Apolipoprotein B mRNA editing in 12 different mammalian species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins[1]

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Abstract Two different isoproteins are encoded by the apolipoprotein (apo) B gene, apoB-48 and apoB-100. ApoB-48, core component of intestinally derived chylomicrons, has an accelerated plasma turnover as compared with the full-length protein apoB-100. A posttranscriptional modification of the apoB mRNA by conversion of cytidine into uridine at nucleotide position 6666 changes the genomically encoded glutamine codon CAA at amino acid residue 2153 into a translational stop codon UAA. This mRNA editing explains the formation of the truncated isoform apoB-48. In the present investigation editing of apoB mRNA in liver and intestine from 12 different mammalian species was measured by a quantitative primer extension analysis of reverse-transcribed and polymerase chain reaction- (PCR) amplified apoB mRNA in order to determine whether *i)* editing of apoB mRNA is generally restricted to the intestine or may also be found in the liver of other species than rodents, and *ii)* hepatic expression of apoB mRNA editing influences lipoprotein concentrations in plasma. Intestinal apoB mRNA was edited at high levels in all species, 40% in sheep, 73% in horse, 82% in pig, 84% in dog, 84% in cat, 87% in guinea pig, 88% in rat, 89% in mouse, and >90% in human, monkey, cow, and rabbit. In liver apoB mRNA was edited to 18% in dog, to 43% in horse, to 62% in rat, and to 70% in mouse. Low levels of editing below 1% were detected in liver of rabbit and guinea pig. In contrast, hepatic apoB mRNA from human, monkey, pig, cow, sheep, and cat liver was not edited. The results of the primer extension analysis were confirmed by cloning and sequencing of the PCR products from dog, horse, cat, guinea pig, sheep, and cow for all of which the apoB cDNA sequence had not been established by previous investigations. Primer extension analysis of apoB mRNA from dog intestine and dog liver indicated C/U editing at C_{6655} in addition to C_{6666}. Cloning and sequencing of apoB cDNA from dog liver and intestine confirmed additional C/U editing at C_{6655} which changes ACA for threonine at amino acid residue 2149 into AUA for isoleucine. Synthesis and secretion of apoB-48-containing lipoproteins from liver was demonstrated by pulse labeling of freshly isolated horse hepatocytes and immunoprecipitation with apoB-specific antibodies or density gradient ultracentrifugation. The concentrations of VLDL, LDL, and HDL in all species were determined after fractionation by density gradient ultracentrifugation. The ratio (VLDL + LDL)/HDL was calculated for humans (1.92), pig (1.40), cow (1.04), monkey (0.91), sheep (0.65), cat (0.47), horse (0.44), rat (0.41), rabbit (0.32), dog (0.26), and mouse (0.25). Therefore, in four (dog, horse, rat, and mouse) out of six species (dog, horse, rat, mouse, cat, rabbit) with low ratios of below 0.5, apoB mRNA was edited in liver. In contrast, none of the species with high amounts of apoB-containing plasma lipoproteins expressed apoB mRNA editing in liver. Taken together, these results indicate that *i)* editing of apoB mRNA is not intestine-specific but is also found in liver of many mammalian species, and *ii)* editing of apoB mRNA in liver appears to be one important genetic determinant for plasma concentrations of apoB-containing lipoproteins.—**Greeve, J., I. Altkemper, J-H. Dieterich, H. Greten, and E. Windler.** Apolipoprotein B mRNA editing in 12 different mammalian species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins. *J. Lipid Res.* 1993. **34:** 1367–1383.

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Apolipoprotein (apo) B has two different isoforms, apoB-100 and apoB-48 (1). ApoB-100, a 512 kDa protein, is the essential core component of very low density lipoproteins (VLDL) which are secreted by the liver (1). After triglyceride hydrolysis most of the VLDL remnants are rapidly taken up by the liver, but some are further metabolized to low density lipoproteins (LDL) which remain in plasma with a half-life of approximately 20 h (1, 2). In humans, LDL contain about 60–70% of the total plasma cholesterol (1, 2). ApoB-48 represents the amino terminal half of apoB-100 and is the core protein of

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

[1]This investigation is dedicated to Professor Gustav Paumgartner, University of Munich, on the occasion of his 60th birthday.

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chylomicrons which are secreted by the intestine (1). After triglyceride hydrolysis the chylomicron remnants are rapidly and completely cleared by the liver with an average plasma half-life of less than 10 min (1, 3). Thus, the two isoforms of apoB differ substantially in their metabolic pathways.

The generation of the truncated isoform apoB-48 is mediated by a unique posttranscriptional RNA modification (4, 5). At nucleotide position 6666 in the mRNA of apoB the genomically encoded cytidine is changed into uridine (6, 7). This RNA editing creates a translational stop codon UAA from the glutamine codon CAA at amino acid residue 2153 and explains the formation of apoB-48 in the intestine (4, 5). The editing reaction is mediated by a novel enzyme that specifically deaminates this cytidine residue in the apoB mRNA (7, 8). In humans, the expression of the apoB mRNA editing enzyme is restricted to the intestine (4, 5). In rats, the editing activity is also highly expressed in liver and is regulated by hormonal, developmental, and nutritional factors (9–11). Thus, apoB mRNA editing represents an unprecedented modulation of gene expression.

Rodents are generally assumed to be an exception in that they express the editing activity both in liver and intestine (11). Indeed, in the other species investigated, i.e., human (4, 5), pig (12), and monkey (13), mRNA editing was not found in liver. These findings, however, do not rule out that apoB mRNA editing limits the accumulation of apoB-100-containing lipoproteins. Editing of apoB mRNA in liver may be one fundamental determinant for plasma levels of apoB as the two isoforms differ so much in plasma half-lives. Unfortunately, there is no obvious experimental system to test this hypothesis. As has been demonstrated for the LDL-receptor (14), expression of the apoB mRNA editing activity in liver of transgenic animals would enable a determination of the impact editing in liver may have on lipoprotein metabolism. However, this approach requires cloning of the apoB mRNA editing enzyme which has not been achieved so far. Alternatively, in the present investigation interspecies comparison, a more conventional approach has been used to address the question as to whether editing of apoB mRNA influences concentrations of lipoproteins. Editing of apoB mRNA in liver and intestine as well as plasma concentrations of VLDL, LDL, and HDL were measured in 12 different mammalian species in order to determine whether *i)* editing of apoB mRNA in liver may also be found in species other than rodents and *ii)* editing of apoB mRNA in liver is reflected in low plasma levels of apoB-containing lipoproteins. Our results provide evidence that in many species editing of apoB mRNA is, in fact, not restricted to the intestine but also highly expressed in liver and appears to correlate with low concentrations of apoB-containing lipoproteins.

EXPERIMENTAL PROCEDURES

Tissue specimens

Human liver was obtained from three different patients (ages 37, 52, and 57) with end-stage liver disease who underwent orthotopic liver transplantation. Human small intestine was obtained from two patients (ages 45 and 53) who had small bowel resection. Human blood was donated by healthy volunteers (mean age 23). Liver, small intestine, and blood from rhesus monkey was removed from three animals (mean age 5.8 years) that were killed for neuroanatomical studies at the Institute for Psychology, Hungarian Academy of Science, Budapest, Hungary. Liver, small intestine, and blood from cow (n = 3), pig (n = 3), and horse (n = 6) were removed from animals that had just been killed at the slaughterhouse (Hamburg). Liver, small intestine, and blood from sheep (n = 3) and dog (n = 5) were taken from animals provided by Zentrale Versuchstierhaltung, Universitäts-Krankenhaus Eppendorf that had been killed for anatomic studies (sheep) or after a 6-month follow-up after undergoing laparoscopic cholecystectomy (dog). Liver, small intestine, and blood from cats (n = 4) were from animals that were killed by a veterinarian on instructions from their respective owners. Liver, small intestine, and blood from rabbit (n = 3) and guinea pig (n = 3) were obtained from animals that were killed at the Zentrale Versuchstierhaltung, Universitäts-Krankenhaus Eppendorf, for collection of immune serum (rabbit) or for studies of the isolated heart (guinea pig). Liver, small intestine, and blood from rats (n = 3) and mice (n = 3) were from animals provided by Zentrale Versuchstierhaltung specifically for this investigation. All animals were in the fasted state and were killed according to the rules of Deutsches Tierschutzgesetz. All aspects of the present investigation were approved by the ethics committee of Universitäts-Krankenhaus Eppendorf. All tissues were obtained within 5 min of removal, washed in sterile phosphate-buffered saline, snap-frozen in liquid nitrogen, and stored at −80°C until used for RNA preparation.

Oligonucleotides

The following oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer and were purified on NAP 10 columns (Pharmacia):

Oligo 1: CTG ACT GCT CTC ACA AAA AAG TAT AGA, corresponding to human apoB cDNA sequence from nucleotide position 6552–6578.

Oligo 2: CAC GGA TAT GAT AGT GCT CAT CAA GAC, corresponding to human apoB cDNA sequence (antisense) from nucleotide position 6786–6760.

Oligo 3: A ATC ATG TAA ATC ATA ACT ATC TTT AAT ATA CTG A, corresponding to human apoB cDNA sequence (antisense) from nucleotide position 6708–6674.

Oligo 4: TGC CAA AAT CAA CTT GAA TGA AAA AC, corresponding to rat apoB cDNA sequence from nucleotide position 6614–6639.

Oligo 5: CAA GCA TTT TTA GCT TTT CAA TGA TT, corresponding to rat apoB cDNA sequence (antisense) from nucleotide position 6765–6740.

Oligo 6: CAA GAA TTT TTA ATT TTT CCA TGA TT, corresponding to rabbit apoB cDNA sequence (antisense) from nucleotide position 6765–6740.

Oligo 7: GAT TAT CTG AAT TCA TTC AAT TG, corresponding to human apoB cDNA sequence from nucleotide position 6498–6520.

Oligo 8: AGG ATG CAG TAC TAC TTC CAC, corresponding to human apoB cDNA sequence (antisense) from nucleotide position 6870–6850.

Oligo 9: GTA AAA CGA CGG CCA GT, M13 universal primer.

Oligo 10: AAC AGC TAT GAC CAT G, M13 reverse sequencing primer.

