

# C→U editing of apolipoprotein B mRNA in marsupials: identification and characterisation of APOBEC-1 from the American opossum *Monodelphus domestica*

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## ABSTRACT

The C→U editing of RNA is widely found in plant and animal species. In mammals it is a discrete process confined to the editing of apolipoprotein B (apoB) mRNA in eutherians and the editing of the mitochondrial tRNA for glycine in marsupials. Here we have identified and characterised apoB mRNA editing in the American opossum *Monodelphus domestica*. The apoB mRNA editing site is highly conserved in the opossum and undergoes complete editing in the small intestine, but not in the liver or other tissues. Opossum APOBEC-1 cDNA was cloned, sequenced and expressed. The encoded protein is similar to APOBEC-1 of eutherians. Motifs previously identified as involved in zinc binding, RNA binding and catalysis, nuclear localisation and a C-terminal leucine-rich domain are all conserved. Opossum APOBEC-1 contains a seven amino acid C-terminal extension also found in humans and rabbits, but not present in rodents. The opossum APOBEC-1 gene has the same intron/exon organisation in the coding sequence as the eutherian gene. Northern blot and RT-PCR analyses and an editing assay indicate that no APOBEC-1 was expressed in the liver. Thus the far upstream promoter responsible for hepatic expression in rodents does not operate in the opossum. An APOBEC-1-like enzyme such as might be involved in C→U RNA editing of tRNA in marsupial mitochondria was not demonstrated. The activity of opossum APOBEC-1 in the presence of both chicken and rodent auxiliary editing proteins was comparable to that of other mammals. These studies extend the origins of APOBEC-1 back 170 000 000 years to marsupials and help bridge the gap in the origins of this RNA editing process between birds and eutherian mammals.

## INTRODUCTION

RNA in a variety of organisms undergoes C→U editing (1,2). In the mitochondria and to a lesser extent the chloroplasts of plants, protein coding mRNAs are extensively edited (3–5). This RNA editing corrects sequence and structural anomalies that would otherwise prevent protein translation or RNA processing. Mitochondrial transcripts in the slime mold *Physarum polycephalum* also undergo C→U RNA editing (6). More restricted forms of C→U RNA editing alter the tRNA for glycine (tRNA<sup>Gly</sup>) in the mitochondria of marsupials to aspartate tRNA (tRNA<sup>Asp</sup>) (7,8). In eutherian (placental) mammals the nuclear transcript for apolipoprotein (apo)B mRNA also undergoes RNA editing (9,10). This editing converts a glutamine codon (CAA) to a stop codon (UAA). It truncates the reading frame of the genomically encoded protein giving rise to two proteins of different size and function in lipid transport and metabolism. They are designated apoB100 and apoB48 for the large genomically encoded form and for the small form generated from the edited mRNA, respectively.

Some eutherian mammals also make apoB48 in the liver (11–13). Hepatic editing of apoB mRNA is under metabolic control so that increased synthesis of apoB48 occurs under conditions where there is increased lipogenesis and the need for triglyceride export from the liver.

In species such as birds that do not edit apoB mRNA (12) dietary lipid is secreted from the intestine as triglyceride-rich apoB100-containing particles, called portamicros, into the portal vein, which drains intestinal lipid directly into the liver. The evolutionary origin of apoB mRNA editing appears to coincide with the origin of apoE and the development of the intestinal lymphatics, which drain intestinal lymph by the thoracic duct into the systemic circulation. The editing of apoB mRNA in mammals provides a mechanism for the rapid clearance of lipids of intestinal origin analogous to the delivery of portamicros into the portal vein in birds. After the circulation of chylomicrons to the periphery and the formation of chylomicron remnants these remnants rapidly circulate back to the liver and are cleared. It is not known at what stage of evolution between birds and eutherian mammals apoB mRNA editing arose.

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The apoB mRNA editing enzyme APOBEC-1 (14) is a site-specific cytidine deaminase (15–19). The cytidine deaminase family include the *Escherichia coli* cytidine deaminase (ECCDA). The crystal structure of ECCDA has been established (20). Similarities in amino acids sequence and the spacing of the residues that form the active site in conjunction with molecular modelling and mutagenesis have shown that APOBEC-1 is related in quaternary and tertiary structure to ECCDA (21). Both enzymes form a homodimer with composite active sites constructed with contributions from each monomer (20,22). Significant gaps are present in the APOBEC-1 sequence, compared to ECCDA (21). The location of the gaps in ECCDA suggest how APOBEC-1 has been reshaped to accommodate an RNA substrate.

The evident similarity in tertiary structure and domain organisation between ECCDA and APOBEC-1 suggest the possibility that APOBEC-1 may have arisen in bacteria and come forward, giving rise to C→U RNA editing in plant mitochondria and chloroplasts and marsupial mitochondria as well as apoB mRNA editing. In the present study we set out to establish whether the editing of apoB mRNA is present in marsupials and hence close the gap between birds and eutherian mammals in order to come closer to the evolutionary origin of this phenomenon and to determine whether the editing of tRNA in marsupial mitochondria is mediated by APOBEC-1 or an enzyme encoded by a gene closely related in nucleotide sequence to APOBEC-1.

We find that marsupial APOBEC-1 is closely related to eutherian APOBEC-1, but with features of the gene that place it closer in structure to human and rabbit APOBEC-1 than to the rodent enzyme. Like human and rabbit, the opossum APOBEC-1 gene is not expressed in the liver. Thus, the rodent far upstream promoter that is expressed in the liver may have been a later evolutionary event. Also, a short C-terminal extension is found in humans, rabbits (23) and opossum, but not in rodents. We have found no evidence for the involvement of an enzyme closely related to APOBEC-1 in the editing of marsupial mitochondrial tRNA.

## MATERIALS AND METHODS

### cDNA cloning of opossum apoB and APOBEC-1 mRNAs

Total RNA was isolated from the small intestine of the American opossum *Monodelphis domestica* by the guanidium thiocyanate/CsCl method and poly(A)<sup>+</sup> RNA purified from the total RNA using oligo(dT)-latex (Qiagen) according to the manufacturer's instructions. An aliquot of 2.5 µg of poly(A)<sup>+</sup> was subjected to first strand synthesis by Superscript II reverse transcriptase (Life Technologies Inc.) with an oligo(dT)<sub>24</sub>-*NotI* primer adapter (Stratagene). The replacement synthesis of second strand cDNA was performed as described (24). The resulting double-stranded cDNA was blunt ended by T4 DNA polymerase and digested with the restriction enzyme *NotI*. The cDNA was size fractionated by 5–20% (w/v) potassium acetate density gradient centrifugation. The cDNA from each fraction was ligated to a BlueScript vector (Stratagene) digested with *SmaI* and *NotI* and transformed into *Escherichia coli* DH5α by electroporation. The resulting cDNA sublibraries were screened with <sup>32</sup>P-labelled APOBEC-1 or apoB cDNA as probe under

the low stringency conditions described below. Replica filters were hybridised in 25% formamide, 6× SSPE (1× SSPE is 10 mM sodium phosphate, pH 7.4, 180 mM NaCl, 1 mM EDTA), 5× Denhart's, 1% SDS, 100 µg/ml denatured salmon sperm DNA and 1 × 10<sup>6</sup> c.p.m./ml labelled probe at 42°C for 16 h. After hybridisation filters were washed with 2× SSC and 0.1% SDS at room temperature for 15 min and three times at 55°C for 30 min. The signals were detected by autoradiography.

