An additional editing site is present in apolipoprotein B mRNA

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ABSTRACT

Human intestinal apolipoprotein (apo) B mRNA undergoes a C to U RNA editing at nucleotide 6666 to generate a translation stop at codon 2153, which defines the carboxy-terminal of apo B48. Here we show that two of eleven human intestinal cDNAs spanning residue 6666 were edited from a genomically-encoded C to a T at residue 6802 as well as at residue 6666. This additional editing converts Thr (ACA) codon 2198 to Ile (AUA). Synthetic RNA including the nucleotide 6802 was edited *in vitro* by intestinal extracts at 10 – 15% of the editing efficiency of nucleotide 6666. A sequence is identified as important for recognition by the editing activity. No secondary structural homology was identified between the two edited sites. No other sequence in the region between 6411 and 6893 nucleotides of apo B mRNA was found to be edited *in vivo* or *in vitro*. Apo B RNA editing extracts from intestine did not edit maize cytochrome oxidase II mRNA.

INTRODUCTION

Messenger RNA transcribed in mitochondria of kinetoplastid protozoa (*trypanosomes*, *Leishmania* and *Crithidia*) undergoes editing at multiple sites (1–4). Most usually editing involves the addition of multiple U residues, but Us may also be deleted. The editing reaction is considered to involve endonuclease, poly U polymerase, and RNA ligase activities. The specificity of the reaction is templated by anti-sense guide RNAs. The mRNA in the mitochondria of a variety of plants including wheat, maize, rice and pea, also undergoes editing at multiple sites to create a U at the place where a genomic C is templated (5–7). The back reaction of U to C conversion has also been observed (8,9).

Apolipoprotein (apo) B100 mRNA undergoes site-specific modification at nucleotide 6666 to form uridine, thereby generating from a glutamine codon (CAA) a stop translation codon (UAA) that defines the carboxy-terminal of apo B48 (10,11). The biochemical mechanism of this editing reaction is considered to be deamination at the 4 position of cytidine to form uridine (12). Apo B100 mRNA is the only known example of a nuclear transcript that undergoes RNA editing. No other editing site in apo B mRNA has been identified to date.

The sequence requirements for the editing of apo B mRNA *in vitro* reside within 55 nucleotides spanning the RNA modification site (13). 26 nucleotides spanning the conversion site when transcribed as an apo B minigene are edited in transfected tissue culture cells (14). The specific sequence requirements for editing have been examined in the 9 nucleotide sequence encompassing the edited site and appear to be relatively relaxed (15). Indeed, mutation of A-6667 to C immediately downstream of the C normally edited at position 6666 allows this second C to be edited *in vitro*. Therefore the present study was undertaken to determine whether this apparently relaxed specificity for editing allows alternative editing sites in apo B mRNA, to be used *in vivo* and *in vitro*. We have also examined maize mitochondrial mRNA for editing *in vitro* with apo B mRNA editing extract.

MATERIALS AND METHODS

Preparation of RNA

Plasmids ECE-RS and ECE-SX used were as described by Davies *et al* (14). RNA was transcribed as described by Driscoll *et al* (13).

Oligonucleotides

The following oligonucleotides were synthesised on a Milligen 7500 DNA synthesiser and gel-purified.

ND1	(5541)	CGAGACCTAGAGTGGTTGTTACCCTTTGAT
ND2	(6293)	ACGAAAACATTTCATACTATTTTTGGTTCT
ND3	(6421)	TCGACTTCGTGTAGTTATAACTAGTTAAAC
ND4	(6443)	AGTTAAACATTCTTTTATGTCTCGTCGGGA
ND5	(6450)	CATTCTTTTTATGTCTCGTCGGGACCCTTTTGA
ND6	(6524)	CTCTGTTCAAAGTGTACGGTTCCTCTTTGA
ND7	(6534)	AGTGTACGGTTCCTCTTTGACTGACGAGAG
ND8	(6602)	ACGTAATCTACTACGGTTTTAGTTGAAATTACT

