assay used to measure editing. RNA was prepared from 12 different tissues, the region of apoB mRNA flanking the editing site was amplified by PCR; then, the primer extension editing assay was performed. The presence of ddGTP in the reaction causes termination of extension when a CTP is in the template strand, the template being apoB mRNA, thereby allowing differentiation between edited (apoB-48) and unedited (apoB-100) transcripts. Results from the editing assays are shown in **Figure 3**. We were not surprised to find the highest level of editing in the small intestine

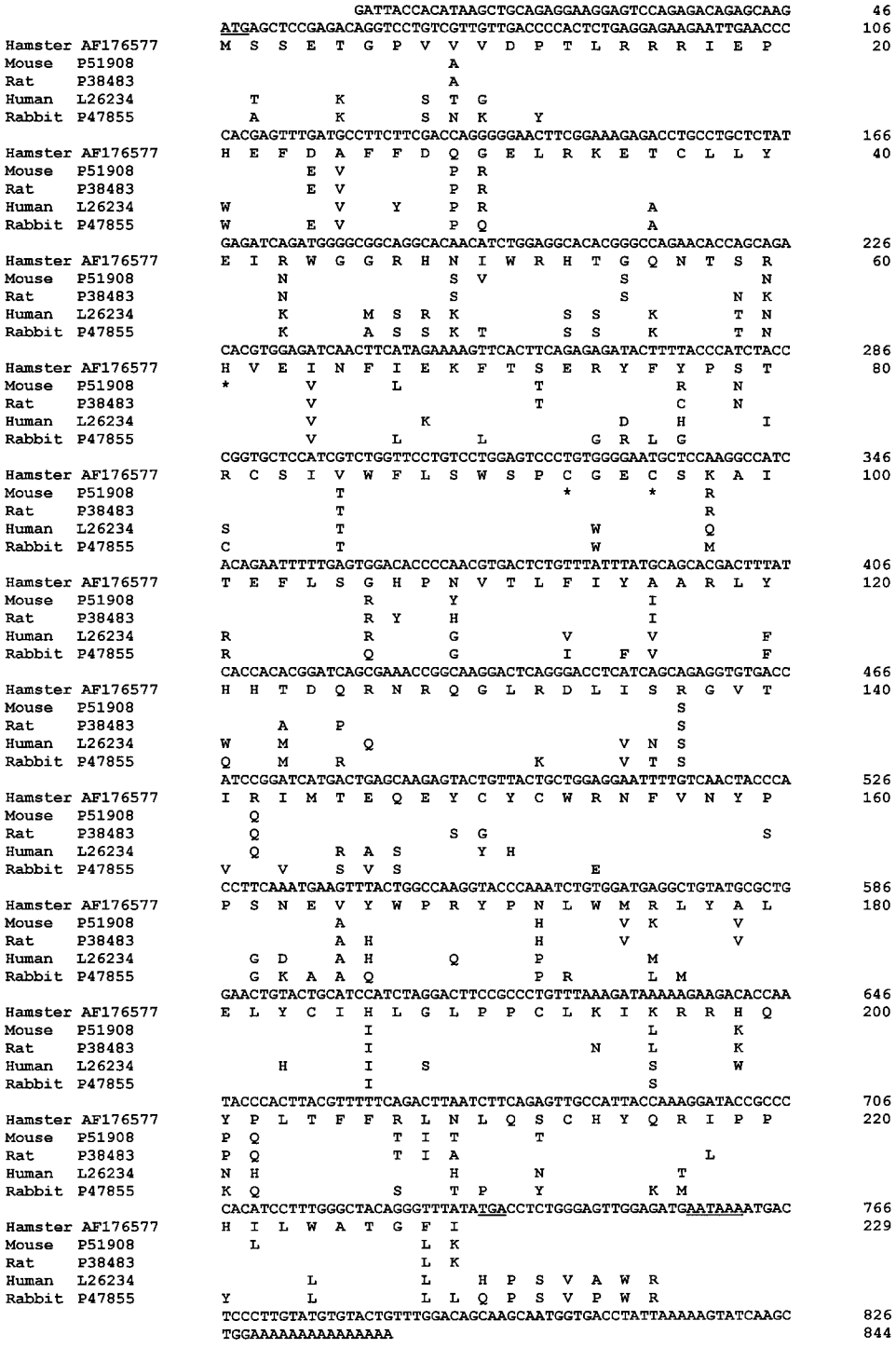

FIGURE 1 Nucleotide and deduced amino acid sequence of hamster apolipoprotein B mRNA editing catalytic subunit 1 (apobec-1) and alignment with mouse, rat, human, and rabbit apobec-1. The translational initiation and termination codons as well as the polyadenylation signal are *underlined*. The conserved His^{61}, Cys^{93}, and Cys^{96} residues are indicated with an asterisk. Only amino acid residues that are different from hamster are shown for the other species. GenBank accession numbers for each of the sequences used for alignment are also indicated.

and colon RNA samples. However, there also appeared to be very small amounts of editing in the stomach and pancreas because an apoB-48 product was barely visible. Of particular interest is the finding that hamsters apparently do not edit apoB mRNA in the liver or at least the editing is at an extremely low level, below detection by primer extension analysis. To the best of our knowledge, this represents the first published report using primer extension analysis to establish that hamsters do not edit apoB mRNA in the liver. These findings support those of other researchers indicating that hamsters do not synthesize apoB-48 protein in the liver.