With rat apoB as the probe 30 positive signals were obtained by screening a sublibrary which contains inserts >5.0 kb in length. Four positive clones were finally isolated and analysed by restriction enzyme digestion and sequencing. The longest apoB clone had a 5.8 kb insert. It was sequenced on both strands. The sequence surrounding the apoB mRNA editing site is shown. The entire sequence is available on the DDBJ/EMBL/GenBank database (accession no. AB027413).

With rat APOBEC-1 cDNA as the probe for screening, eight positive clones were obtained from a sublibrary. This had inserts of between 0.8 and 2.3 kb. The cDNA clones were characterised by cleavage with various restriction enzymes and sequenced on both strands. The sequence revealed two different kinds of cDNA, one of which encoded a protein highly homologous to APOBEC-1 and another of a protein lacking 45 amino acids at the C-terminus of the protein.

### Characterisation of opossum apoB mRNA editing site

Opossum genomic DNA was isolated from the liver cells as described (24). Aliquots of 5 g of fresh liver were minced and homogenised with a glass homogeniser (Wheaton) in 10 ml of phosphate-buffered saline. Crude nuclei were sedimented by centrifugation at 1000 g at 4°C for 5 min. The pellet was dissolved in 10 ml of PK buffer (0.1 M Tris-HCl, pH 7.5, 12.5 mM EDTA, 0.15 M NaCl, 1% SDS). After incubation with proteinase K (20 mg/ml), genomic DNA was extracted with phenol/chloroform and precipitated with ethanol. The opossum liver and small intestine poly(A)<sup>+</sup> RNA were subjected to reverse transcription with Superscript II reverse transcriptase and random hexamers according to the manufacturer's protocol. Single-stranded cDNA and genomic DNA was amplified with a pair of primers flanking the opossum apoB mRNA editing site, OP/APOB-OUF (5'-GTTGTAAATGTGAAGGAGAGCC-3') and OP/APOB-OUR (5'-GGTTGATTAGTTTTGTGATTGATTC-3'), by 30 cycles (denaturation at 94°C for 30 s, annealing at 62°C, 1 min, extension at 72°C, 1 min). The PCR products were analysed by agarose gel electrophoresis to ensure the size of the products and absence of amplification products. About 100 ng of PCR products were reamplified in 50 µl of a standard reaction mixture with 0.01 µM internal primer, OP/ApoB-INF (5'-TAATGCCAAAATCAGCCTC-3') and 1 µM OP/ApoB-OUR for 40 cycles (denaturation at 94°C for 30 s, annealing at 58°C for 1 min, extension 72°C for 1 min). The single-stranded PCR products were purified by ultrafiltration using Centricon 100 (Amicon) spin columns to remove free nucleotides and primers according to the manufacturer's instructions and concentrated to 10 µl. Sequencing reactions were carried out using the Sequenase v.2.0 sequencing kit (Amersham) with end-labelled primer OP/APOB-INF.

### Northern blot analysis

Total RNA was prepared from various tissues of an adult opossum as described before. Aliquots of 20 µg of total RNA from each tissue or 5 mg of poly(A)<sup>+</sup> RNA were fractionated on a 1.5% MOPS-formaldehyde gel for APOBEC-1 and 0.8% for apoB and transferred to nylon membranes. The signals were detected under the high stringency conditions described below. The membranes were hybridised with <sup>32</sup>P-labelled opossum APOBEC-1 or apoB cDNA in a solution containing 6× SSPE, 5× Denhardt's solution, 1% SDS, 50% formamide and 100 µg/ml denatured salmon sperm DNA at 42°C for 16 h. The filter was washed once with 2× SSC and 0.1% SDS at room temperature for 15 min and twice with 0.2× SSC and 0.1% SDS at 65°C for 30 min and then exposed to an X-ray film, Kodak XAR-5, at 70°C for 16 h to 1 week.

### Characterisation of opossum APOBEC-1 gene

Genomic DNA (40 µg) was digested with *Eco*RI and fractionated on a 0.6% agarose gel. The DNA fragment between 4.3 and 6.3 kb was excised from the gel and cloned into a pUC vector to construct a genomic library. About 4.0 × 10<sup>5</sup> recombinant clones from the library were screened with <sup>32</sup>P-labelled oligonucleotides corresponding to various regions of the opossum APOBEC-1 cDNA. DNA fragments generated by restricted digestion from the genomic DNA were subcloned into a BlueScript vector for sequencing. All exon/intron junctions except the exon encoding the 3'-untranslated region and first part of the coding sequence, which is not included in the genomic clone, were sequenced on both strands.

### Construction of a mutant APOBEC-1 cDNA

The opossum APOBEC-1 fusion protein with 45 amino acids from the C-terminus of the rat enzyme was constructed by a PCR procedure. Primers Op1-5' (5'-AGAACATATGAATTC-TAAGACAGGTCCATC-3') and Op574-3' (5'-ACTCCCTA-GGATTATGCAGTGGAGTTCCAGG-3') were used to amplify a 571 bp fragment with opossum cDNA as template. The PCR fragment was digested with *Nde*I and *Bln*I. Another pair of primers (5'-ATCATCCTAGGACTTCCACCTGTTTAA-3') and P27BAM3' (5'-GGCCAGGATCCTCATTTC AACCT-GTGGC-3') were used for PCR amplification with rat APOBEC-1 cDNA, followed by restriction enzyme digestion with *Bam*HI and *Bln*I. The resulting fragments were ligated into pET vector digested with *Nde*I and *Bam*HI. All the PCR-derived fragments were sequenced to exclude PCR artefacts.