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DD3	(6674)	AGTCATATAATTTCTATTAATTACTAAATGTACTAA
ND10	(6806)	GGTACTAGATGTAAACAAATAACTTTTATA
ND11	(6881)	ACACCTATGATTCATGTTTAGTCTTAGGTCTAT
Cox2D18	(642)	GTTGCTCTTCCTCAAATG
Cox2E18	(704)	GGATAGCAGCATCTTCGT
ZMCox5	(391)	TGACAATATCAGGCTTATGAGTAT
ZMCox3	(798)	CGCCTTTACGTTAAGAGCCCACTC

The oligonucleotides ND1-ND11, and Cox2D18, and Cox2E18 are complementary to human apo B mRNA (11) and maize *CoxII* mRNA (5,17) respectively. The 3′ end of the oligonucleotides is given in brackets. Oligonucleotides were labelled with γ-[^{32}P]-ATP (3000 Ci/mmol) and T4 kinase (Pharmacia) to specific activity of 10^9 dpm/μg. Labelled oligonucleotides were separated from free ^{32}P on 'Nick' columns (Pharmacia) according to the manufacturer's instructions.

In vitro conversion assay

Enterocyte S100 cytosolic extracts were prepared from 200−300 g Sprague Dawley rats as described (16). Editing *in vitro* was performed as described previously (13) and modified (16) as follows. The assay contained 50 mM EDTA pH 8.0, 10 mM HEPES pH 8.0, 10% glycerol, 50 μM 2-mercaptoethanol, 50 mM KCl, 0.5 nM RNA and 10 μg/ml protein. Mixtures were incubated for 2 h at 30°C, and the RNA was recovered and analysed by primer extension.

For primer extension γ-[^{32}P]-ATP labelled oligonucleotides (40−50 fmol) were mixed with 0.5 nM RNA in 100 mM Tris HCl pH 8.2 containing 12 mM $MgCl_2$ and 20 mM DTT. Mixture was denatured at 85°C for 10 min and was annealed at 42°C. After 30 min 1 mM each dATP, dCTP, dTTP and 500 μM ddGTP and 0.2−0.3 U/μl super reverse transcriptase (Anglian Biotechnology) were added and the mixture was further incubated for 60 min. The reactions were ethanol precipitated and analysed on a 7.5% polyacrylamide 7 M urea gel. Dried gels were subjected to autoradiography at −70°C for 6−12 h. Autoradiographs were scanned and quantitated by densitometry (14).

Preparation *Zea mays* RNA

Mitochondria were prepared from *Zea mays* (maize) as described (17). Maize mitochondrial DNA was prepared (17). The DNA was treated with RNase A and proteinase K. The oligonucleotides ZMCox5 and ZMCox3 used for PCR amplification of maize cytochrome oxidase 2 gene (*CoxII*) are shown above. PCR amplification was performed with Taq polymerase as previously described (14). The PCR cycle was initiated at 94°C for 3 min and 30 cycles of 91°C for 1 min, 55°C for 1.5 min and 72°C for 2 min performed. The reaction was terminated by incubation at 72°C for 5 min. The reaction product was isolated on DE81 paper and cloned into the pBluescript KS vector cut with EcoRV as previously described (13). The DNA sequence of the *CoxII* gene was confirmed by the dideoxynucleotide method (11,13). cRNA was transcribed with T7 RNA polymerase. The synthetic *CoxII* transcript was incubated with rat enterocyte extract as described above. Possible editing at nucleotides 638 and 703 of CoxII mRNA was assayed with oligonucleotides Cox2D18 and Cox2E18 as for apo B transcripts described above (6,17).

Clones and sequence

The screening of human cDNA libraries and DNA sequencing have been described previously (11).