Tissue distribution of apobec-1 and apoB mRNA. Because the editing assay detected low levels of editing activity in several tissues, we chose to examine the tissue-specific expression of apobec-1. Initially, we had set up ribonuclease protection assays (RPA) to detect apoB and apobec-1 mRNA in the various tissues, using the newly cloned cDNA fragments as probes. Low levels of apobec-1 mRNA expression were detected in the intestine but not in any other tissues (data not shown). In contrast, apoB mRNA was detected in both liver and intestine, at relatively high expression levels, but no clear RPA bands for other tissues were observed (data not shown).

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hamster:   AF176576   cctcagcagattcatgattatctGAATGCATCTGACTGGGAGAGACAAGTAGCCAATGCC
human:     M15421     --A-----AGC A--------------T---TCA-T--------------TT-AC-----
rat:       M21842     ------------------------------------------------------TGG----
pig:       M62614     --A-----AG--A------------G-A---TCAGT-----A--------TTTG-G----
rabbit:    M17780     --A-----AG--A------------G-A---TCA-T--------------TT---G----

hamster:   AF176576   AAGGAAAAATTGACTGCTTTCATGGAAAATTATAGAATTACAGACAATGATGTACTAATT
human:     M15421     -----G---C--------C---CAA----G--------------A------A---A----
rat:       M21842     ----------A---T-------------C--------------T---------------
pig:       M62614     --AA-G---CAT-G--A----------G----------------A----------G----
rabbit:    M17780     -----G---C-A---A------CAA---------A---------G------A---A--C-

hamster:   AF176576   GCATTAGATAGTGCCAAAATCAACTTCAATGAAAAACTTTCTCAACTTGAGACATATGTG
human:     M15421     ---------GA---------------T-----------A--------GC--------A--
rat:       M21842     --C-----------------------G-----------C-----------------C-C-
pig:       M62614     --------C-A-------------C-T-----------AA----G--AC-A---------
rabbit:    M17780     -----G----A---------------A-----------G---------C-----------
                         *
hamster:   AF176576   ATACAATTTGATCAGTATATTAGAGACAATTATGATCCACAGGATTTAAAAAAAACTATT
human:     M15421     ----------------------A---T-G-------TT---T-----G----T-G-----
rat:       M21842     --------------------------T---------G-------C-------G-------
pig:       M62614     ----------------------A---T---------TT---T-----T--G-C-G-----
rabbit:    M17780     ----------------------A---T----T-----T---T-----T----T-G----A