Oligo 11: GCG AAT TCC TGA TGA CTG CTC TCA CAA AAA AG, corresponding to human apoB cDNA sequence from nucleotide position 6552–6572 plus an additional Eco Rl restriction enzyme recognition site at the 5' end.

Oligo 12: GCG GAT CCA CGG ATA TGA TAG TGC TCA T, corresponding to human apoB cDNA sequence (antisense) from nucleotide position 6786–6866 plus an additional Bam Hl restriction enzyme recognition site at the 5' end.

Oligo 13: GCG AAT TCC TGC CAA AAT CAA CTT GAA TGA, corresponding to rat apoB cDNA sequence from nucleotide position 6614–6634 plus an additional Eco Rl restriction enzyme recognition site at the 5' end.

Oligo 14: CAT CAC GTA GAT CAT AAT TAT TTA TAA TAT AAT GA, corresponding to guinea pig apoB cDNA sequence (antisense) from nucleotide position 6708–6674.

Plasmids

Plasmid pDH30, which contained the human apoB cDNA sequence from nucleotide position 6649–6703 with a C at nucleotide position 6666 cloned into the HindIII-AccI site of the plasmid bluescriptKS, and plasmid pDH31, which contained the identical insert with a T at nucleotide position 6666, were kindly provided by Dr. Rudi Hauptmann, Bender Corporation, Vienna, Austria.

Preparation of total RNA

Total RNA from liver and intestine was prepared using guanidinium isothiocyanate (15) with subsequent ultracentrifugation through cesium chloride (16) essentially as described (17). Briefly, 2 g tissue was homogenized in 20 ml 4 M guanidinium isothiocyanate, 0.1 M Tris-HCl, pH 7.5, containing 1% β-mercaptoethanol using a motor-driven tissue homogenizer, and were subsequently centrifuged for 10 min at 10,000 rpm in a Beckmann JA13 rotor. The supernatant was supplemented with sarcosyl to 2% final concentration, heated to 65°C for 2 min, and layered over 12 ml 5.7 M CsCl using siliconized SW 28 polyallomer tubes. Ultracentrifugation was performed in a Beckmann SW 28 rotor for 24 h at 25,000 rpm. The RNA pellet was redissolved in 1 ml 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 0.1% SDS, extracted twice with phenol–chloroform–isoamyl alcohol 25:24:1, once with chloroform–isoamyl alcohol 24:1, and precipitated in 0.3 M Na-acetate (pH 7.5), 70% ethanol for 30 min at 0°C. The resulting pellet was redissolved in 200 μl 20 mM Tris-HCl, 2 mM EDTA, and the RNA concentration was determined by absorbance at 260 and 280 nm. The normal yield was 2–4 mg RNA, the 260/280 ratio was 1.8–2.0.

cDNA synthesis and PCR-amplification

Ten μg total RNA in 20 μl 20 mM Tris-HCl, 150 mM KCl, 10 mM $MgCl_2$ was digested for 30 min at 37°C with 20 units DNasel, RNase-free, (Boehringer, Mannheim), extracted with phenol–chloroform–isoamyl alcohol 25:24:1 and chloroform–isoamyl alcohol 24:1, and was subsequently precipitated with ethanol. Two μg total RNA was denatured for 5 min at 65°C and annealed for 1 h at 42°C with 20 pmol apoB antisense oligonucleotide in 25 μl 50 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM DTT, and 1 mM each of dATP, dGTP, dCTP, and dTTP. Reverse transcription was performed for 2 h with 2 units super reverse transcriptase (Pharmacia). After addition of 20 pmol upstream apoB sense oligonucleotide and 3 units Taq-polymerase (Pharmacia), PCR amplification was performed in 50 μl 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 3 mM $MgCl_2$, and 0.5 mM each of dATP, dGTP, dCTP, and dTTP using a Perkin-Elmer Cetus DNA Thermal Cycler. Normally, amplification was for 25 cycles, each consisting of 1 min at 92°C, 2 min at 52°C, and 2 min at 72°C. In order to ensure specific PCR amplification of apoB mRNA, controls for each determination were performed in which the reverse transcriptase was omitted. Five μl of the PCR reaction and its corresponding control were analyzed side by side on a 1.5% agarose gel. Amplification of apoB mRNA from human, monkey, sheep, and dog with oligonucleotides 2 and 1 gave PCR products of 235 bp. Amplification of rat and mouse apoB mRNA with oligonucleotides 5 and 4 resulted in PCR products of 152 bp. Amplification of apoB mRNA from cow, pig, horse, and guinea pig with oligonucleotides 2 and 4 produced PCR products of 173 bp. Amplification of apoB mRNA from rabbit with oligonucleotides 6 and 4 gave PCR products of 152 bp. Amplification of cat apoB mRNA with oligonucleotides 8 and 7 resulted in PCR products of 373 bp.

Primer extension analysis for detection of apoB mRNA editing

PCR products were purified by passage over Nick spin columns (Pharmacia) with subsequent ethanol precipitation. Fifty ng PCR products were analyzed by primer extension as described (8). Briefly, after denaturing for 5 min at 95°C, PCR products were annealed at 42°C for 30 min in 8 μl 50 mM Tris-HCl (pH 8.2), 6 mM MgCl, 10 mM DTT, and 0.5 mM each of dATP, dCTP, dTTP, and ddGTP with 100 fmol oligonucleotide 3 that had been labeled with T4-polynucleotide kinase (Pharmacia) and γ^{32}P-ATP (Amersham International, 3000 Ci/mmol) to a specific activity of 1×10^9 dpm/μg. Extension was for 60 min with 2 units reverse transcriptase. This generates extension products of 43 nucleotides for cDNA from unedited apoB mRNA and of 54 nucleotides for cDNA from edited apoB mRNA. For rat and mouse, primer extension resulted in an extension product of 48 nucleotides for edited RNA due to a T to C base change at position 6661 (9, 12). The extension products were separated on a 8.5% polyacrylamide, 7 M urea sequencing gel and analyzed by autoradiography. Quantification of editing was performed by liquid scintillation counting of the excised extension products on a Packard TriCarb 22000 CA liquid scintillation counter using a quench program with an external standard. For guinea pig, whose apoB cDNA sequence has several base changes in the region 6674–6708, primer extension analysis was performed with oligonucleotide 14 complementary to the guinea pig sequence.

Cloning and sequencing of PCR products from dog, horse, guinea pig, cat, cow, and sheep

PCR products from liver and intestine of dog, cat, and sheep were reamplified by PCR (10 cycles) with oligonucleotides 11 and 12. PCR products from liver and intestine of horse, cow, and guinea pig were reamplified by PCR (10 cycles) with oligonucleotides 12 and 13. Amplified products were extracted with phenol–chloroform and precipitated with ethanol. Subsequently, PCR products were digested with 30 units Bam Hl and 30 units Eco Rl for 3 h at 37°C in 50 μl of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, extracted with phenol–chloroform, and precipitated with ethanol. Two μg plasmid DNA (pT7T3, Pharmacia) was digested with 30 units Bam Hl and 30 units Eco Rl for 3 h at 37°C in 20 μl of 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, extracted with phenol–chloroform, and precipitated with ethanol. Subsequently, DNA was digested with 0.5 units calf intestinal phosphatase for 20 min at 37°C in 25 μl of 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine. Calf intestinal phosphatase was digested with 10 μg proteinase K for 20 min at 37°C in 50 μl of 60 mM Tris-HCl (pH 8.8), 100 mM NaCl, 1 mM EDTA, heat-inactivated for 30 min at 70°C, and subsequently extracted with phenol–chloroform and precipitated with ethanol. Fifty ng vector DNA and 25 ng PCR products were ligated with 1 Weiss unit T4 DNA ligase for 12 h at 16°C in 10 μl of 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 1 mM ATP. Competent *E. coli* DH5α cells were transformed with half the ligation mix as described (17) and selected for ampicillin resistance. Individual clones were grown in liquid medium containing 50 μg/ml ampicillin and plasmid DNA was prepared by alkaline lysis (17). Plasmid DNA was analyzed by digestion with Bam Hl/Eco Rl. Recombinant clones were sequenced using a commercially available kit (Sequenase 2.0, United States Biochemical Corporation, USA), following exactly the manufacturer's instruction for sequencing double-stranded plasmid DNA. As sequencing primers M13 universal primer and T7 sequencing primer (Pharmacia) were used. Sequencing reactions were analyzed by electrophoresis in a 7% polyacrylamide-7 M urea sequencing gel followed by autoradiography.

Preparation of primary horse hepatocytes

Horse hepatocytes were isolated by perfusion of a horse liver fragment with collagenase essentially as described previously (18–21). After slaughtering the horse by decapitation (Slaughter House Hamburg, Germany) a fragment of horse liver, 5 cm × 5 cm × 5 cm, was immediately removed and was perfused via the larger veins with 200 ml ice-cold 0.9% NaCl solution containing 20 units heparin/ml. The liver fragment was put on ice and transferred to the laboratory within 60 min. The liver fragment was perfused at 37°C with 250 ml Minimal Essential Medium (GIBCO, BRL), equilibrated with 95% O_2/5% CO_2, which contained insulin at 0.75 μg/ml, glucagon at 0.35 μg/ml, heparin at 20 units/ml, 2 mM L-glutamine, 5 mM EGTA, penicillin at 100 units/ml, streptomycin at 100 μg/ml, 10 mM HEPES, 1 mM Na-pyruvate. Subsequently, the liver fragment was perfused at 37°C with William's Medium (GIBCO, BRL), equilibrated with 95% O_2/5% CO_2, which contained collagenase at a concentration of 0.5 mg/ml (Collagenase Typ IV, Sigma), 5.5 mM glucose, insulin at 0.75 μg/ml, glucagon at 0.35 μg/ml, 2 mM L-glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, 10 mM HEPES, 1 mM Na-pyruvate, and 3 mM $CaCl_2$. After collagenase-perfusion the liver capsule was removed and isolated cells were eluted with ice-cold Minimal Essential Medium (GIBCO, BRL), containing insulin at 0.75 μg/ml, glucagon at 0.35 μg/ml, 2 mM L-glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, 10 mM HEPES, and 1 mM Na-pyruvate. The cell suspension was filtered through a 250-μm mesh nylon filter and centrifuged at 50 *g* for 3 min at 4°C. The cell pellet was resuspended in the same medium and washed three times under the