### In vitro apoB RNA editing assay

APOBEC-1 and mutant cDNAs under the control of the T7 promoter were expressed in a 50 µl reaction using the TNT T7 Quick Coupled Transcription/Translation System (Promega) with [<sup>35</sup>S]methionine (Amersham) as recommended by the manufacturer. The *in vitro* translation products were separated on a 12.5% SDS-polyacrylamide gel and detected by fluorography. Aliquots of the translation lysate and a lysate of Sf9 cells expressing rat APOBEC-1 were incubated in an *in vitro* editing assay reaction with either human, opossum or rat synthetic apoB RNA in the presence of 20 µg chicken small intestine S100 extract. Following the *in vitro* editing assay (25,26), the RNAs were analysed by primer extension as described previously.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs isolated from opossum liver, kidney and small intestine RNA were treated with 1 U/µg RNA of RNase-free DNase I. The RNAs were subjected to RT-PCR as described above, with specific primers either for APOBEC-1, OP/BEC-F1 (5'-TTAACCCCTCAAGAACTCCGTAA-3') and OP/BEC-R1 (5'-GTAATCTTCTTCCCCTTGTGG-3'), or apoB, OP/APOB-OUF and OP/APOB-OUR (5'-AATCCAGACCACAGCCCTACT-3'). The thermal profile used was 33 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 30 s. The amplified fragments were analysed on a 3% agarose gel.

### 3' Rapid amplification of cDNA ends (3'-RACE)

3'-RACE was conducted using a 3'-RACE kit (Life Technologies Inc). One microgram of total RNAs was reverse transcribed using Superscript II reverse transcriptase and 250 ng of oligo(dT) primer DT2 (5'-GGCCACGCGTGCAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTV-3'). First strand cDNA was amplified with the anchor primer and the apoB primer OP/APOB-OUF by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 1 min. The PCR products were analysed on a 3% agarose gel.

## RESULTS

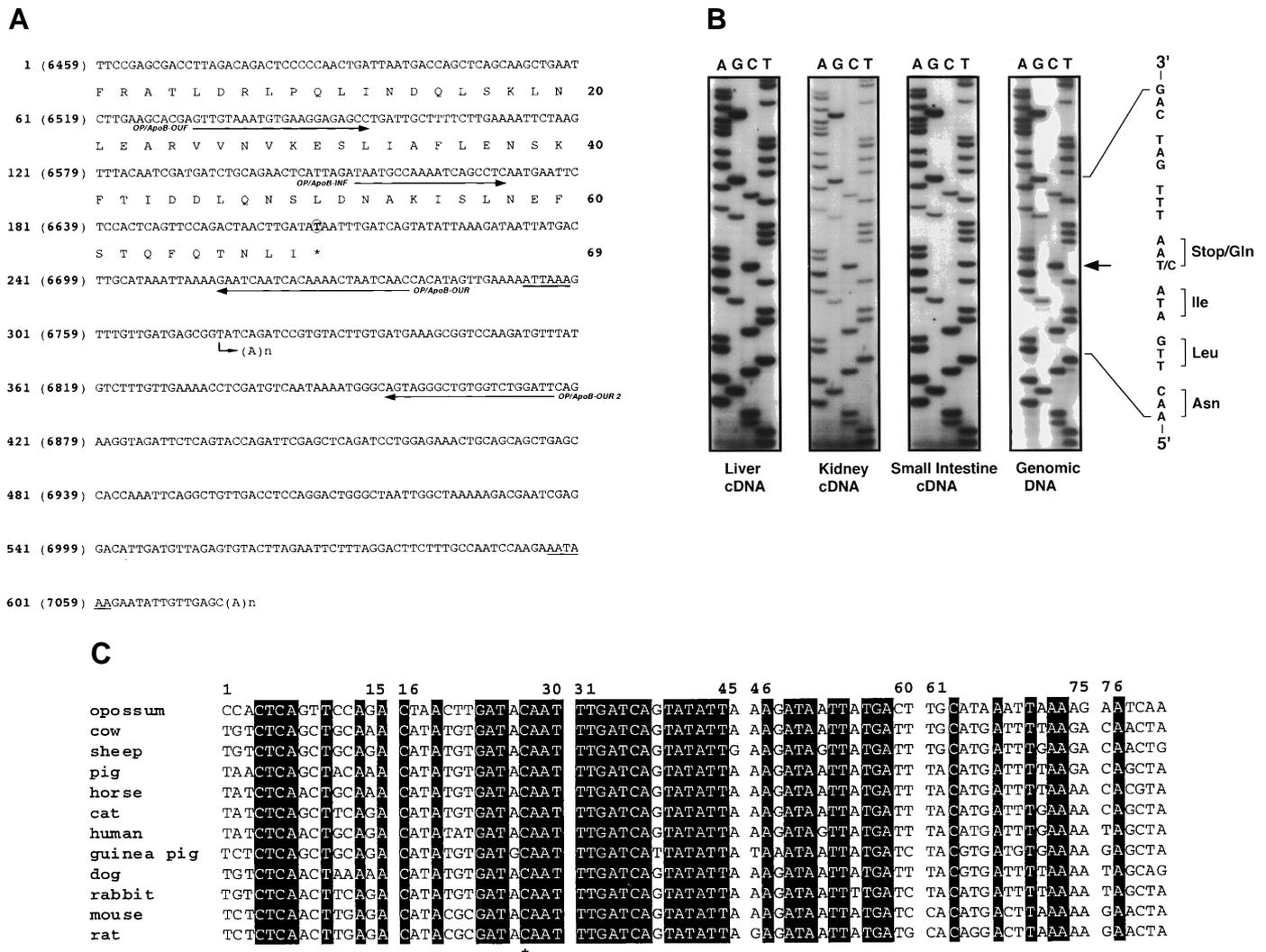
### Marsupial apoB mRNA is edited

To evaluate marsupials for the editing of apoB mRNA we prepared and screened a cDNA library from the intestine of the American opossum, *Monodelphis domestica*. The opossum small intestinal cDNA library was screened at low stringency with a rat apoB probe incorporating the mRNA editing site. Four positive clones were identified. Analysis of these clones by restriction enzyme mapping and partial sequencing showed that all were derived from the apoB mRNA and overlapped each other. The longest cDNA (5.7 kb) corresponded to the human apoB mRNA between nucleotide positions 1356 and 7075, which includes the RNA editing site at position 6666. The four cDNAs had a stop codon at position 6666, a poly(A) tail 406 bp downstream of the stop codon and a polyadenylation signal 15 bp upstream of the poly(A) addition site (Fig. 1A and B). The cDNA clones presumably represent selective priming from this poly(A) tail.

The authenticity of the RNA editing in the opossum mRNA was verified by sequencing PCR-amplified genomic DNA. Direct sequencing of the PCR product showed that the genomic DNA encoded the unedited glutamine codon (CAA) corresponding to the stop codon (TAA) in the isolated cDNAs (Fig. 1B). No other sequence differences between the genomic DNA and the cDNAs, such as the alternative editing sites previously identified in human and dog, were found in the sequenced region.

To examine whether apoB mRNA is edited in the opossum liver, as is found in rat, mouse, horse and dog liver, cDNA from the opossum was amplified by PCR and directly sequenced alongside the opossum small intestinal RT-PCR product. The PCR primers were chosen to be internal to the polyadenylation signal at positions 6753–6758. The results demonstrate that the majority of the opossum small intestinal mRNA is edited, but liver and kidney mRNA are not (Fig. 1B).