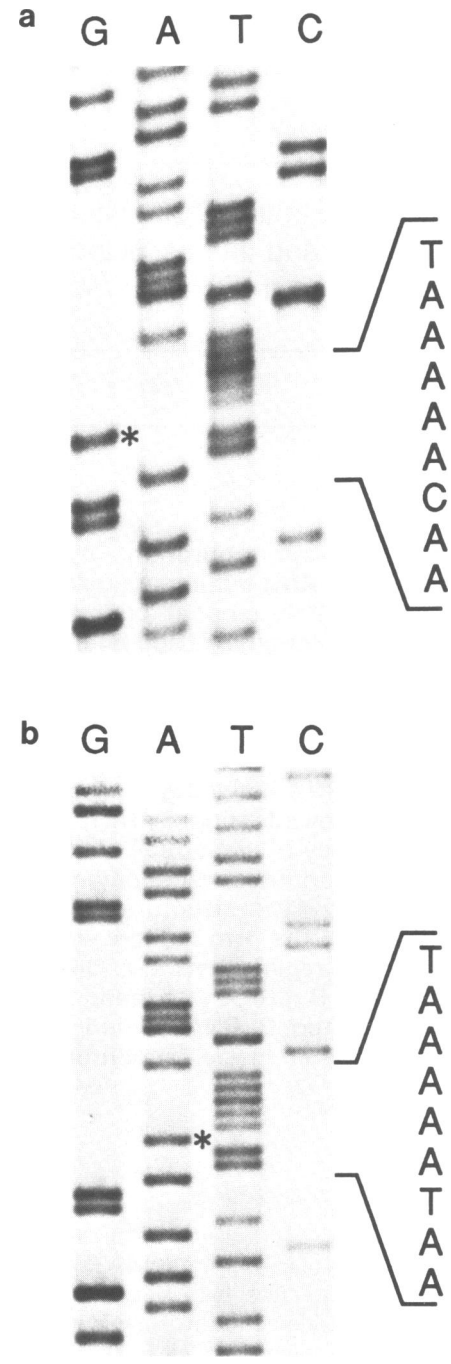

Figure 1. DNA sequence showing the edited nucleotide at position 6802. a) genomic sequence; b) edited cDNA sequence. The modified nucleotide has an asterisk.

RESULTS

Apo B mRNA contains an additional edited nucleotide

The sequence of the human apo B gene and of apo B cDNAs that encode the carboxy-terminal of apo B48 have previously been described (11). Comparison of the sequence of small intestinal cDNAs encompassing the edited C at nucleotide 6666 revealed a C to T substitution at nucleotide 6802 in two out of eleven clones isolated from cDNA libraries prepared from different individuals as previously described (Fig. 1). Each of the cDNAs with the substitution at position 6802 was also edited at position 6666.

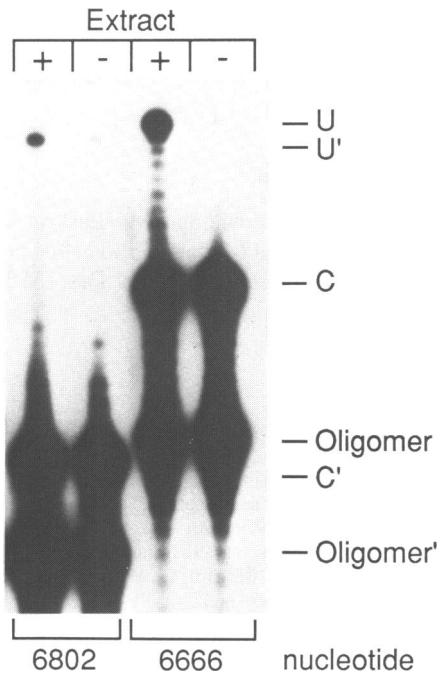

Figure 2. Sites assayed for editing *in vitro* in apo B and *CoxII* mRNAs. The CAA and CAG triplets assayed are underlined. Solid triangles mark the 3′ end of oligonucleotides used in primer extension analysis. Asterisks mark the edited sites in apo B and *CoxII* mRNAs. An open circle marks the secondary edited site in apo B mRNA.