hamster:   AF176576   GCTCAGATTATTGATCGaatcattgaaaagctaaaaatgct
human:     M15421     ---A-T---------GA------------AT------GT--
rat:       M21842     ---------------A-------------------------
pig:       M62614     ---AG----------GA--------C--CAT-G-----T--
rabbit:    M17780     ---AGT-----A----A------G-----AT-------T--
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FIGURE 2 Hamster apolipoprotein (apo)B cDNA sequence flanking the editing site and alignment with human, rat, pig, and rabbit apoB sequences. Nucleotide residues that are different from hamster are shown for the other species. GenBank accession numbers for each of the sequences used for alignment are also indicated. The editing site is indicated with an asterisk above the cytidine residue and the mooring sequence is *underlined.* Primer regions used for polymerase chain reaction (PCR) amplication are shown in lower-case type.

Because the editing assay detected editing activity in tissues other than the small intestine, we then selected the more sensitive RT-PCR method to search for apoB and apobec-1 expression. For apobec-1, the most important RT-PCR product was found in the small intestine; however, much lower levels of apobec-1 mRNA were also present in other tissues, including a very slight but visible band in hamster liver RNA (**Fig. 4**). RT-PCR of apoB mRNA showed that the highest level of apoB mRNA was present in the liver and small intestine (**Fig. 5**). Slight bands were also present in the colon, kidney, stomach and pancreas samples (Fig. 5). Because the purpose of RT-PCR was to examine tissue distribution and not to quantify apoB and apobec-1 mRNA abundance, we did not utilize a method such as competitive RT-PCR for these studies.

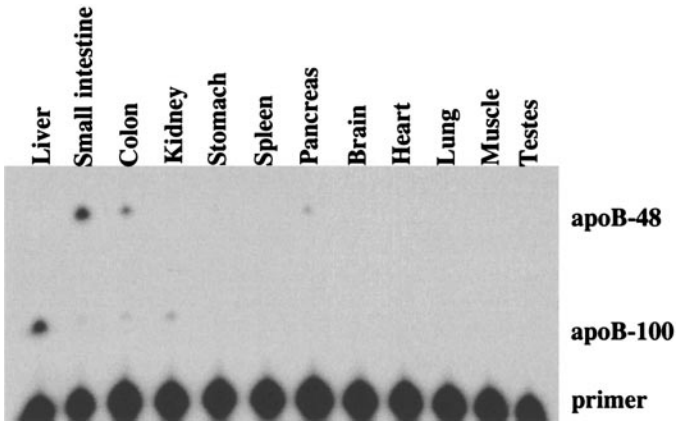

FIGURE 3 Apolipoprotein (apo)B mRNA editing activity in various tissues in hamsters as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and subsequent primer extension analysis. RNA was isolated from each of the indicated tissues, digested with RQ1 DNase, and apoB sequence was amplified by RT-PCR with the primers RP1–3′ and RP1–5′. Primer extension using ddGTP to differentiate apoB-48 and apoB-100 transcripts was used to determine editing activity. Primer extension products were separated on sequencing gels. ApoB-48, edited apoB transcripts containing UAA; apoB-100, unedited apoB transcripts containing CAA; primer, free labeled oligo.

Zinc deficiency depressed intestinal apoB mRNA editing activity. As mentioned earlier, the editing reaction has been shown to be a zinc-dependent reaction in vitro (Barnes and Smith 1993, Navaratnam et al. 1993a). We reported previously that rats consuming a zinc-deficient diet exhibited modest decreases in plasma apoB-48 and hepatic editing levels (Reaves et al. 1999). To examine zinc dependency in vivo, we fed hamsters a zinc-deficient diet for 7 wk. Because no hepatic editing activity was detected, only intestinal editing was measured. Hamsters consuming the zinc-deficient diet exhibited signs of zinc deficiency such as depressed food intake, reduced growth rate, lowered plasma zinc and reduced hepatic zinc. These findings were reported previously in a study that examined the effect of zinc status on apolipoprotein A-I gene expression (Wu et al. 1998). Interestingly, the zinc-deficient hamsters exhibited a reduction in intestinal apoB mRNA editing (**Fig. 6**). Although the reductions were not drastic, they were significant. Zinc-deficient hamsters that consumed a zinc-adequate diet for the last 2 d of treatment (ZDA group) had higher editing levels than ZD hamsters and were not different from ZA hamsters (Fig. 6). The food intake of ZDA