Sequence alignment of the opossum apoB cDNA with that of human and other species is shown in Figure 1C. Overall, the



**Figure 1.** ApoB mRNA is edited in opossum small intestine. (A) cDNAs for apoB mRNA encompassing the RNA editing site were identified and characterised from an opossum small intestinal cDNA library. The nucleotide sequence of apoB mRNA and the C-terminal portion of apoB48 are shown. A termination codon was demonstrated in all cDNAs identified corresponding to Glu2153 in the human apoB mRNA. The arrows denote PCR primers used. The asterisk denotes the RNA editing site. The sequence is numbered from one and according to the human cDNA sequence. (B) The sequences encoding apoB mRNA from opossum genomic DNA, liver cDNA and small intestine cDNA were analysed by DNA sequencing. T at position 6666 was identified solely in the small intestinal cDNA (arrowed). The nucleotides encompassing the RNA editing site were amplified by PCR on DNA and RT-PCR on RNA specimens. (C) Comparison of the RNA editing site sequence between opossum and eutherian mammals. Completely conserved nucleotides are shown in black boxes.

level of sequence identity between opossum and human apoB mRNA between positions 1356 and 7075 was 63%, with a predicted amino acid sequence identity compared to the human protein of 55%. In the region between nucleotides 6640 and 6720 spanning the RNA editing site the nucleotide sequence homology rises to 72%, but the amino acid sequence homology remains the same (results not shown).

### Cloning and characterisation of the opossum APOBEC-1 cDNA

To identify the opossum APOBEC-1 cDNA we prepared and screened an opossum small intestinal cDNA library. Eight positive clones were isolated and sequenced in their entirety.

The cDNAs fell into two groups. The longest cDNA in one group was 0.8 kb and had an open reading frame of 705 nt, which coded for the entire opossum APOBEC-1 protein of 235 amino acids from the initiating methionine codon (Fig. 2A). The AUG corresponds to the initial methionine in other APOBEC-1 mRNAs. (An in-frame stop codon was found 10 nucleotides upstream.) The 3'-untranslated region contained a polyadenylation signal (AAUAAA) overlapping the termination codon and a poly(A) tail 3 nt downstream of this signal (Fig. 2A).

The cDNAs from the second group contained a 1.3 kb insert (Fig. 2A and B). This cDNA encoded a form of APOBEC-1 with an insertion sequence of 435 bp at position 598 of the

**A**

1 CCCTCACAGCTCCAGAAACGATTCGGGTGAAAAGAAGCCATGAATTCTAAGACAGGTCCA  
 \* M N S K T G P 7  
 61 TCAGTAGGAGACGCCACTCTAAGGAGGAGATCAAACCTGGGAATTTGTGGCCTTCTTT  
 S V G D A T L R R R I K P W E F V A F F  
 121 AACCTCAAGAACTCCGTAAGAGACATGTCTCCTATGAAATTAATGGGGTAATCAA  
 N P Q E L R K E T C L L Y E I K W G N Q 47  
 181 AATATTTGGGCACACTCCAATCAAATACATCTCAACATGCTGAAATCAACTTCATGGAA  
 N I W R H S N Q N T S Q H A E I N F M E 67  
 241 AAATTCAGTGCAGAAAGACATTTAACTCTTCTGTAGATGCTCCATTACCTGGTTCCCTC  
 K F T A E R N F N S S V R C S I T W F L 87  
 301 TCTTGGAGCCCTTGTGGGAGTGTTCGAAGCCATAAGAAAGTTTTGGATCATTACCCC  
 S W S P C W E C S K A I R K F L D H Y P 107  
 361 AATGTGACTCTTGCCATTTTTATTTCTCGGCTTTATTTGGCACATGGATCAACAGCATCGT  
 N V T L A I F I S R L Y W H M D Q Q H R 127  
 421 CAAGGACTCAAGGAACTTGTCCACTCTGGAGTGACTATCCAGATTATGAGCTACTCAGAG  
 Q G L K E L V H S G V T I Q I M S Y S E 147  
 481 TATCATTACTGTTGGAGAAATTTGTTGACTATCCACAAGGGGAAGAAGATTACTGGCCC  
 Y H Y C W R N F V D Y P Q G E E D Y W P 167  
 541 AAATACCCCTACCTTTGGATTATGTTATATGCTGGAAGTCCACTGCATAATTTTAGGG  
 K Y P Y L W I M L Y V L E L H C I I L G 187  
 601 CTTCCACCTTGTAAAGATTTGAGGGAGTCATCCCAATCAGCTTGGCCTCTTCAGCCT  
 L P P C L K I S G S H S N Q L A L F S L 207  
 661 GATTTGCAAGACTGCCATTACCAAAGATTCCATATAATGTTCTGTAGCCACAGGGCTG  
 D L Q D C H Y Q K I P Y N V L V A T G L 227  
 721 GTCCAGCCATTTGTGACCTGGAGATGAATAAAATGTTTTTTTTCTTTTGTGTTCTT(A)<sub>n</sub>  
 V Q P F V T W R \* 235

**B**

598 GTGAGTTATCTTAGATAGCCAAGGAATAACATCTGGAATAACCATCCTTATTTCTTGC  
 V S Y L R \* 191  
 658 CTCTTTCTGGGGCTTTTGGTACTCACGATACTCAATATTTATTAGCCTTAGCCTAATT  
 718 GTTTTATATGCTTACTTGTCTTGGGGGTGAGAGTAGAACAAGGCACTTTTATTTGGGA  
 778 ATAAATGGATAAAGGATGCAGACATATGGGTAAGTAAATCTTCACTCCTTCCATAA  
 838 TACCAATATTTGTAGGTGTACATGTATGTGGATTGTTAGGTAATCTTACAGATGACCA  
 898 TTAATAGACTTATCTGATAATTAACACAAATTAAGATTAATAAATCAACATTAATAAT  
 958 TGCTAAAATGTCAAAAATTAACAGTAATAATGATATGATCCTAATTTCTTGTCTATTCT  
 1018 ATTTTGTCTTCCAGGGCTTCCACCTTGTAAAGATTTTCAGGGAGTCATTCCAATCAG  
 1078 CTTGGCCTCTTCAGCCTTGATTGCAAGACTGCCATTACCAAAGATTCCATATAATGTT  
 1138 CTTGTAGCCACAGGGCTGGTCCAGCCATTGTGACCTGGAGATGAATAAAATGTTTTTTT  
 1198 TTCTTTTGTGTTCTT(A)<sub>n</sub>