above conditions. The cells were counted and the viability was estimated by trypan blue exclusion. Viable cells (1.5×10^6) were seeded onto 35-mm plastic dishes in 3 ml William's Medium containing 10% fetal calf serum, insulin at 0.75 μg/ml, glucagon at 0.35 μg/ml, 2 mM L-glutamine, penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 10 mM HEPES. After 4 h incubation at 37°C the medium was replaced with 1 ml serum-free, methionine-free Minimal Essential Medium (GIBCO, BRL) containing 50 μCi[^{35}S]methionine but no other additives. After 6 h the medium was removed and immediately leupeptin, aprotinin, benzamidine, and phenylmethylsulfonyl fluoride, each at a concentration of 10 μg/ml, were added. The medium was concentrated on a Centricon 30 (Amicon) at 3000 *g* to about 200 μl. One ml phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 2 mM EDTA, 0.3% SDS, and 10 μg/ml of each leupeptin, aprotinin, benzamidine, and phenylmethylsulfonyl fluoride were added and concentrated to 300–500 μl. The supernatant was immediately used for immunoprecipitation or was stored at −20°C. The cell monolayer was lysed by addition of 1 ml "immune-precipitation buffer" (1% DOC, 100 mM HEPES, 40 mM EDTA, 150 mM NaCl, 0.5% BSA) at 70°C. The lysed cells were transferred to an Eppendorf tube and the cell culture dish was washed with 500 μl of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 2 mM EDTA, 0.3% SDS, and leupeptin, aprotinin, benzamidine, and phenylmethylsulfonyl fluoride, each at a concentration of 10 μg/ml. After incubation for 5 min at 70°C, the cell lysate was centrifuged in a microfuge and the supernatant was immediately immunoprecipitated or stored at −20°C.

Immunoprecipitation

Immunoprecipitation was performed as described previously (22). Cell supernatant or cell lysate (1.2 ml), prepared as described above, was incubated with 10 μl of a rabbit anti-human apoB polyclonal antibody (Darco) or a goat anti-human apoB polyclonal antibody (Boehringer Mannheim) for 12 h at 4°C with continuous agitation. Ten mg protein A-Sepharose (Sigma) was incubated for 12 h with continuous agitation in 1 ml Minimal Essential Medium (GIBCO, BRL) containing 10% fetal calf serum, and subsequently for 30 min with 1 ml of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 1% bovine serum albumin. The protein A-Sepharose was added to the immunoprecipitation mix and the incubation was continued for 2 h. Subsequently, the protein A-Sepharose was washed four times with 1.2 ml of 150 mM NaCl, 1% DOC, 0.7% NP-40, 50 mM HEPES (pH 7.5), 0.5% bovine serum albumin, and twice with phosphate-buffered saline. To the pelleted protein A-Sepharose, 100 μl 2× SDS-PAGE sample buffer was added and heated to 100°C for 5 min. The supernatant was applied to a 3–12.5% linear gradient SDS-PAGE. After electrophoresis the gel was fixed for 30 min in 10% glacial acetic acid, 20% methanol, and subsequently soaked in Amplify® (Amersham International) for 15 min. The gel was dried under vacuum and exposed to X-ray film with intensifying screens at −80°C.

Density gradient ultracentrifugation of supernatant from isolated horse hepatocytes

Three ml of supernatant from horse hepatocytes was supplemented with leupeptin, aprotinin, benzamidine, and phenylmethylsulfonyl fluoride, each to a final concentration of 10 μg/ml, and were adjusted to a density of d 1.21 g/ml by adding KBr. Three ml KBr solution of d 1.063 g/ml and 3 ml of d 1.019 g/ml solution were overlaid. Ultracentrifugation was performed in a SW 40 rotor (Beckmann) at 40,000 rpm for 24 h at 4°C as described (23). The gradient was harvested from the bottom into 11 fractions and the density of each fraction was determined with a densitometer. The individual fractions were desalted on a Centricon 30 (Amicon) and analyzed on a 3–12.5% linear gradient SDS-PAGE as described above.

Analysis of plasma lipoproteins

Triglyceride and cholesterol concentrations were measured by commercially available kits (Triglyceride GPO-PAP and Monotest Cholesterin, Boehringer Mannheim). Lipoproteins were separated by buoyant density gradient ultracentrifugation as described (23). Briefly, 3 ml of serum was adjusted to a density of 1.21 g/ml with KBr and overlaid stepwise with 3 ml of KBr solution of d 1.063 g/ml and an additional 3 ml of d 1.019 g/ml, and centrifuged in an SW 40 rotor (Beckmann) for 24 h at 40,000 rpm at 4°C. Gradients were fractionated from the bottom into 20 fractions and the cholesterol concentration in each fraction was measured with Monotest Cholesterin (Boehringer Mannheim). The density in each fraction was measured with a densitometer. The HDL fraction was defined as the density range from d 1.08–1.20 g/ml, the LDL fraction was defined as the density range from d 1.02–1.06 g/ml, and the VLDL fraction was defined as the density range $d < 1.02$ g/ml which possibly also contained some IDL. The cholesterol concentration within these three density ranges was integrated and expressed as mg cholesterol/dl serum as a measure for plasma concentrations of HDL, LDL, and VLDL, respectively. The presence of apoB-100 in the density range of d 1.02–1.06 g/ml was confirmed by SDS-PAGE in a 3–12.5% linear gradient and direct staining with brilliant blue or by immunostaining with a goat anti-human apoB polyclonal antibody (Boehringer, Mannheim) with substantial cross-reactivity against apoB of other species after electroblotting onto nitrocellulose as described (24).

RESULTS

Establishing a quantitative assay for apoB mRNA editing in vivo

Primer extension analysis for editing of apoB mRNA, as originally described by Driscoll et al. (25) for an in vitro system, was used to determine the extent of apoB mRNA editing in vivo. As primer extension for apoB mRNA using hepatic or intestinal total RNA resulted in weak signals that could not be quantitated reliably, the editing site of apoB mRNA was selectively amplified by PCR. In order to exclude that PCR leads to bias amplification of edited and unedited cDNA, different ratios of plasmid DNA containing human apoB sequences with C ("Go"-DNA) or T ("Stop"-DNA) at nucleotide position 6666 were amplified by PCR and analyzed by primer extension. Autoradiography of the sequencing gel demonstrated good representation of both products (**Fig. 1**). Liquid scintillation counting of the excised extension products showed a linear relationship between input plasmid DNA and finally recovered extension products (Fig. 1). Linearity was even demonstrated at low concentrations of "Stop"-DNA (0.25; 0.5; 0.75; 1.0; 1.5; 2.5%) as shown on the expanded scale (Fig. 1). Thus, PCR representatively amplifies any given ratio of edited and unedited apoB cDNA and was therefore used to increase the signal of the primer extension analysis for subsequent quantitation by liquid scintillation counting.

Primer extension analysis for apoB mRNA editing in liver and intestine

ApoB mRNA from liver and intestine of 12 different mammalian species, at least three different animals each, was reverse transcribed and selectively amplified by PCR. Oligonucleotides specific for the human sequence did not amplify apoB mRNA from all species due to sequence heterogeneity. However, by different combinations of oligonucleotides specific for human, rat, or rabbit apoB cDNA (12, 26) PCR products of the expected sizes were obtained for all 12 species (**Fig. 2**). For every individual PCR amplification a separate control reaction was performed in which the reverse transcriptase was omitted. Absence of PCR products in the control reactions demonstrated the RNA specificity of PCR amplification (Fig. 2). Traces of genomic DNA were excluded by Southern blotting and hybridization with radiolabeled oligonucleotide 3 where no signals for the control reactions were generated (results not shown). Thus, reverse transcriptase-coupled PCR selectively amplifies apoB mRNA and was therefore used to increase the template for the subsequent analysis of apoB mRNA editing.

Primer extension demonstrated almost complete editing of apoB mRNA in the intestine of human (98%), monkey (97%), pig (82%), cow (95%), cat (84%), rabbit (90%), guinea pig (87%), dog (84%), horse (73%), rat (88%), and mouse (89%) (**Fig. 3**). To a lesser extent apoB mRNA was edited in sheep intestine (40%). In contrast to intestine, apoB mRNA editing in liver varied substantially among the species investigated. In liver from human, monkey, pig, cow, sheep, and cat, apoB mRNA editing was not detected. In liver of rabbit and guinea pig, apoB mRNA was edited at low levels of less than 1%. Hepatic apoB mRNA was edited to high degrees in dog (18%), horse (43%), rat (62%), and mouse (70%). All these values represent the mean of three independent determinations from at least three different animals.