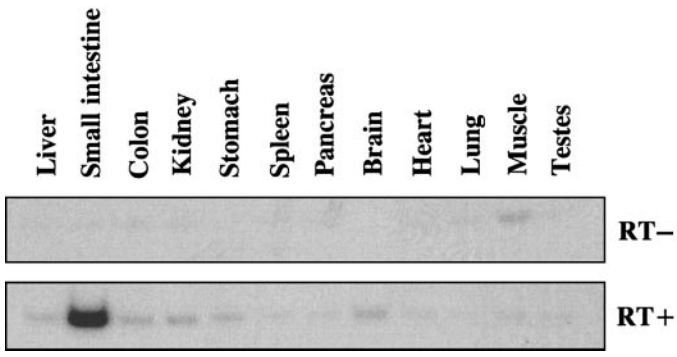

FIGURE 4 Tissue distribution of apolipoprotein B mRNA editing catalytic subunit 1 (apobec-1) mRNA in hamsters as determined by reverse transcriptase-polymerase chain reaction (RT-PCR). PCR was performed using [α-^{32}P]dCTP; PCR products were electrophoresed on native 5% polyacrylamide gels. Control reactions for each sample were also conducted without reverse transcription (RT−).

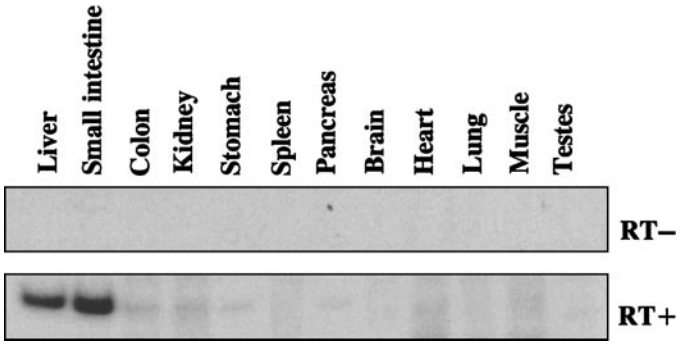

FIGURE 5 Tissue distribution of apolipoprotein (apo)B mRNA in hamsters as determined by reverse transcriptase-polymerase chain reaction (RT-PCR). PCR was performed using [α-^{32}P]dCTP, and PCR products were electrophoresed on native 5% polyacrylamide gels. Control reactions for each sample were also conducted without reverse transcription (RT−).

hamsters was not different from that of ZD hamsters during the 2-d replenishment, remaining significantly lower than that of ZA hamsters (Wu et al. 1998).

DISCUSSION

Sequence alignment of hamster apobec-1 with other species illustrated a high amino acid similarity overall. Perhaps a more important finding, however, is that certain residues and/or motifs appear to be well conserved. For example, the putative zinc-coordinating residues His^{61}, Cys^{93} and Cys^{96} of the active site are intact in the hamster sequence as is the Glu^{63} residue believed to be involved in proton transfer functions (Betts et al. 1994). Experiments using point mutations of these residues (Navaratnam et al. 1995, Yamanaka et al. 1994) have substantiated the relevance and essentiality of these amino acids. The leucine-rich region of the carboxyl-terminus, thought to play a role in dimerization, is also relatively well conserved. The region that spans residues 173–210 is highly conserved in hamsters, mice, rats and humans. In fact, each leucine residue is conserved in these four species at every instance except residue 196. At this position, both hamsters and humans have residues other than leucine. It should also be noted that as in the other species, the hamster sequence also contains proline residues, suggesting that this region more closely resembles that of a leucine-rich motif, previously shown to facilitate protein-protein interactions (Roth 1991), instead of a classic leucine-zipper motif. Although the precise function of this region is yet to be defined clearly, the importance of its presence appears to be validated by its conservation across species.