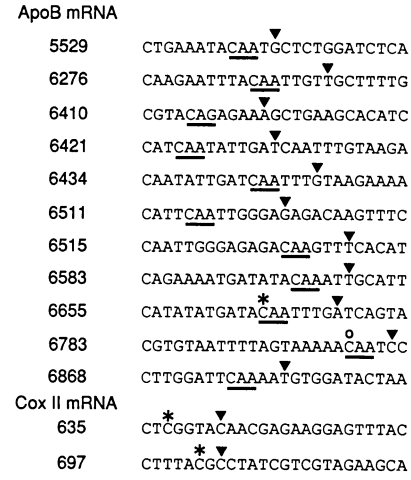

Figure 3. *In vitro* editing of apo B mRNA at nucleotide 6666 and 6802. U, C and oligomer denote the edited and unedited nucleotide and priming nucleotide for position 6666. U′, C′ and oligomer′ are the corresponding edited and unedited nucleotides and primer for the editing site at position 6802.

Apo -B alternative editing site

6662	GATACAATTTGATCAGTATATTAAAG6687	wild type
6798	aAaACAATccatgatcTAcATTtGtt6823	alternative
	uUgUGUUGGGUuagucAUaUAAuUuc	G:U base pairing

Figure 4. Comparison of the primary and alternative editing site in apo B mRNA. The nucleotides shown correspond to the 26-base RNA that confers editing on non-contiguous apo B mRNA sequences (14). The RNA sequence complementary to the major editing site is shown. The consensus sequence for editing is shown in uppercase letters. This is increased by allowing for G:U base pairing with the proposed complementary RNA sequence.

This single base change substitutes Thr at amino acid codon 2198 (ACA) with Ile (ATA). The C to T base change was not identified in genomic clones taken from three separate intestinal libraries. We therefore considered that it might represent an additional RNA editing site involving a C to U substitution.

Editing *in vitro* of apo B RNA

Previously we have established a sensitive primer extension assay for the editing of apo B RNA and have developed a system *in vitro* for RNA editing (13). The putative new editing site at position 6802 was examined for editing *in vitro* on a 482-nucleotide RNA representing an Rsa I restriction fragment spanning position 6666 (nucleotides 6411 to 6893). The nucleotide at position 6802 was edited *in vitro* with 11 to 15% of the efficiency of position 6666 as judged by scanning densitometry (Fig. 2). The additional editing site at position 6802 and the *bona fide* editing site at position 6666 have in common the sequence CAA. Nine other sites containing CAA (and in one case CAG) were assayed for editing *in vitro* on cRNA transcribed from the Rsa I fragment or from a Sal I, Xba I fragment (nucleotides 5289 to 7674) (Fig. 3). Oligonucleotides were designed to screen these additional CAA sequences for editing as described under Materials and Methods. None of these additional sites was edited *in vitro* (results not shown).

Maize *CoxII* RNA is not edited *in vitro* by apo B RNA editing activity

Plant mitochondrial mRNAs undergo numerous RNA editing events in which genomically-encoded Cs are converted to U or to a U-like base or occasionally U is converted to C (5–9, 17). In order to investigate whether unedited maize cytochrome oxidase 2 (*CoxII*) mRNA can be edited by the apo B mRNA editing activity a template of the maize gene was prepared. The absence of edited nucleotides in the DNA template was verified by DNA sequencing. After incubation *in vitro* with the apo B RNA editing extract maize *CoxII* RNA was assayed by primer extension at two nucleotides, 638 and 703, with oligonucleotides described in Materials and Methods (Fig. 3). The *CoxII* RNA was not edited by the apo B RNA editing activity (results not shown).