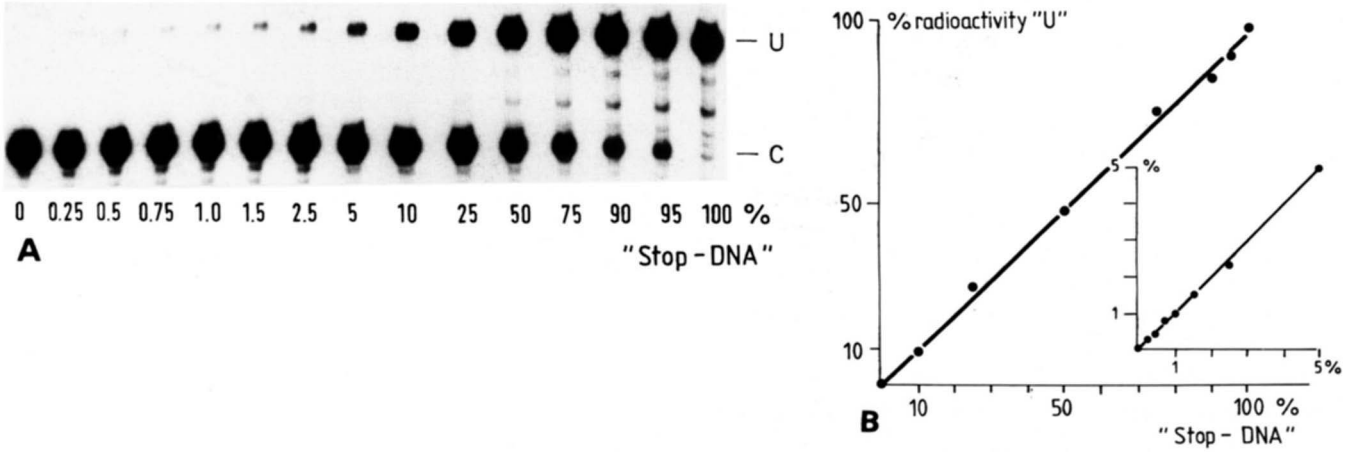

Fig. 1. Quantitative primer extension analysis of PCR-amplified apoB cDNA. Plasmid DNA of pDH30 containing apoB cDNA sequence with C at nucleotide position 6666 ("Go-DNA") and pDH31 containing the identical insert with T at nucleotide position 6666 ("Stop-DNA") were mixed at increasing ratios and amplified by PCR. PCR products were purified on Sephadex G 50 spin columns and analyzed by primer extension for C/T at nucleotide position 6666. The extension products were separated on a sequencing gel (A), excised from the gel, and quantitated by liquid scintillation counting. B: The percentage of radioactivity for extension of edited cDNA (U) was plotted against the percentage of input plasmid DNA containing the stop codon TAA ("Stop-DNA").

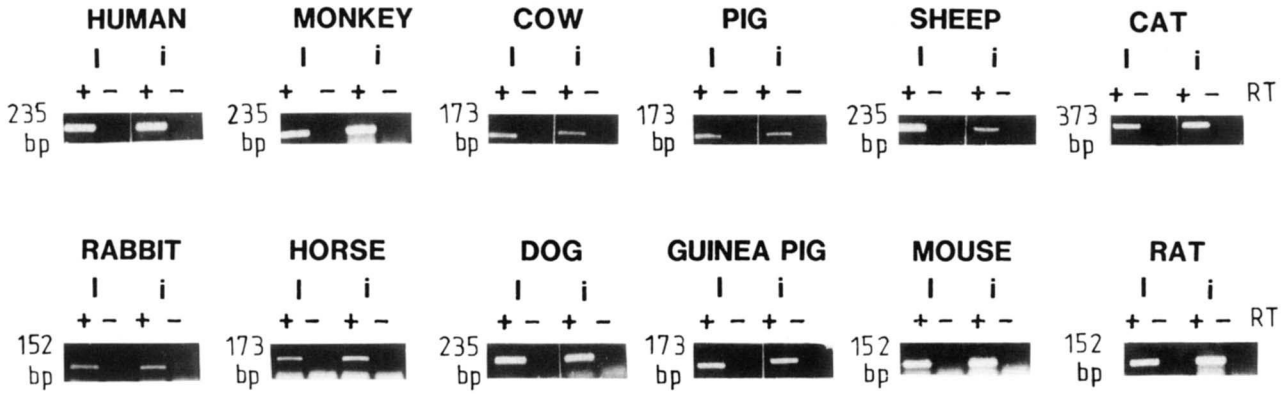

Fig. 2. PCR of apoB mRNA from liver and intestine of 12 mammalian species. Total RNA was isolated from liver (l) and intestine (i) by CsCl density gradient ultracentrifugation after homogenization in 5.7 M guanidinium isothiocyanate. After digestion with RNase-free DNase I, apoB cDNA was synthesized using as primer oligonucleotide 2 (human, monkey, sheep, dog, cow, pig, horse, guinea pig), oligonucleotide 5 (rat, mouse), oligonucleotide 6 (rabbit), or oligonucleotide 8 (cat). ApoB cDNA was amplified by PCR (25 cycles) using as upstream primer oligonucleotide 1 (human, monkey, sheep, dog), oligonucleotide 4 (rat, mouse, cow, pig, horse, guinea pig, rabbit), or oligonucleotide 7 (cat). The PCR products were separated on a 1.5% agarose gel which was stained with ethidium bromide. For each amplification of apoB cDNA (+) a separate control (−) was performed which was lacking the reverse transcriptase for cDNA synthesis. The lengths of the PCR products in base pairs are shown.

Additional editing in dog apoB mRNA at nucleotide position C_{6655}

Primer extension analysis of dog apoB mRNA resulted in incomplete termination at position C_{6655} for edited apoB mRNA generating a third extension product which terminated at position C_{6648} of dog apoB cDNA sequence (**Fig. 4**). This additional extension product was demonstrated in apoB mRNA from dog liver and dog intestine

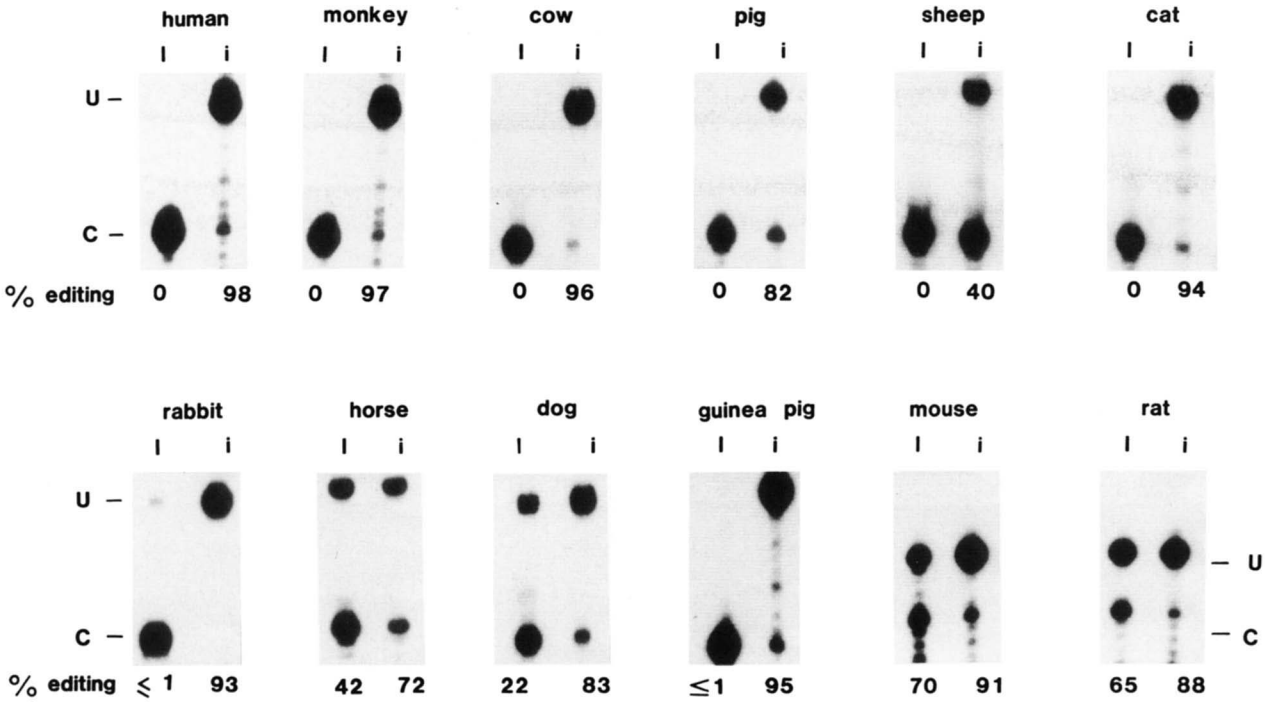

Fig. 3. Primer extension analysis for editing of apoB mRNA from liver and intestine of 12 mammalian species. PCR products were purified on Sephadex G 50 spin columns and analyzed by primer extension. Representative autoradiographs for primer extension analysis of PCR amplified apoB cDNA from liver (l) and intestine (i) of 12 different mammalian species are shown. For all species primer extension was performed with oligonucleotide 3 except for guinea pig for which oligonucleotide 14 was used. Extension products for unedited apoB cDNA (43 nucleotides) and edited apoB cDNA (54 nucleotides) were separated on a 8.5% polyacrylamide, 7 M urea sequencing gel, and exposed to X-ray film. For rat and mouse the extension products for edited apoB cDNA had a length of 48 nucleotides due to a T to C base change at nucleotide position 6661 as compared with the human apoB cDNA sequence. Extension products were excised and quantitated by liquid scintillation counting. The quantification for the demonstrated primer extension analysis is given.

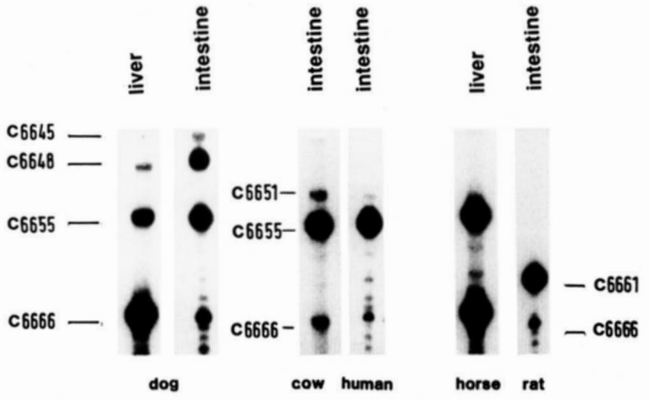

Fig. 4. Primer extension analysis of apoB mRNA from dog indicates additional editing at C_{6655}. PCR-amplified apoB cDNA from dog liver and intestine, cow and human intestine, horse liver, and rat intestine was analyzed by primer extension after two subsequent spin column chromatographies and an additional purification on glass beads (GeneClean®). Primer extension of apoB cDNA from dog liver and intestine resulted in incomplete termination at nucleotide position 6655 generating a third extension product of 61 nucleotides which ends at the next upstream C in the dog apoB cDNA sequence at nucleotide 6648. Primer extension analysis of apoB cDNA from cow intestine resulted in a third additional extension of 58 nucleotides which ends at the next upstream C in the cow apoB cDNA sequence at nucleotide position 6651. In contrast to cow and dog, primer extension of human, horse, or rat apoB gave no additional extension product or none that was clearly above a possible read-through level (human).