For the cloned hamster apoB cDNA fragment, the most interesting region is just downstream of the editing site proposed to contain the "mooring sequence" (Smith et al. 1991). Indeed, the region is perfectly conserved in each of the species shown in Figure 2, thereby lending further credence to its importance. In fact, when considering the sequences in Figure 2 as well as those reported by Greeve et al. (1993), out of a total of 11 different species, only the guinea pig contains a one-nucleotide deviation in the sequence spanning nucleotides 6666–6681. The significance of this single nucleotide difference seems minimal, however, because the guinea pig has relatively high intestinal editing (~87%).

For apoB mRNA editing to occur, apobec-1, the catalytic subunit, complementary factor(s) and apoB mRNA must all be present. Most data point to the presence or absence of apobec-1 as determining whether a tissue edits apoB mRNA; however, there are exceptions. There are some tissues that edit apoB mRNA but do not synthesize and secrete apoB-48 protein (Teng et al. 1990). Obviously, some tissues express apoB mRNA but do not edit; however, it is not clear whether there are tissues that express apobec-1 and apoB mRNA but do not have editing activity. In these studies, possibly the most difficult finding to interpret was the apparent lack of editing activity in the liver and kidney in which both apobec-1 and apoB mRNA were detected, albeit at very low levels. One explanation could be that the level of editing activity was