DISCUSSION

The present study establishes that in addition to C to U conversion of nucleotide 6666 and creation of a stop codon at position 2153, apo B mRNA undergoes editing *in vivo* and *in vitro* at nucleotide 6802. This editing site introduces a Thr → Ile substitution at amino acid codon 2198 of apo B mRNA. However, in two out of eleven of the cDNAs in which sequencing established that the new site had been edited, editing was additional to rather than alternative to editing in of a stop codon at upstream position 2153, and would produce no functional amino acid change in the encoded protein. Editing *in vitro* of nucleotide 6802 showed 11 to 15% of the efficiency of editing at position 6666.

The sequences shared by the two edited sites are shown in Fig. 4. The region of homology between the two edited sites corresponds to the 26-nucleotide sequence (positions 6662–6687)

that is highly conserved in mammals and which we have shown to confer editing on non-contiguous apo B RNA sequences transiently expressed in rat hepatoma cells (14). Chen *et al* have previously used mutagenesis to examine the nine nucleotides (positions 6663 to 6671) spanning the editing site at position 6666 and concluded that the sequence requirements for editing in this intermediate region are relatively relaxed (15). However, comparison of the two edited sites suggests that the sequence requirements for editing may extend downstream and include most of the conserved 26-nucleotide segment previously identified by us (14).

Secondary and tertiary structure are more important than primary structure in many RNA processing reactions. For example RNA secondary structure is required for the specific interactions involving the HIV rev protein, the iron response element in ferritin and transferrin RNAs and the phage R17 coat proteins (18–22). A potential stem-loop structure conserved between humans, rabbits, rats and mice with the editing site at position 6666 in the loop is found at the primary editing site (14). This stem-loop is not conserved at the secondary editing site at position 6802 (Navaratnam and Scott, unpublished results). While it is possible that this region of apo B mRNA base pairs with other RNA sequences within apo B mRNA in *cis* to form secondary structure, no basis for this is found in the 55-base minimal RNA spanning position 6666 that undergoes editing *in vitro* (Navaratnam and Scott, unpublished results).

The possibility of stable interaction between RNA sequences is increased by chemically stable G:U base pairing in the stems of tRNAs and rRNAs (4). We find that the introduction of G:U base pairing increases the consensus between the two editing sites in the apo B RNA. G:U base pairing between RNA species in *trans* has been implicated in the editing of kinetoplast mRNAs, where guide RNAs that allow such base pairing are considered to template editing (2,4). Similar guide RNAs have been speculated as being involved in the C to U editing that occurs in plant mitochondrial mRNAs (8). It is possible that apo B mRNA editing also uses a guide RNA as part of its recognition system.

The editing of kinetoplast mRNA is considered to progress in a 3′ to 5′ direction (1–4). It is possible that the apo B RNA editing enzyme, having edited one site on the mRNA, also moves to the next site. However, we find no evidence of editing 5′ of position 6666 which, if it occurred and created a stop codon, could be deleterious to the function of the apo B protein. The finding of a relatively inefficient editing site that alters a residue 3′ of the stop codon generated at codon 2153 would seem to have little functional significance. However, it has not been excluded that under some circumstances $Thr_{2198} \rightarrow Ile$ might occur independently of editing at position 6666 and affect the structure and function of apo B100, the larger hepatic form of apo B. This is unlikely to be frequent in the intestine, where more than 98% of apo B mRNA is usually edited at position 6666 (11).

The editing sites in plant mitochondrial *CoxII* mRNA bear no primary or secondary structure homology to either of the two editing sites in apo B mRNA (8,17). It is not surprising, therefore, that *CoxII* RNA was not edited. Clearly the apo B RNA editing activity and the plant mitochondrial activity do not share substrate specificity. It remains to be established whether plant mitochondrial mRNA undergoes the site-specific cytidine deamination, which we have identified as the mechanism of apo B mRNA editing (12).

The discovery of a second RNA editing site in the apo B mRNA, and the consensus sequence provided by comparison of the two sites may help in the search for further target sequences in this and in other RNAs.

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