(Fig. 4). C_{6648} represents the next upstream C to position 6655 in the dog apoB cDNA sequence (**Table 1**). A similar, but less abundant, additional extension product which terminated at position C_{6651} was obtained by primer extension analysis of intestinal apoB from cow (Fig. 4). C_{6651} represents the next upstream C to position 6655 in human and cow apoB cDNA sequence (Table 1).

Primer extension analysis of apoB cDNA from human resulted in an additional extension product ending at C_{6651} that was not clearly distinct from possible background levels. Primer extension analysis from none of the other species investigated (horse and rat are shown as examples) resulted in additional extension products (Fig. 4). Further purification of the PCR products by additional spin column chromatography and DNA purification on glass beads (GeneClean®) did not diminish the intensity of these additional extension products. This excluded contamination with dGTP as a possible explanation. Therefore, the primer extension analysis indicated that beside C_{6666}, C_{6655} might also be edited to U in apoB mRNA from dog and possibly at much lower efficiency also from cow or even human.

In order to prove C/U editing at nucleotide position C_{6655} in dog apoB mRNA, the PCR products from dog liver and intestine were cloned in plasmid pT7T3 and individual recombinant clones were sequenced. Four out of 13 clones with intestinal apoB cDNA inserts contained T both at position 6666 and 6655, 6 clones had T at position 6666 and C at position 6655, and 3 clones were not edited and contained C both at position 6666 and 6655 (**Fig. 5**). Sequence analysis of 14 recombinant clones from dog liver demonstrated in 1 clone T both at position 6666 and 6655, in 1 clone T at position 6666 and C at position 6655 and in 12 clones C both at position 6666 and 6655. No clone had C at position 6666 and T at position 6655. Beside position 6666 and 6655 no other sequence heterogeneity was observed. Since all 15 cDNA clones from dog liver and intestine which were not edited at C_{6666} contained C at position 6655, allelic heterogeneity at nucleotide position 6655 in dog genomic DNA is highly unlikely. These results confirmed that *i)* apoB mRNA is edited to

TABLE 1. ApoB cDNA sequence between nucleotide position 6640–6720 from dog, cat, horse, cow, sheep, and guinea pig

ApoB cDNA Sequence from Nucleotide Position 6640 to 6720

```
                6640        50        60        70        80        90      6700        10        20
                 |         |         |         |         |         |         |         |         |
human:          TATCTCAACTGCAGACATATATGATAC*AATTTGATCAGTATATTAAAGATAGTTATGATTTACATGATTTGAAAATAGCTA
dog:            -G--------AA-A------G----------------------------A----------G------T--------AG
cat:            -------G--T---------G----------------------------A----------------------C-----
horse:          -------------A------G----------------------------A----------------T----C-CG--
cow:            -G-----G-----A------G----------------------------A---------G--------T--G-C-A---
sheep:          -G-----G------------G----------------------G----------------G-----------G-C-A--G
guinea pig:     -C-----G------------G----G-----------T-------T-A---A-------C---G----G------G-----
```

PCR-amplified apoB cDNA from dog, cat, horse, cow, sheep, and guinea pig, of which the apoB cDNA sequence around the editing site had not been established by previous investigations, were cloned into plasmid pT7T3 and sequenced. The nucleotide sequence of human apoB cDNA between position 6640–6720 is shown above and the nucleotide differences of the species are aligned beneath. The hyphens represent nucleotides identical to the human sequence. The editing position C_{6666} is indicated with an asterisk.

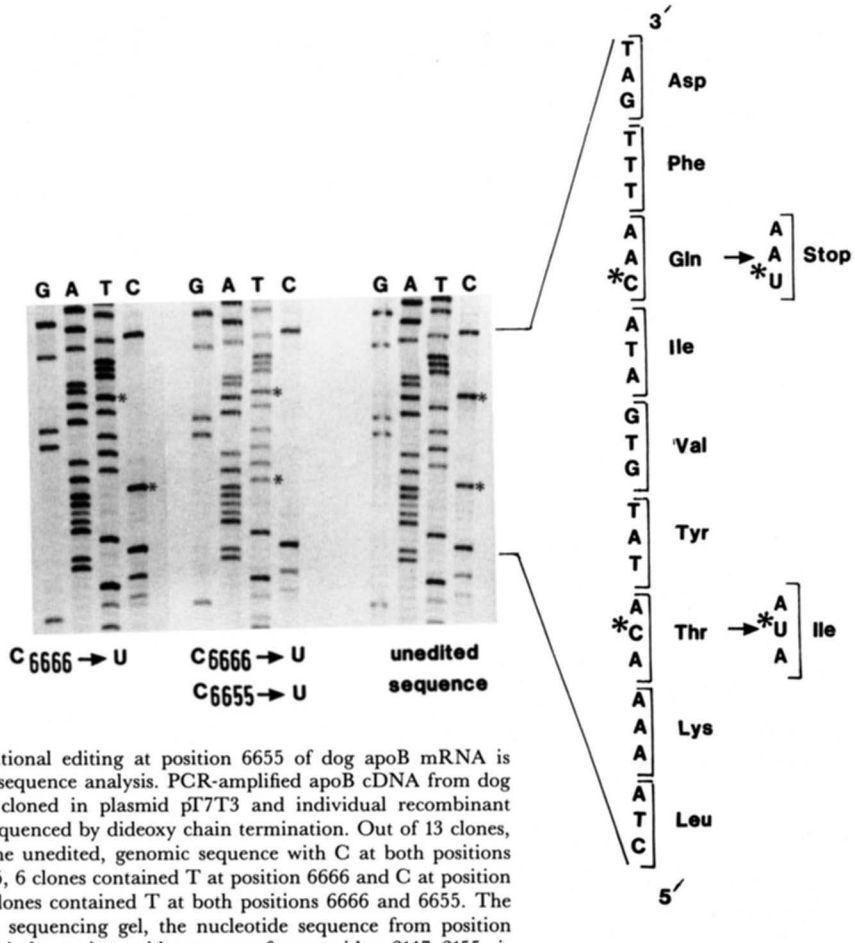

Fig. 5. Additional editing at position 6655 of dog apoB mRNA is confirmed by sequence analysis. PCR-amplified apoB cDNA from dog intestine was cloned in plasmid pT7T3 and individual recombinant clones were sequenced by dideoxy chain termination. Out of 13 clones, 3 contained the unedited, genomic sequence with C at both positions 6666 and 6655, 6 clones contained T at position 6666 and C at position 6655, and 4 clones contained T at both positions 6666 and 6655. The corresponding sequencing gel, the nucleotide sequence from position 6648–6674 and the amino acid sequence from residue 2147–2155, is shown. The edited bases are indicated by an asterisk.

about 80–85% in dog intestine and about 15–20% in dog liver; and *ii)* dog apoB mRNA is edited at C_{6655} in addition to position 6666. Editing at position 6655 is only observed in addition to editing at position 6666 and does not represent an alternative editing site. This additional editing site changes ACA for threonine at codon 2149 into AUA for isoleucine.

Cloning and sequencing of apoB cDNA from intestine and liver of horse, cat, guinea pig, cow, and sheep

In contrast to human, monkey, pig, rabbit, rat, and mouse for which the apoB cDNA sequence around the editing site has been established by previous investigations (12, 13, 26), apoB cDNA from dog, horse, guinea pig, cat, cow, and sheep has not been sequenced. Therefore, in addition to dog, the PCR products from intestine and liver of horse, guinea pig, cat, cow, and sheep were cloned in plasmid pT7T3 and sequenced. The apoB cDNA sequence of these six species and the human sequence from position 6640 to 6720 is shown in Table 1. Overall, there is a high degree of sequence homology in this region between these species. The region downstream of the editing position 6666, which contains the recognition motif for the apoB mRNA editing enzyme, is highly conserved between all species as has been noted previously (12, 26). This sequence homology allowed us to perform primer extension analysis with oligonucleotide 3 on apoB cDNA from all these different species. Only guinea pig contained several mismatches for oligonucleotide 3. Therefore, as has been stated in the Materials and Methods section, the primer extension assays for apoB cDNA of guinea pig were performed with oligonucleotide 14 which is completely complementary to the guinea pig sequence.

Five out of 12 clones from horse liver were edited and contained a T at position 6666 while the remaining 7 clones were not edited and had the genomically encoded C at position 6666. All 6 clones from horse intestine were edited and contained a T at position 6666. Besides C_{6666} no other sequence heterogeneity was found in this part of horse apoB. Therefore, cloning and sequencing confirmed 40% editing of apoB mRNA in horse liver as determined by primer extension in this particular animal. All 7 clones from cow liver were unedited and 9 out of 10 clones from

cow intestine were edited containing T at position 6666. No other heterogeneity was found in cow apoB, all clones from cow intestine contained the genomically encoded C at position 6655. In guinea pig 10 clones from liver were unedited and 10 clones from intestine were edited. Three out of 5 clones from cat intestine were edited and 2 were not edited. All 5 clones from cat liver were not edited. No heterogeneity apart from position 6666 was found in this part of cat apoB. Three clones from sheep intestine were edited and 3 were not edited while all 5 clones from sheep liver were not edited. Also in sheep apoB cDNA, no other sequence heterogeneity apart from C_{6666} was found.