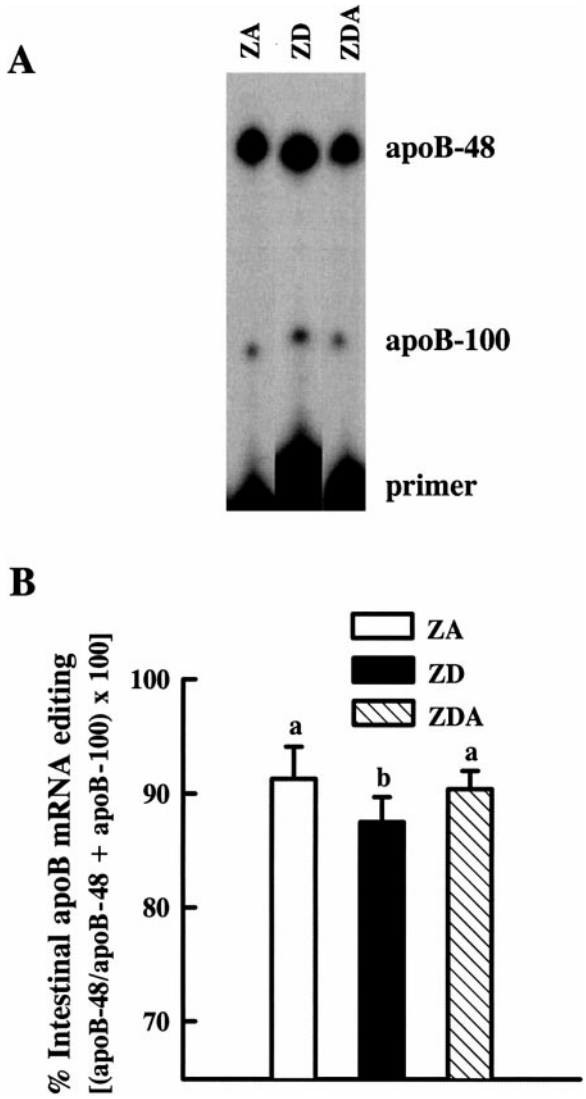

FIGURE 6 Percentage of intestinal apolipoprotein (apo)B mRNA editing in zinc-adequate (ZA), zinc-deficient (ZD) and zinc-replenished (ZDA) hamsters. RNA was isolated from the small intestine of the hamsters in each treatment group, digested with RQ1 DNase, and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with the primers RP1–3′ and RP1–5′. Quantitative primer extension using ddGTP to differentiate transcripts was used to determine the percentage of editing activity. (*A*) Primer extension products were separated on 6% polyacrylamide/8 mol/L urea sequencing gels. Representative samples from each treatment group are shown. apoB-48, edited apoB transcripts containing UAA; apoB-100, unedited apoB transcripts containing CAA; primer, free labeled oligo. (*B*) Values are means ± SD, $n = 8$ (ZA and ZD) and $n = 4$ (ZDA). Different letters indicate different means, $P < 0.05$. Film exposure times that yielded a linear response were used for densitometric analyses.

extremely low and was below the level of detection for this assay. In the liver, apobec-1 may be a limiting factor, whereas in the kidney, apoB mRNA may be limiting. Other possibilities could be that the sensitivity of RT-PCR allows for detection of apobec-1 mRNA at levels that would expectedly yield very low levels of protein. Post-transcriptional mechanisms that inhibit production of apobec-1 protein could also be involved. Examples exist in which apoB mRNA has been detected in several tissues without apoB protein. The sensitivity of RT-PCR is illustrated by our RPA findings (data not shown) in which apobec-1 mRNA was seen only in the small intestine. Apobec-1 detection will obviously differ depending on which method is used; hence it is often difficult to compare the results from different studies. For example, in humans, Hadjiagapiou et al. (1994) detected apobec-1 mRNA in stomach and testis tissue in addition to the intestine by using RT-PCR analysis. However, Lau et al. (1994) reported that apobec-1 mRNA was expressed only in the small intestine of humans as indicated by Northern blot analysis. Most other studies have not reported both apobec-1 expression and editing activity. We felt it was important to investigate this in hamsters.

We chose to use RT-PCR analysis because of its sensitivity, although this assay poses some difficulties that are not encountered in Northern blots. Because we were not intending to use this method to quantitate mRNA abundance, no internal reference or competitor was used during RT-PCR. In view of its reported sensitivity, we used rTth to amplify apobec-1 mRNA. Apparently the use of SuperScriptII or rTth can result in slightly different results in the RT− reactions. For example, in the RT− reactions of apoB mRNA using SuperScriptII, no band is evident. However, when the same RNA samples were used for RT-PCR of apobec-1 with rTth, slight bands were visible in some of the RT− reactions. Therefore, these bands are most likely related to rTth and could be from a "leaking" of reverse transcriptase activity through the incubation on ice or during PCR.

Apobec-1 and/or apoB mRNA was present in tissues that would apparently not require apoB protein synthesis. Why apobec-1 and/or apoB mRNA would be expressed in these tissues is unknown, but this phenomenon has also been observed by other researchers in several different species (Hadjiagapiou et al. 1994, Teng et al. 1990 and 1993).