**C**

Nuclear Targeting Signal

opossum 1 MNSKTPGSVGDATLRRRIKPEWEEVAFENFOELRKEACLLYEIKWGNQNIWRHSNQNTSQ  
 human 1 MTSEKGPSTGDETLRRRIKPEWEEVDFVDFDERELKKEACLLYEIKWGMRSKIWRSSGKNTTN  
 rabbit 1 MASEKGPSNKDVTLRRRIKPEWEEVDFVDFDERELKKEACLLYEIKWGCASSKIWRSSGKNTTN  
 rat 1 MSSEKGPVAVDPTLRRRIKPEHEEVDFVDFDERELKKEACLLYEINWGRHSIWRHSIQNTNK  
 mouse 1 MSSEKGPVAVDPTLRRRIKPEHEEVDFVDFDERELKKEACLLYEINWGRHSVWRHSIQNTSN

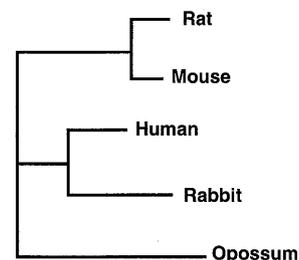
opossum 60 HAELNPEKFFTAERHFNSVSRCSITWFLSWSPCECSKATRKFLDHPNVTIATTSRLY  
 human 61 HVEVNEKFFTSERDFHPSISCSITWFLSWSPCECSQATREFLSRHPGVTLVTVARLF  
 rabbit 61 HVEVNEKFFTSEGRLPSTCCSITWFLSWSPCECSMATREFLSQHPGVTLVTVARLF  
 rat 61 HVEVNEKFFTTERYFCPNTRCSITWFLSWSPCECSRAATEFLSRYPHVTLVTVARLY  
 mouse 61 HVEVNEKFFTTERYFRPNTRCSITWFLSWSPCECSRAATEFLSRHPVTLVTVARLY

Catalytic Center

opossum 120 WHMDQQHROGLKDLVHSGVTIQIMSYSEYHCWRNFVDFPQGEEDYWPKYFYLWIMLYVL  
 human 121 WHMDQQNROGLKDLVNSGVTIQIMRASYYHCWRNFVDFPFGDEAHWPQYPLWMMIYAL  
 rabbit 121 QHMDRRNROGLKDLVTSGVTVRMSVSEYCYCWRNFVDFPFGKAAQWPRYPPRMLIYAL  
 rat 121 HHADPRNROGLKDLISSGVTIQIMTEQESYGCWRNFVDFSPSNEAHWPYRPHLWVRIYVL  
 mouse 121 HHTDQRNROGLKDLISSGVTIQIMTEQESYCYCWRNFVDFPPSNEAYWPRYRHLWVRIYVL

opossum 180 ELHCIIILGLPPCLKISGSHSNQALFSLDLDCHYQKIFYNVVLVATGLVQPFVTR  
 human 181 ELHCIIILSLPPCLKISRWRQNHLEFFRHLQNHCHYQTIIPHILLATGLIHPSVAWR  
 rabbit 181 ELYCIIILGLPPCLKISRHRQKQLEFFFSLTPQYCHYKMIIPYIILLATGLLQPSVPWR  
 rat 181 ELYCIIILGLPPCLNLRKQPOLFFFTALOSCHYQRIPHILLWATGLK-----  
 mouse 181 ELYCIIILGLPPCLKILRRKQPOLFFFTLQTCYQRIPHILLWATGLK-----

**D**



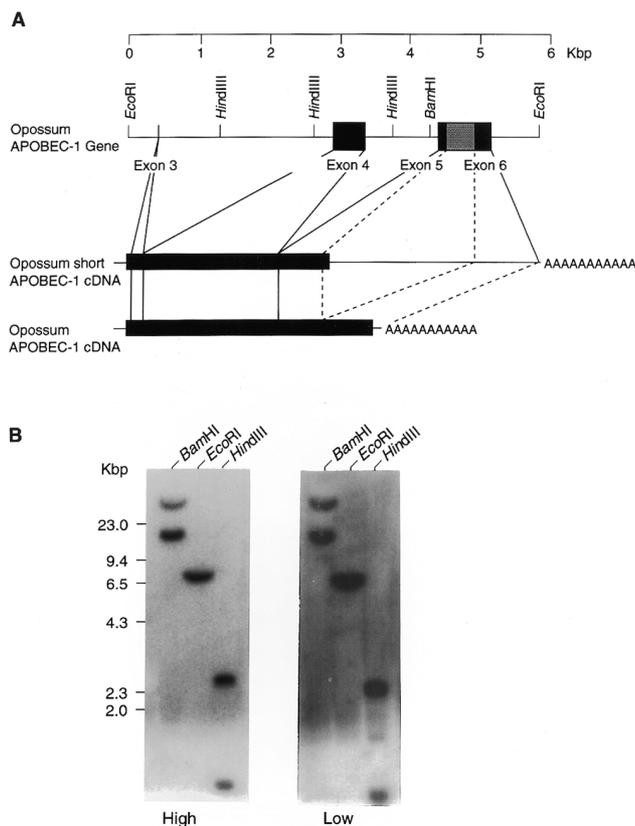
APOBEC-1 cDNA and predicted a protein 45 amino acids shorter than human APOBEC-1. At each end of the insertion sequence typical splice donor and acceptor sequences were found. Otherwise the nucleotide sequence of this clone was identical to APOBEC-1, suggesting that the insertion sequence is an unspliced intron.

Opossum APOBEC-1 has a 67% amino acid homology to human, 65% to mouse and 63% to rabbit and rat APOBEC-1, respectively (Fig. 2C). Construction of phylogenetic trees was based on the amino acid sequences and used the maximum likelihood procedure (Fig. 2D). These analyses separate marsupials from rodents, lagomorphs and primates, but do not resolve the eutherian branching pattern.

Structural motifs previously identified in the APOBEC-1 of placental mammals are conserved in opossum APOBEC-1. The zinc ligands that form the active site, the proton-shuttling glutamate and spacing between the amino acids are preserved. The phenylalanine residues in the active site that bind the RNA substrate and a short insert sequence SITWF, which contains one of these phenylalanine residues, were also found. Overall, the features of the active site, pseudoactive site and linker peptide between the active site and pseudoactive site identified as similar between ECCDA and APOBEC-1 are all retained. At the N-terminus the putative nuclear localisation signal sequence is present. At the C-terminus of opossum APOBEC-1 all 11 leucine residues present in placental APOBEC-1 were found. These leucine residues have been variously proposed to be involved in homo- or heterodimerisation, transport between the nucleus and cytoplasm and to contribute to the structure of the active site. The C-terminus of opossum APOBEC-1 is extended by seven amino acids, like the human and rabbit APOBEC-1 compared to rat and mouse APOBEC-1, which have shorter C-termini.