Horse liver secretes apoB-48-containing lipoproteins

Secretion of apoB-48-containing lipoproteins has been described for rat liver (27, 28). In order to establish that editing of apoB mRNA in liver is of general physiological significance, it is necessary to demonstrate secretion of apoB-48-containing lipoproteins from liver of species other than rodents. Therefore, hepatocytes were isolated from horse liver by perfusion with collagenase, cultivated for 4 h and then pulse-labeled with [^{35}S]methionine in methionine-free, serum-free medium as described (20). ApoB was immunoprecipitated by a rabbit anti-human apoB polyclonal antibody or a goat anti-human apoB polyclonal antibody and analyzed by SDS-PAGE. Predominantly apoB-48 was immunoprecipitated from cell lysate as well as cell supernatant of freshly isolated horse hepatocytes (**Fig. 6**). ApoB-48 isolated from chylomicrons of rat mesenteric lymph duct (24) ran exactly at this position in our gel system. The apoB mRNA of these cells was edited to about 70% as measured by primer extension. However, when isolated horse hepatocytes were pulse-labeled after 72 h in culture, both apoB-100 and apoB-48 were immunoprecipitated from the cell lysate (Fig. 6). Concomitantly, editing of apoB mRNA in cultivated horse hepatocytes decreased to about 30–40%. This result, synthesis of predominantly apoB-48 from freshly isolated horse hepatocytes and of apoB-100 and apoB-48 from horse hepatocytes cultivated for 72 h, was confirmed in three independent experiments using liver fragments from three different horses. When the supernatant of pulse-labeled horse hepatocytes that had been cultivated for 72 h was subjected to buoyant density gradient ultracentrifugation, both apoB-100 and apoB-48 banded at a density of d 1.07–1.04 g/ml and to a less extent at d $<$ 1.02 g/ml (**Fig. 7**). When the supernatant of pulse-labeled, freshly isolated horse hepatocytes was subjected to density gradient ultracentrifugation, predominantly apoB-48 banded at the same density ranges. These results demonstrated that horse liver synthesizes apoB-48 and secretes it as lipoprotein particles.

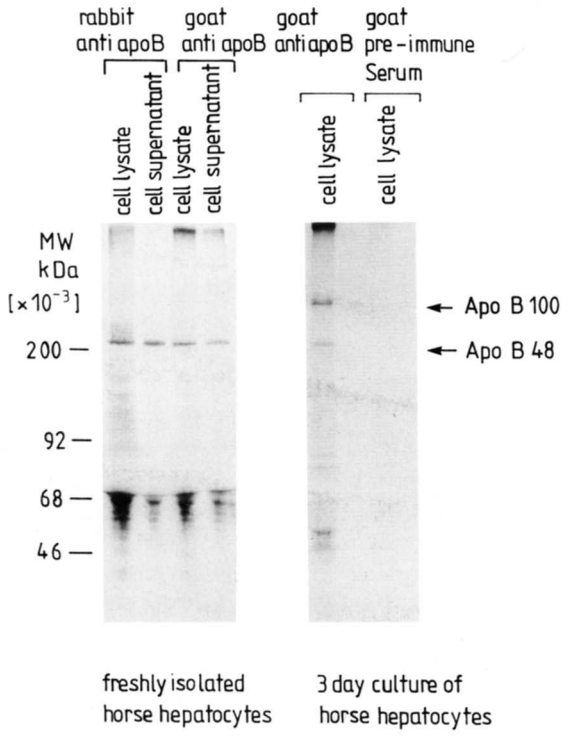

Fig. 6. Immunoprecipitation of apoB from cell lysate and cell supernatant of horse hepatocytes pulse-labeled with [^{35}S]methionine. Horse hepatocytes were isolated from horse liver by perfusion with collagenase, cultivated for 4 h or 72 h, and subsequently pulse-labeled with [^{35}S]methionine for 6 h. ApoB was immunoprecipitated from cell lysate and cell supernatant with a rabbit anti-human apoB polyclonal antibody or a goat anti-human apoB polyclonal antibody and analyzed on a 3–12.5% linear gradient SDS-PAGE.

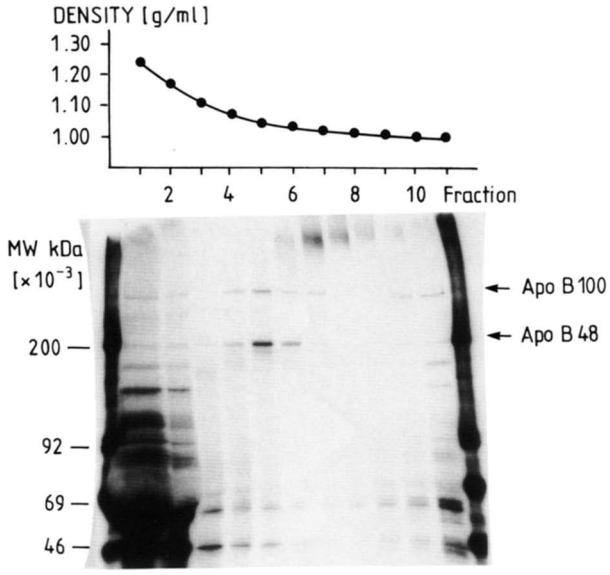

Fig. 7. Density gradient ultracentrifugation of supernatant from horse hepatocytes pulse-labeled with [^{35}S]methionine. Horse hepatocytes were isolated from horse liver by perfusion with collagenase, cultivated for 72 h, and pulse-labeled for 6 h with [^{35}S]methionine. Three ml supernatant was subjected to density gradient ultracentrifugation. The gradient was fractionated from the bottom and the density in each fraction was determined with a densitometer. The fractions were desalted by filtration on centricon 30 (Amicon) and analyzed on a 3–12.5% linear gradient SDS-PAGE.

TABLE 2. Plasma lipoprotein concentrations of 12 mammalian species

Species	Triglycerides	Total Cholesterol	VLDL Cholesterol	LDL Cholesterol	HDL Cholesterol
			mg/dl		
Human (n = 4)	110 ± 13.1	192 ± 11.5	12.5 ± 2.7	101 ± 14.1	59 ± 15.0
Monkey (n = 3)	90 ± 7.2	125 ± 11.1	7.0 ± 1.6	57 ± 1.5	70 ± 5.9
Pig (n = 3)	40 ± 4.7	115 ± 7.6	5.4 ± 0.7	58 ± 1.5	45 ± 1.3
Cow (n = 3)	60 ± 4.9	113 ± 16.9	3.4 ± 1.5	50 ± 9.7	51 ± 18.3
Sheep (n = 3)	20 ± 5.5	84 ± 2.1	8.8 ± 4.5	26 ± 1.3	53 ± 1.9
Cat (n = 3)	62 ± 4.0	204 ± 5.3	10.5 ± 5.4	44 ± 0.7	116 ± 7.0
Rabbit (n = 3)	29 ± 7.3	42 ± 7.5	7.2 ± 0.6	3.0 ± 1.4	32 ± 1.2
Guinea pig (n = 3)	52 ± 10.1	46 ± 8.7	5.6 ± 4.0	29 ± 4.0	2.8 ± 0.4
Dog (n = 3)	40 ± 6.2	228 ± 19.7	3.2 ± 1.4	43 ± 6.7	178 ± 15.0
Horse (n = 3)	25 ± 4.3	88 ± 10.5	2.2 ± 1.6	22 ± 4.3	55 ± 14.2
Rat (n = 3)	31 ± 5.4	101 ± 37.8	8.7 ± 1.4	18 ± 16.6	65 ± 23.3
Mouse (n = 3)	20 ± 3.9	92 ± 9.6	4.5 ± 1.3	15 ± 6.8	79 ± 11.4

Serum total cholesterol, triglycerides, and VLDL-, LDL-, and HDL-cholesterol were measured after separation by density gradient ultracentrifugation in at least three individual animals from each species. All values represent the mean ± SD.

Correlation of apoB mRNA editing with lipoprotein distribution in plasma

Cholesterol and triglyceride concentrations were measured in the serum of all animals whose liver and intestine were analyzed for apoB mRNA editing (**Table 2**). Lipoproteins were separated by density gradient ultracentrifugation and analyzed for cholesterol concentration (**Fig. 8** and Table 2). Well-separated peaks for HDL (d 1.08–1.2 g/ml), LDL (d 1.02–1.06 g/ml), and VLDL (d < 1.02 g/ml) were found in plasma of human, monkey, pig, sheep, rabbit, horse, dog, rat, and mouse (Fig. 8). In guinea pig serum HDL (d 1.08–1.2 g/ml) was not detected, but there were two typical peaks for LDL (d 1.02–1.06 g/ml) and VLDL (d < 1.02 g/ml) (Fig. 8). In

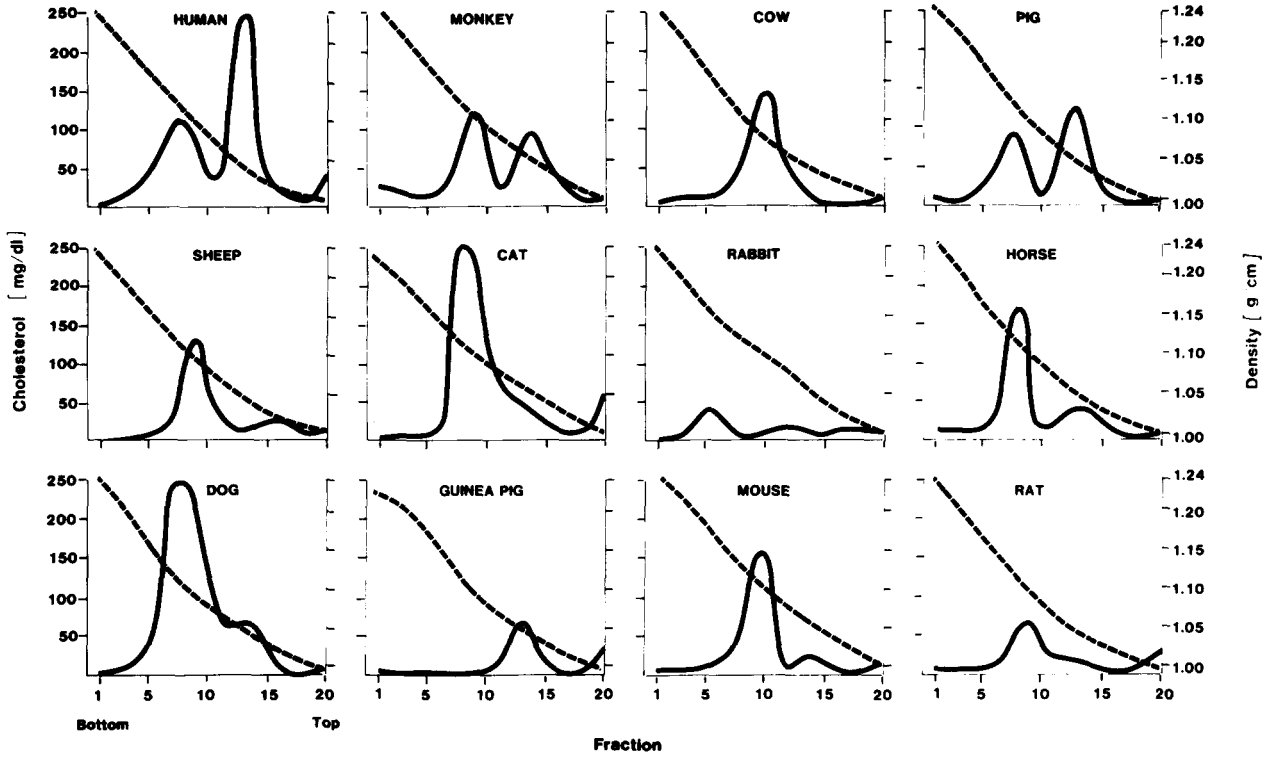

Fig. 8. Plasma lipoprotein distribution in 12 mammalian species. Serum (3 ml) of 12 different mammalian species was adjusted to a density of 1.21 g/ml with KBr, overlaid with 3 ml d 1.063 g/ml and further 3 ml of d 1.019 g/ml and centrifuged in a Beckmann SW 40 rotor at 40,000 rpm for 24 h at 16°C. Gradients were fractionated from the bottom into 20 fractions. The cholesterol concentration and density were measured in each fraction. The cholesterol and the density distribution of one representative ultracentrifugation analysis for each species are shown.