Dietary treatments reported in this study have been used routinely in our laboratory to induce zinc deficiency in hamsters. We found that changes in plasma and hepatic zinc concentrations were comparable between hamsters consuming a zinc-deficient diet for 7 wk and rats consuming a zinc-deficient diet for 2.5 wk (Wu et al. 1998). Impaired absorption of dietary lipids has been established in ZD rats (Koo and Turk 1977, Koo et al. 1986). In the intestinal absorptive cells, nascent chylomicrons appear to be irregular in shape and larger in the zinc-deficient than in zinc-adequate rats (Koo et al. 1985). Because of these findings, and the fact that editing has been shown to be zinc dependent in vitro, we examined the influence of dietary zinc on intestinal editing levels. Although hepatic editing has been shown to be influenced by several nutritional regimens (Baum et al. 1990, Funahashi et al. 1995, Leighton et al. 1990, Reaves et al. 1996 and 1999), intestinal editing activity has been found to be resistant to dietary manipulations. In the past, we reported that copper deficiency in rats increased the plasma ratio of apoB-48 to total apoB, as well as hepatic editing activity. However, possibly due to high variances, the intestinal editing activity was not different among the treatments (Reaves et al. 1996). One explanation for these observations is that high intestinal editing activity is the result of highly saturated apobec-1 enzymatic activity. As a result, small changes in the level of apobec-1 will not lead to detectable changes in editing activity. In this study, however, our ZD hamsters exhibited a small but significant reduction in intestinal editing. Refeeding a ZA diet to ZD hamsters for the last 2 d resulted in the restoration of intestinal editing activity to a level comparable to that of ZA hamsters. The food intake for the ZDA group was not increased during the 2-d repletion, as was reported previously (Wu et al. 1998). Our previous work in rats suggested that differences in food consumption were not responsible for returning ZDA hepatic editing to ZA levels (Reaves et al. 1999). In a previous publication, we reported the measurement of mRNA abundance of the well-described zinc-responsive metallothionein (MT)-II gene as a means of assessing zinc levels in the intestinal mucosal cells. Tissue samples used for the determination of editing activity in this study and those used for MT-II analysis were derived from the same hamsters. The previous work indicated that intestinal MT-II mRNA levels were reduced by 71% in ZD hamsters compared with ZA hamsters. In the ZDA group, MT-II mRNA was elevated to 259% of ZA values (Wu et al. 1998). These data strongly suggest that intestinal zinc levels were different among groups and that zinc levels in the intestinal mucosal cells were restored by the zinc replenishment. In view of the metallothionein data as well as the food intake data, this would suggest that differences in intestinal editing are due to differences in cellular zinc levels.

We did not report the hamster plasma apoB-48/apoB-100 ratios for several reasons. ApoB-48 in hamsters is derived from intestine. We observed very low levels of apoB-48 in hamster plasma (unpublished observation). These low levels of apoB-48 with the relatively high levels of apoB-100 make it difficult to quantify apoB-48/apoB-100 ratios in an accurate manner. We realize that there are several other aspects of apoB metabolism that might be affected by zinc deficiency. Alterations in synthesis, degradation, secretion and/or circulatory half-life of apoB-48 derived from the intestine could all be affected potentially by zinc status. However, we chose to focus our efforts on apoB editing.

It should be noted that our current findings differ from those of a study by Nassir et al. (1996) in which neither hepatic nor intestinal editing levels were changed significantly by the consumption of a zinc-deficient diet in rats. However, we reported recently that rats consuming a zinc-deficient diet do indeed exhibit reductions in hepatic editing levels compared with zinc-adequate controls (Reaves et al. 1999). As pointed out in our previous study, there are many differences in the experimental design between the two studies that could explain distinct observations regarding hepatic editing. However, it may be extremely difficult to compare findings from these two studies with the current work on hamsters because of fundamental differences in lipoprotein metabolism that set these species apart.

In summary, we demonstrated that hamsters have no or extremely low levels of hepatic editing activity. We also reported the cDNA sequence of hamster apobec-1 and apoB mRNA sequence flanking the editing site. Moreover, our findings indicate that intestinal editing activity was reduced in hamsters consuming a zinc-deficient diet. Although it may not be common for humans to consume a diet as low in zinc as the diets these studies, there are additional factors in humans that may promote zinc deficiency. Dietary factors that have been shown to promote zinc deficiency include diets high in phytates (O'Dell and Savage 1960), certain types of fiber (Reinhold et al. 1976) and casein (Harzer and Kauer 1982). In addition, other factors such as age (Prasad et al. 1993), exces-

sive alcohol use, liver diseases, malabsorption syndromes, renal disease, enteral or parenteral alimentation, and sickle cell disease (as reviewed by Evans 1986) may also increase the risk of zinc deficiency. A diet low in zinc and/or any number of the above factors could contribute to compromised zinc status in humans. Moreover, severe zinc deficiency has been observed in humans with a rare, autosomal recessive disease termed acrodermatitis enteropathica (Barnes and Moynahan 1973). Therefore, data derived from the zinc-deficient hamster model can provide useful information concerning apolipoprotein B metabolism in humans.

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