### Cloning and characterisation of the opossum APOBEC-1 gene

An opossum genomic DNA library was prepared in a pUC vector. A 5.8 kb *EcoRI* restriction fragment containing the opossum APOBEC-1 gene was isolated and characterised by restriction mapping and DNA sequencing. The four protein coding exons corresponding to the human and mouse genes were located within this fragment (Fig. 3A; 27). The fragment did not contain the first protein coding exon, corresponding to the second exon of the human gene, which encodes the first six amino acids of APOBEC-1. The cDNA and genomic coding



**Figure 3.** Organisation of the APOBEC-1 gene. (A) The intron/exon organisation of the APOBEC-1 gene is shown together with the cDNAs derived from the full-length construct and from aberrantly spliced mRNA. The exons are numbered in accordance with the human gene organisation. (B) Southern blots of opossum genomic DNA hybridised and washed under the high and low stringency conditions as described in Materials and Methods. Under those conditions, genes with >60% of overall identity are detectable. The 5.7 kb apoB cDNA (nt 1356–7075) was used as a probe.

sequence were identical. All of the sequences at the intron/exon junctions followed the GT/AG rule (data not shown). The position of the intron/exon boundaries and amino acids interrupted were identical to the human and mouse genes. The only deviation from this was the identification of the cDNA in which the last intron of the APOBEC-1 gene was not spliced. This led to the generation of shorter APOBEC-1 coding sequence. Sequence analysis of the opossum gene confirmed

**Figure 2.** (Opposite) cDNA sequence of opossum APOBEC-1 and encoded protein. (A) The opossum APOBEC-1 cDNA was cloned from an opossum small intestinal library. The translated reading frame corresponding to eutherian APOBEC-1 is shown. The asterisk denotes a stop codon upstream of the initiating methionine. The arrows denote the position of an unspliced in-frame intron. The polyadenylation signal and poly(A) tail are indicated. (B) Sequence of the unspliced intron found in some cDNAs. The GT and AG splice donor and acceptor sites are shown in bold. Amino acids in the open reading frame are shown. The asterisk denotes the termination codon for the protein. Italics denote continuation of the sequence of exon 6 after unspliced intron 5. The polyadenylation signal and site are shown. (C) Comparison of marsupial and eutherian APOBEC-1 protein sequences. Black boxes denote residues identical in all species. Grey boxes denote residues identical between the opossum and the consensus for other mammals. The catalytic centre and putative nuclear targeting signal are shown. (D) Phylogenetic analysis of APOBEC-1 sequences. APOBEC-1 sequences from human (P26234), mouse (U22262), rabbit (U10695), rat (L07114) and opossum (AB027195) were analysed (Database accession nos are given in parentheses). For each of the 15 possible five species trees the maximum likelihood value was computed assuming a Tamura–Nei model of sequence evolution. To account for rate heterogeneity a discrete  $\gamma$  distribution with eight categories was assumed. Because the first, second and third codon positions are known to evolve at different rates, codon positions were analysed separately, as were the amino acid sequences. For the four sequence set the PUZZLE program output three trees that are not significantly different from each other. The best tree is the tree shown. The branching pattern of rodents (rat and mouse), lagomorphs (rabbit) and primates (human) cannot be resolved. Opossum is a separate group.

that the deviant mRNA was generated by failure to splice the last intron.

To test for the existence of genes related to APOBEC-1, such as might be involved in the editing of opossum mitochondrial tRNA<sup>Gly</sup>, Southern blotting was performed at high and low stringency (Fig. 3B). Restriction fragments corresponding to the APOBEC-1 gene were identified under both conditions of hybridization. No additional hybridization was demonstrated at low stringency, indicating that no gene closely related in nucleotide sequence to that of APOBEC-1 is found in the opossum nuclear genome. The marsupial mitochondrial genome contains no classical cytidine deaminase (8).

### Sites of expression of apoB and APOBEC-1 mRNA

Northern blot analysis was performed to determine the tissue distribution of opossum apoB and APOBEC-1 mRNA expression (Fig. 4A and B). The mRNA for apoB was detected in the opossum liver and small intestine at high levels and at a much lower level in the kidneys (Fig. 4B). This result was confirmed by RT-PCR (Fig. 4C). No hybridization signal was observed in other tissues. In both the small intestine and liver two major mRNA species were identified at ~14 and 7.5 kb. The four cDNA clones of opossum small intestinal apoB each have poly(A) tails and polyadenylation signals between nucleotides 7055 and 7060 at 15 nt upstream of the poly(A) tail, indicating that these cDNAs are derived from the shorter mRNA. To confirm this result we performed 3'-RACE analysis using a primer upstream of the editing site (Fig. 4D). The 3'-RACE product indicated that two short apoB mRNAs are generated in liver and kidney as well as the small intestine (Fig. 4D). Neither of the short mRNAs were edited in the liver or kidney (Fig. 1B). In the opossum intestine the three mRNAs are produced by the use of different polyadenylation signals. Previously, similar results have been demonstrated in human and rabbit, where short and long edited mRNAs for apoB are expressed in the small intestine (10). In human and rabbit liver the short mRNA is either absent or present at very low levels. These results give rise to the question as to the origin and function of the shorter apoB mRNA in opossum liver. The shorter mRNA in the liver probably has no in-frame stop codon at its 3'-end because the mRNA is not edited. However, its high abundance relative to the apoB100 mRNA suggests that it could compete with the longer apoB100 mRNA for translation. Further studies are required to elucidate the relationship of this mRNA to the editing process and to the origins of opossum apoB48. Its abundance in poly(A)<sup>+</sup> RNA and detection with a 5' apoB cDNA probe indicate it is not a degradation product.

The mRNA for APOBEC-1 was demonstrated exclusively in the small intestine by northern blotting (Fig. 4A). Prolonged exposure of the northern blot revealed no signal in any other tissue examined including the brain, heart, lung, liver, spleen and kidney. These results were confirmed by RT-PCR of small intestine and liver (Fig. 4A and C). ApoB mRNA editing is not present in the opossum liver or kidney (Fig. 1B). Surprisingly, APOBEC-1 cDNA was detected by RT-PCR in the kidney (Fig. 4C).

Three different sized hybridising bands were observed on northern blots probed for APOBEC-1. These were 1.0, 1.7 and 3 kb (Fig. 4A). The 1.0 and 1.7 kb mRNAs correspond to the two classes of cDNA identified. The 1.0 kb species was much the most abundant. However, the moderate expression of the

1.7 kb mRNA suggests that this mRNA species, which contains the unspliced last intron, is due to differential rather than aberrant splicing. The 3 kb transcript could be the consequence of alternative splicing or alternative polyadenylation or come from a far upstream promoter. This has not been investigated.