cow and cat the peaks for HDL and LDL appeared to overlap (Fig. 8). To investigate whether LDL are the major lipoproteins in the density range of 1.02–1.06 g/ml even in these two species, the protein composition of the density range 1.02–1.10 g/ml was analyzed by SDS-PAGE and direct staining or immunoblotting with a rabbit anti-human apoB polyclonal antibody or a goat anti-human apoB polyclonal antibody that crossreacted with apoB of these species. In cow and cat, apoB-100 was the predominant apolipoprotein the density range 1.02–1.06 g/ml (results not shown). Therefore, also in cow and cat, the lipoproteins in the density range 1.02–1.06 g/ml were defined as apoB-containing lipoproteins, even if no discrete peaks were separated by density gradient ultracentrifugation.

The amount of cholesterol in the density ranges 1.08–1.2, d 1.02–1.06, and d < 1.02 g/ml was integrated and expressed in terms of mg/dl as a measure for serum concentrations of HDL, LDL, and VLDL (plus some potential IDL), respectively. The results are given in detail in Table 2. As a measure for the proportion of apoB-containing lipoproteins, the ratio of (VLDL + LDL)/HDL was calculated (**Table 3**). The recovery of cholesterol in these three defined density ranges was between 90 and 100%. Only in cat was recovery slightly lower (83%) due to a substantial amount of cholesterol in the density range 1.06–1.08 g/ml which was excluded from the calculation. The highest concentration of LDL was observed in humans (101 mg/dl), followed by monkey (57 mg/dl), pig (58 mg/dl), and cow (50 mg/dl). Low concentrations of LDL were detected in rabbit (3 mg/dl), mouse (15 mg/dl), rat (18 mg/dl), horse (22 mg/dl), sheep (26 mg/dl), and guinea pig (29 mg/dl). The highest concentrations of HDL were found in cat (116 mg/dl) and dog (178 mg/dl), both of which had relatively low levels of LDL with 44 mg/dl and 43 mg/dl, respectively. HDL was not found in plasma of guinea pig. Apart from cat, dog, and guinea pig, HDL levels of all other species were in a similar range from 45 mg/dl (pig) to 79 mg/dl (mouse). Low ratios of (VLDL + LDL)/HDL were observed in dog (0.26), horse (0.44), cat (0.47), rabbit (0.32), rat (0.41), and mouse (0.25) (Table 3). High ratios of (VLDL + LDL)/HDL were found in human (1.92), monkey (0.91), pig (1.40), and cow (1.04). Guinea pig ex-

TABLE 3. ApoB mRNA editing in liver and intestine and the ratio of (VLDL + LDL)/HDL of 12 mammalian species

Species	ApoB mRNA Editing		(VLDL + HDL)/HDL
	Liver	Intestine	
	%		
Human (n = 4)	0 (0; 0; 0; 0)	98 (97; 97; 98; 99)	1.92
Monkey (n = 3)	0 (0; 0; 0)	97 (95; 97; 98)	0.91
Pig (n = 3)	0 (0; 0; 0)	82 (80; 82; 85)	1.40
Cow (n = 3)	0 (0; 0; 0)	95 (91; 96; 98)	1.04
Sheep (n = 3)	0 (0; 0; 0)	40 (38; 40; 43)	0.65
Cat (n = 4)	0 (0; 0; 0; 0)	84 (70; 78; 94; 95)	0.47
Rabbit (n = 3)	<1 (0.4; 0.4; 1.3)	90 (85; 93; 93)	0.32
Guinea pig (n = 3)	<1 (0.4; 0.7; 0.8)	87 (82; 84; 95)	12.3
Dog (n = 5)	18 (11; 14; 18; 22; 24)	84 (81; 83; 87)	0.26
Horse (n = 6)	43 (29, 35; 42; 44; 50; 57)	73 (62; 68; 72; 90)	0.44
Rat (n = 3)	62 (43; 65; 78)	88 (86; 88; 91)	0.41
Mouse (n = 3)	70 (61; 70; 80)	89 (84; 91; 93)	0.25

Editing of apoB mRNA in liver and intestine was measured by primer extension analysis of PCR-amplified apoB cDNA in at least three different individual animals. The mean and all individual data points are given. The ratio (VLDL + LDL)/HDL was calculated from the plasma lipoprotein concentrations in these 12 mammalian species as presented in Table 2.

hibited the highest ratio (12.3) due to the absence of HDL-cholesterol (Table 3). In sheep, low levels of LDL and relatively high concentrations of VLDL resulted in a ratio of 0.65. Overall, none of the species with high ratios (human, monkey, pig, cow) had editing of apoB mRNA in liver (Table 3). In contrast, four (dog, horse, rat, mouse) out of six species (dog, horse, rat, mouse, rabbit, cat) with a low ratio of less than 0.5 express apoB mRNA editing in liver (Table 3).

DISCUSSION

In the present investigation the expression of apoB mRNA editing in intestine and liver was studied in 12 different mammalian species and was correlated to the species-specific lipoprotein distribution in plasma. In all species intestinal apoB mRNA was found to be almost completely edited. In liver from dog, horse, rat, and mouse, high levels of apoB mRNA editing were demonstrated whereas low levels were found in liver from guinea pig and rabbit. In liver from human, monkey, pig, cow, sheep, and cat, apoB mRNA editing was not detected. Cloning and sequencing of the PCR products independently confirmed the results of the primer extension analysis. In dog apoB mRNA additional C/U editing at position 6655 was detected which changes the codon ACA for threonine at amino acid residue 2149 into AUA for isoleucine. Hepatic secretion of apoB-48-containing lipoproteins was demonstrated by [^{35}S]methionine pulse labeling of freshly isolated horse hepatocytes and immunoprecipitation or density gradient fractionation. Measurement of plasma lipoprotein concentrations indicated that species without apoB mRNA editing in liver have a higher proportion of apoB-containing lipoproteins as compared with those species that do express apoB mRNA editing in liver. Therefore, *i)* editing of apoB mRNA is not intestine-specific but a general modulation for apoB gene expression, and *ii)* editing of apoB mRNA in liver appears to be one important determinant for plasma concentrations of apoB-containing lipoproteins, especially of LDL.

Primer extension analysis for detection of apoB mRNA editing using PCR-amplified apoB cDNA proved to be a rapid and quantitative assay for measurement of apoB mRNA editing in vivo. PCR amplification enables quantification by liquid scintillation counting of the excised extension products. This circumvents gel scanning for quantification which leads to incorrect quantification when comparing extension products that differ markedly in intensity. Scanning with a densitometer is the most likely explanation why previously primer extension analysis of PCR-amplified apoB mRNA was reported to overestimate low levels of editing and to underestimate high levels of editing (29). By different combinations of oligonucleotides that were synthesized according to human, rat, or rabbit apoB cDNA sequence (12, 26) apoB mRNA from all species was amplified as demonstrated by PCR products of the expected sizes. The amplification was specific for apoB mRNA because no products were generated in control reactions without reverse transcriptase. Absence of genomic DNA that might have been coamplified was confirmed by Southern blotting of the PCR products and their respective controls and hybridization with radiolabeled oligonucleotide 3 which produced no hybridization signals for the controls.

In all species intestinal apoB mRNA was edited to high degrees. The lowest proportion of editing was found in intestine from sheep (40%). As for all other species, genomic DNA contaminating the PCR of sheep intestine was ruled out by the absence of products in the controls without reverse transcriptase, which was demonstrated by ethidium staining or hybridization with oligonucleotide 3. The reason for the relative low editing in sheep intestine and its physiological consequences remain unclear. Further, more detailed studies on lipoprotein metabolism in sheep appear to be required to address this issue. Overall, our findings are in good agreement with previous studies in human (10, 30), pig (12), and rat intestine (29, 30) where apoB mRNA was found to be edited between 80 and 90%. Since apoB mRNA editing occurs posttranscriptionally coincident with splicing and polyadenylation (31), the amount of editing may be slightly underestimated depending on the proportion of unedited apoB pre-mRNA in the preparation of total RNA. This assumption is corroborated by studies using cDNA libraries from human or rabbit intestine for determination of apoB mRNA editing where nearly 100% editing was detected (4, 5). Therefore, apoB-48 is the predominant product of the apoB gene in the intestine of all species investigated. Absence of interspecies variation indicates that editing of intestinal apoB mRNA has an important function that must be conserved. Since apoB-48 is the characteristic component of chylomicrons and chylomicron remnants with their rapid plasma turnover, editing of apoB mRNA generating apoB-48 may well be the essential constituent of this biochemical vena portae. This assumption is supported by the coordinated increase of apoB mRNA editing in the prenatal intestine during fetal development. Editing of apoB mRNA reaches adult levels just before birth as demonstrated for fetal intestine from human (10, 30), pig (12), and rat (29, 30). Thus, editing of apoB mRNA appears to be required for the metabolism of dietary lipids.

In contrast to the intestine, great variation was observed in the hepatic expression of apoB mRNA editing. No editing was detected in liver of human, monkey, pig, cow, sheep, and cat. These findings are in agreement with previous determinations of apoB mRNA editing in human (4, 5), pig (12), or monkey liver (13), where also no editing activity was found. The sensitivity is limited by

the primer extension analysis that can detect editing at a level of approximately 0.1%. Editing of apoB mRNA in human liver has been detected by isolation of 4 clones from a human liver cDNA library that were encoding TAA at amino acid residue 2153 (32). This investigation, however, was not quantitative, and the present investigation indicates that in human liver the amount of editing for apoB mRNA is below 1 in 1000. Our results, however, cannot rule out that editing occurs in liver of these species at levels below this detection limit. Moreover, apoB mRNA editing was identified at very low levels in liver from rabbit (< 1%) and guinea pig (< 1%) and at high levels in dog (18%), horse (43%), rat (62%), and mouse (70%). As detection of apoB mRNA editing in total RNA may lead to an underestimation, as stated above, the extent of edited mature apoB mRNA in the liver of these species may be even higher. Previously, editing of apoB mRNA has been demonstrated in rat (9, 29) and mouse liver (33). Our results demonstrate that hepatic editing is a more widespread phenomenon and, in addition to rat and mouse, high expression of apoB mRNA editing is found in the liver of species so diverse as dog and horse.

To confirm the results of the primer extension analysis for species of which the apoB cDNA sequence has not been established by previous investigations, the PCR products of dog, horse, cat, cow, guinea pig, and sheep were cloned and sequenced. In dog, horse, cat, cow, and sheep the sequence around the editing site 6666 is highly conserved in comparison to the human sequence, as has been demonstrated previously for monkey, pig, rabbit, rat, and mouse (12, 13, 26). Thus, the high degree of sequence homology around the editing site enables primer extension analysis for editing of apoB mRNA using oligonucleotide 3 in all these different species. Only for detection of apoB mRNA editing in guinea pig was a separate oligonucleotide complementary to the guinea pig sequence required due to several base changes as compared to the human apoB cDNA sequence. Overall, the sequence analysis independently confirmed the levels of editing as measured by primer extension.

In dog apoB mRNA the C at position 6655 is edited to U in addition to editing at C_{6666}. This changes ACA for threonine at amino acid residue 2149 into AUA for isoleucine. Editing at C_{6655} in addition to C_{6666} was indicated by the primer extension analysis which generated an additional extension product. It was subsequently confirmed by cloning and sequencing PCR products from dog liver and intestine. Editing at position 6655 was only observed in addition to editing at position 6666. Editing at C_{6655} does not represent an alternative editing site. None out of 15 cDNA clones that contained the genomically encoded C at position 6666 had a T at position 6655. Furthermore, this result de facto excluded allele heterogeneity at position 6655. Similarly, additional C/U editing at nucleotide position 6802 has previously been reported in the apoB mRNA from human intestine where ACA at amino acid residue 2198 is also edited to AUA only in addition to editing at position 6666 (34). However, the additional editing site in dog apoB is the first to be located upstream of position 6666. In contrast to position 6802 which is in the untranslated region of edited apoB mRNA, editing at position 6655 leads to an exchange of threonine into isoleucine. Cloning PCR products from cow intestine and sequencing ten recombinant clones failed to confirm C/U editing at position 6655 in cow apoB mRNA. However, for detection of editing at position 6655 with a very low frequency, 10 clones do not appear to be enough. Yet, the data from the primer extension analysis of apoB from cow intestine are indicative for additional editing at 6655 in cow as well, but cannot serve as a formal proof. Overall, the existence of two additional editing sites in the apoB mRNA, C_{6655} and C_{6802}, indicates that the specificity of the apoB mRNA editing enzyme for position 6666 is not as high as previously thought.

Primary horse hepatocytes synthesized and secreted predominantly apoB-48. The apoB mRNA of these cells was edited to about 70%, higher than determined in horse liver in vivo. ApoB mRNA editing appears to be induced in freshly isolated horse hepatocytes, possibly by the cultivation procedure. After 3 days in culture, horse hepatocytes synthesized and secreted both apoB-48 and apoB-100. Concomitantly, apoB mRNA editing decreased to about 30–40%. From these results, which were independently confirmed in three different preparations of horse hepatocytes, it is difficult to deduce to which proportion horse liver secretes apoB-48-containing lipoproteins in vivo. The results may indicate that a higher proportion of apoB-48 is produced by the liver than estimated by measurement of apoB mRNA editing. In the density gradient ultracentrifugation both apoB-48 and apoB-100 banded predominantly at d 1.06–1.04 g/ml and to a less extent at d < 1.02 g/ml. Accordingly, it has been previously demonstrated that both apoB-48 and apoB-100 are secreted from the rat hepatoma cell line McArdle-RH7777 not only in the density range d < 1.02 but also at higher densities (22, 35). The secretion of apoB-containing lipoproteins from primary horse hepatocytes in the density range of 1.06–1.04 g/ml, that of low density lipoproteins, is most likely explained by the culture conditions in the pulse period which leads to starvation of the cells and consequently to the secretion of lipoproteins less enriched in lipids. Taken together, this series of experiments clearly established that horse liver secretes apoB-48-containing lipoproteins.

As apoB-48 has an accelerated plasma turnover, demonstrated in many previous investigations (1, 3, 36), apoB-48-containing VLDL from liver may also be more rapidly metabolized. This assumption is strongly supported by recent findings in familial hypobetalipoproteinemia. In this recessive genetic disorder, point mutations in one allele of the apoB gene generate non-sense or translational stop codons leading to carboxy terminal

truncated isoforms of apoB such as apoB-89, apoB-67, apoB-61, apoB-54, apoB-46, apoB-37 or apoB-31 (37–43). These genetically encoded isoforms of apoB are competent to form buoyant lipoproteins (22) which are more rapidly cleared from human plasma thereby causing hypobetalipoproteinemia (37, 44–46). Recessive hypobetalipoproteinemia has no adverse consequences but on the contrary was reported to be associated with longevity (47). Recently, a patient with homozygous hypobetalipoproteinemia was described who has a C- to T-nucleotide substitution at codon 2252 in both alleles of apoB generating a premature translational stop codon (48). In this patient exclusively, the truncated apoB-50 is produced which confers normal metabolism of chylomicrons and VLDL but causes a complete lack of LDL (48). Thus, editing of apoB mRNA in liver which mimics this genomic mutation on a posttranscriptional level should also lead to low plasma levels of LDL. Therefore, lipoprotein concentrations in plasma were determined after separation by density gradient ultracentrifugation, which proved to be the best method for comparing lipoproteins from different species. The concentrations of lipoproteins in the 12 mammalian species as determined in this study are in accordance with previous reports on lipoprotein concentrations in monkey (49, 50), cow (49, 51, 52), pig (49, 52, 53), sheep (49), cat (49), rabbit (49, 52), horse (51, 52), dog (49, 52, 54), guinea pig (49, 55, 56), mouse (49, 57, 58), and rat (49, 59). Three distinct peaks for HDL, LDL, and VLDL were observed in human, monkey, pig, sheep, rabbit, horse, dog, rat, and mouse and, therefore, in these species apoB-containing VLDL and LDL are clearly discriminated from HDL. In guinea pig no HDL was detected as has been reported previously (49, 55, 56). In cow, HDL and LDL appear to overlap resulting in a single peak with a density of 1.03–1.13 g/ml as previously reported (49, 51, 52). Analysis by SDS-PAGE and direct staining or immunoblotting demonstrated apoB-100 as the major apolipoprotein in the density range 1.02–1.06 g/ml within this single peak. The determination for bovine HDL and LDL as made in this investigation is supported by previous studies that found only little cross-contamination with a light form of HDL in the density range 1.02–1.05 g/ml (49, 51, 52). In cat a separate shoulder at the end of the predominant HDL peak represents LDL which is in accordance with previous findings (49).

As a measure for the proportion of apoB-containing lipoproteins, the ratio of (VLDL + LDL) to HDL was calculated. Overall, a continuous distribution of this ratio without clustering was found amongst these 12 species. None of the species with a high ratio expresses apoB mRNA editing in liver. Four (dog, horse, rat and mouse) out of 6 species (cat, rabbit, dog, horse, rat, and mouse) with low ratios of below 0.5 were found to express high levels of apoB mRNA editing in liver. However, hepatic expression of apoB mRNA editing is not absolutely required for low levels of apoB-containing lipoproteins, as 3 species, sheep, cat, and rabbit, have a low proportion of apoB-containing lipoproteins although they express little or no apoB mRNA editing in liver. Many other genetic and environmental factors apart from editing of apoB mRNA influence plasma lipoprotein levels. Nevertheless, higher levels of apoB-containing lipoproteins are found in species without editing of hepatic apoB mRNA as compared with species that do express editing activity in liver. This provides some evidence that apoB mRNA editing in liver limits the concentration of apoB-containing lipoproteins in plasma and, vice versa, suppression of editing in liver is a determinant for relative higher concentrations. However, the present investigation is no formal proof for this hypothesis. An interspecies comparison has several limitations. Many factors known to influence lipoprotein metabolism such as age, sex, diet, and hormonal status as well as genetic heterogeneity cannot be controlled for and may interfere in this kind of study. The assumption, however, that editing of apoB mRNA has a regulatory function in lipoprotein metabolism is supported by studies in rat where starvation causes down-regulation and refeeding causes up-regulation of apoB mRNA editing in liver (11, 28, 60). During starvation rat liver, therefore, produces lipoproteins with a slower turnover as compared to periods where abundant lipids have to be metabolized. Whether similar regulations can also be observed in liver from mouse, dog, or even horse remains to be investigated.

In conclusion, our results demonstrate apoB mRNA editing as a general and not a tissue-specific modulation of apoB gene expression which determines the metabolism and subsequently the concentration of apoB-containing lipoproteins. Elucidation of the molecular basis for the tissue-specific expression of apoB mRNA editing appears to be important. Understanding of this regulation may open up possibilities to influence editing of apoB mRNA with potential new perspectives for the treatment of hyperlipidemia and atherosclerosis.

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