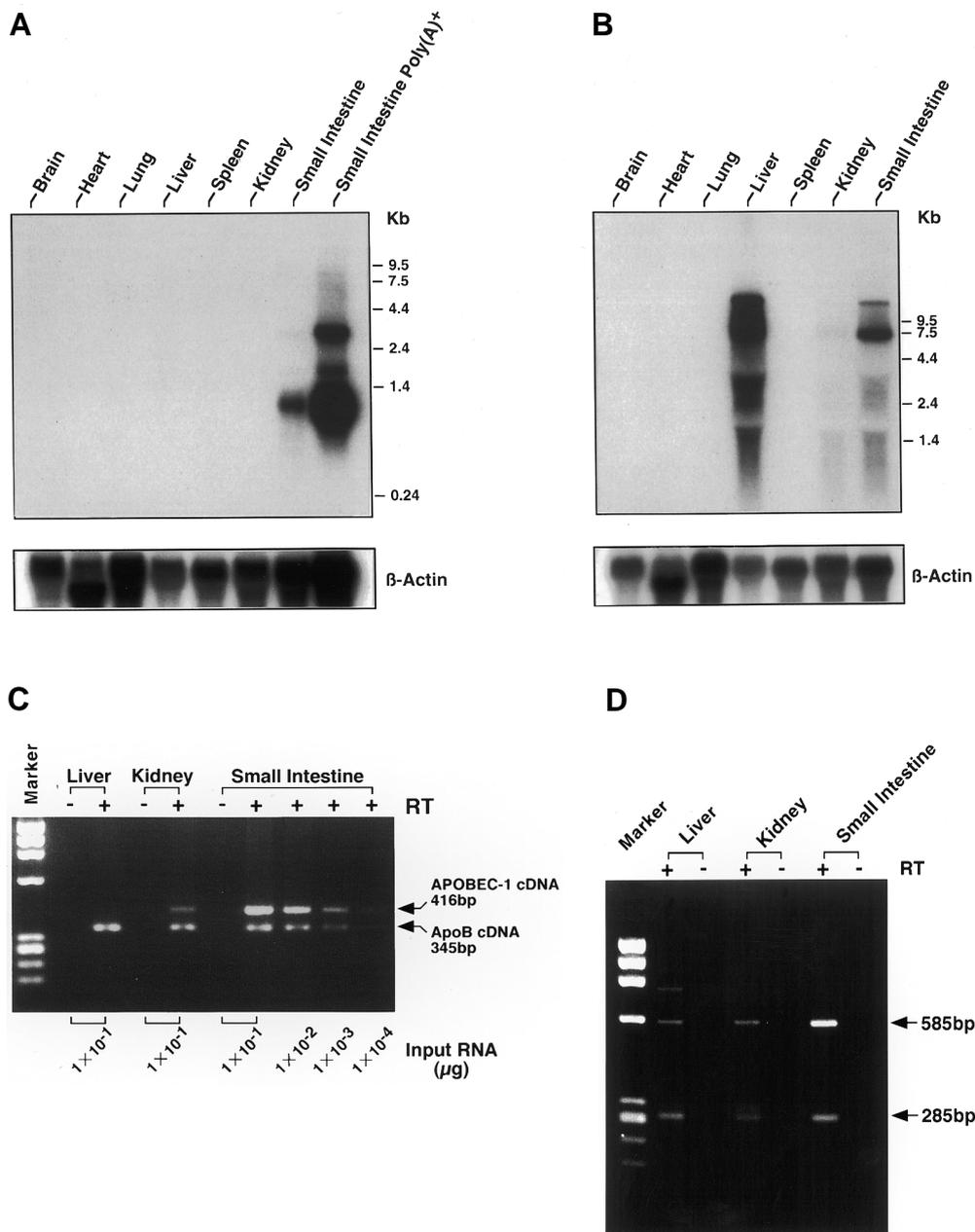
The restricted pattern of tissue expression of APOBEC-1 indicates that it cannot be involved in the processing of mitochondrial tRNA<sup>Gly</sup> in the opossum. This tRNA undergoes RNA editing at the middle C of the anticodon (GCC) (7). This editing reaction is biochemically a deamination and is apparently similar to apoB mRNA editing. Opossum tRNA editing occurs in most tissues at the same level. In view of its confined expression, APOBEC-1 cannot be involved in this process. No APOBEC-1-like gene was demonstrated by low stringency hybridisation whereas the human probe readily picks up the opossum gene on Southern blots (results not shown).

### RNA editing by opossum APOBEC-1

The two forms of opossum APOBEC-1 were evaluated for their ability to edit rat or opossum synthetic apoB mRNA substrates. Full-length and C-terminally truncated opossum APOBEC-1 were generated by *in vitro* transcription and translation. The translated proteins were resolved by SDS-PAGE and fluorography. The size of the expressed proteins corresponded to 27 and 21 kDa, as is predicted by the nucleotide sequence. A hybrid protein comprising a fusion between opossum APOBEC-1 up to the site of differential splicing and rat APOBEC-1 beyond this site was created and translated *in vitro*. Neither the opossum APOBEC-1 nor the opossum-rat hybrid protein gene is alone competent for RNA editing. In the presence of chicken S100 extract containing auxiliary editing factors opossum APOBEC-1 and the hybrid protein produced a similar level of RNA editing to that of rat APOBEC-1 on both the opossum and rat mRNA substrate (Fig. 5). As anticipated, the form of APOBEC-1 lacking most of the leucine-rich region showed no RNA editing and no binding to apoB mRNA as determined by UV cross-linking (data not shown). Together these results indicate that the leucine-rich region is indispensable for RNA binding and consequently RNA editing, as has previously been reported (17,21). The C-terminus of the rat and opossum proteins are interchangeable. Therefore, the extra seven amino acids found in human, opossum and rabbit APOBEC-1 have no function for RNA editing *in vitro*. The auxiliary editing factors found in chicken intestine are fully competent for the editing of apoB mRNA in marsupial and placental mammals.

### DISCUSSION

The ancestors of eutherian (placental) and marsupial mammals form a monophyletic group, which according to paleontological and phylogenetic data diverged from a therian common ancestor as long as 173 000 000 years ago (28). The tRNA for aspartic acid in the mitochondria of American, New Guinean and Australian marsupials carries the anticodon 5'-GCC-3' instead of the normal anticodon for aspartic acid, 5'-GUC-3' (7). The second position of the anticodon in the RNA is post-transcriptionally changed by a C→U RNA editing mechanism. This RNA editing is restricted to a single nucleotide position in the marsupial mitochondrial transcriptome. The gene for the tRNA<sup>Asp</sup> of protherian mammals carries the anticodon 5'-GUC-3',

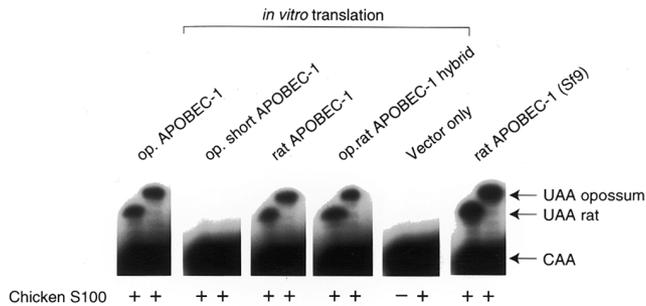


**Figure 4.** Expression pattern of APOBEC-1 and apoB mRNAs. RNA was isolated from opossum tissues as shown and poly(A)<sup>+</sup> RNA prepared from the small intestinal RNA. **(A)** Northern analysis under high stringency conditions using <sup>32</sup>P-labelled APOBEC-1 cDNA and a  $\beta$ -actin control cDNA. Blots analysed under low stringency conditions are not shown. No difference in expression and no extraneous hybridizing bands are revealed. **(B)** Poly(A)<sup>+</sup> RNA was prepared from the tissues and treated as in **(A)** and hybridized with a <sup>32</sup>P-labelled rat apoB cDNA from the 5'-end of the mRNA. Ethidium bromide staining showed that 28S and 18S RNAs were intact. This result is not shown. **(C)** RT-PCR of opossum APOBEC-1 and ApoB mRNAs. Total RNAs from liver, kidney and small intestine were treated with DNase I to exclude contamination by genomic DNA. The amount of RNA indicated 'Input RNA' was subjected to reverse transcription with random hexamers. Single-stranded cDNAs were amplified with specific primers either for APOBEC-1 or ApoB. The amplified fragments were analysed on a 3% agarose gel. **(D)** 3'-RACE analysis of short ApoB mRNA. Total RNAs from liver, kidney and small intestine treated with DNase I were subjected to the 3'-RACE system for rapid amplification of cDNA ends. The PCR products were analysed on a 3% agarose gel.

as do placental mammals. Therefore, this RNA editing evolved in the common ancestor of marsupials. In the present study we have shown that the C $\rightarrow$ U editing of apoB mRNA is also established in American marsupials. However, the editing event and the enzyme responsible for it in marsupials appear to be restricted in expression to a single tissue. Therefore,

APOBEC-1 cannot be responsible for the editing of the mitochondrial aspartic acid tRNA gene. We have found no evidence for a gene closely related to APOBEC-1 in nucleotide sequence in the marsupial genome.

APOBEC-1 and the apoB mRNA editing site are evidently highly conserved between marsupial and eutherian mammals.



**Figure 5.** Editing *in vitro* by opossum APOBEC-1. Full-length APOBEC-1, truncated APOBEC-1 created by aberrant splicing and a construct made up of APOBEC-1 from the opossum sequence at the N-terminus and the rat APOBEC-1 sequence after the site of aberrant splicing were expressed by *in vitro* transcription and translation and used in the *in vitro* editing assay, together with chick S100 small intestinal auxiliary factors. UAA denotes opossum and rat edited and CAA unedited apoB RNA. Input RNA was derived from the opossum or from the rat sequence as indicated.

The difference in nucleotide and amino acid sequence between the APOBEC-1 and apoB mRNA editing site from different mammalian species, including humans, rodents and rabbits, is not greater than the difference between the opossum and the different placental mammals (Fig. 2; 29). The active site catalytic and RNA binding residues, the nuclear localisation signal and leucine-rich domain at the C-terminus of the enzyme are all retained between marsupial and eutherian APOBEC-1 (1,30). In addition, in common with humans and rabbits, marsupial APOBEC-1 has an extended C-terminus seven amino acids longer than that in the rodents, suggesting that this feature was present in the ancestral enzyme. We show here that substitution of the C-terminus of the rodent enzyme for that of the opossum enzyme makes no difference to catalytic activity *in vitro*, suggesting that this C-terminal extension has little functional significance.

The opossum APOBEC-1 gene, like the human, rabbit, guinea pig, monkey, cow, pig, sheep and cat APOBEC-1, is expressed in the small intestine, but not in the liver (29). A single major RNA species of 1.0 kb is demonstrated by northern analysis in the intestine. Of the two minor mRNAs the 1.7 kb species is caused by aberrant splicing and the 3.0 kb species was not investigated. Thus, the acquisition of the far upstream promoter demonstrated in rodents, which confers expression in the liver, appears to have been a later evolutionary event well after their divergence from a common ancestor of placental and marsupial mammals (28,31,32). APOBEC-1 mRNA was detected by RT-PCR in the kidney, but editing was not found within the limits of our editing assay in the kidney. This discrepancy was not investigated further as it seems to represent very low level expression. Otherwise the structure of the gene encoding the APOBEC-1 protein appears to be conserved between marsupials and placentals (27,31). The aberrant splicing of the last intron in marsupial APOBEC-1 mRNA does not give rise to a functional protein and is probably insufficiently frequent to have functional consequences. Like the alternative and aberrant splicing of the human APOBEC-1 gene it may represent some laxity of the

splicing process. Overall, the present study shows similarities between opossum, rabbit and human gene expression and protein structure and differences from rodents. These observations are consistent with the phylogenetic analysis in this study and elsewhere (28), which places the opossum and rodents in separate groups, though not distinguishing eutherian APOBEC-1 into subgroups.

The apoB mRNA editing site is highly conserved at the level of 72% between nucleotides 6640 and 6720. This is greater than the overall homology between the human and opossum apoB gene amino acid coding sequence. The site of RNA editing in both eutherians and marsupials is highly AU-rich, (19,33,34), a characteristic which has been identified as important in facilitating RNA target recognition and editing. Another feature of interest is that alternative polyadenylation of the edited transcript is present in the marsupial and eutherian mRNAs. Surprisingly, however, we show here the existence of a truncated apoB mRNA of considerable abundance in the marsupial liver. This RNA species is apparently not a degradation product. In view of the absence of RNA editing in the marsupial liver, demonstrated here, this mRNA would presumably lack a termination codon and would therefore be non-functional. However, its presumably intact initiation codon could interfere with translation of the normal marsupial hepatic apoB mRNA. The elucidation of these processes requires further investigation.

The present study extends the origins of apoB mRNA editing back from eutherians as far as modern marsupials, ~173 000 000 years ago (28). The gap of 190 000 000 and 140 000 000 years between amphibians and birds, which do not edit apoB mRNA (28), and marsupials has still therefore to be bridged. It is likely that the phenomenon of apoB mRNA editing will be found to have arisen at the same time as the changes in anatomy that occurred during the development of intestinal lymphatics that drain into the systemic circulation rather than directly into the portal vein (12). Identification of this form of RNA editing therefore needs to be sought in other more primitive egg-laying protherians, such as the monotremes platypus and spiny anteater.

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