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Characterization of the apolipoprotein B mRNA editing enzyme: no similarity to the proposed mechanism of RNA editing in kinetoplastid protozoa

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ABSTRACT

Intestinal apolipoprotein B mRNA is edited at nucleotide 6666 by a C to U transition resulting in a translational stop codon. The enzymatic properties of the editing activity were characterised *in vitro* using rat enterocyte cytosolic extract. The editing activity has no nucleotide or ion cofactor requirement. It shows substrate saturation with an apparent K_m for the RNA substrate of 2.2 nM. The editing enzyme requires no lag period prior to catalysis, and does not assemble into a higher order complex on the RNA substrate. In crude cytosolic extract editing activity is completely abolished by treatment with micrococcal nuclease or RNase A. Partially purified editing enzyme is no longer sensitive to nucleases, but is inhibited in a dose dependent manner by nuclease inactivated crude extract. The buoyant density of partially purified editing enzyme is 1.3 g/ml, that of pure protein. Therefore, the apolipoprotein B mRNA editing activity consists of a well defined enzyme with no RNA component. The nuclease sensitivity in crude cytosolic extract is explained by the generation of inhibitors for the editing enzyme. The editing of apo B mRNA has little similarity to complex mRNA processing events such as splicing and unlike editing in kinetoplastid protozoa does not utilise guide RNAs.

INTRODUCTION

After transcription pre-mRNA undergoes a variety of RNA processing events, including capping, splicing, 3'cleavage and polyadenylation, which generate translatable mRNA. RNA editing is a recently described form of RNA processing which leads to posttranscriptional alterations of coding sequences (1–5). RNA editing has been first described in the mitochondrion of kinetoplastid protozoa (*Trypanosoma, Leishmania and Crithidia*) (6). U residues are posttranscriptionally inserted or deleted at multiple, precise sites in several mRNA species. This creates translatable RNA with open reading frames by correcting internal frameshifts or by generating initiation and termination codons (7–9). Small RNA species have been identified containing the complementary sequences for the edited regions (10,11). These RNA species are separately encoded in the mitochondrial genome and have been termed guide RNAs (12). Therefore, a model for this form of RNA editing has been proposed, where guide RNAs serve as a template for the editing reaction providing the necessary sequence information (12). RNA editing has also been described for several mitochondrial genes of plants, where a number of C residues are converted to U (13–15). These C to U changes alter genomically encoded amino acid sequence and explain the ambiguity observed in the use of the genetic code in the genome of plant mitochondria (13–15). The reverse of this process by a U to C transition has also been found in plant mitochondria (16,17). Most recently, RNA editing by C insertions has been discovered in the acellular slime mould *Physarum polycephalum* (18). In addition, RNA editing by a specific C to U transition generates a translational stop codon in the mRNA of apolipoprotein (apo) B and explains the formation of the two isoforms apo B100 and apo B48 (19,20). Therefore, RNA editing as a specific modulation of gene expression occurs in a wide range of biological systems and appears to be an ancient mechanism (3,4). Whether these different forms of RNA editing are ancestrally related or represent independent developments has yet to be established.

Apo B is a major protein component of plasma lipoproteins (21). In the human intestine the apo B mRNA undergoes a C to U transition at nucleotide position 6666 changing the genomically encoded CAA for glutamine into the translational stop codon UAA (19,20). This RNA editing leads to the formation of the truncated isoform apo B48 in the intestine (19,20). In contrast to humans, rodents express the apo B mRNA editing mechanism at high levels in liver as well, where it is hormonally, metabolically and developmentally regulated (22–24).

An *in vitro* system for editing of apo B has been described using rat hepatoma McArdle 7777 S100 cytosolic extract (25) or rat liver nuclear extract (26). The present investigation was undertaken to study the biochemical properties of the apo B editing activity. Cytosolic S100 extract of rat enterocytes was

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identified as a potent source for editing activity *in vitro*. Specifically, the question was addressed whether apo B mRNA editing resembles other mRNA processing events like splicing of pre-mRNA (27,28) or 3'cleavage and polyadenylation of mRNA (29,30) by requirement for (i) energy in form of nucleotide triphospates, (ii) assembly into a high molecular weight complex and (iii) RNA components in form of ribonucleoproteins. It was particularly important to investigate whether guide RNAs similar to those implicated in the editing of mRNA in kinetoplastid protozoa (10–12) are involved in apo B mRNA editing so as to establish whether an evolutionary link exists between these processes.

MATERIAL AND METHODS

Cytosolic extract or rat enterocytes

Intestinal epithelial cells were isolated using sodium citrate as described (31). Briefly, after CO_2 narcosis male Sprague-Dawley rats were killed by cervical dislocation and the small intestine was removed and washed with 0.9% NaCl solution. The intestine was filled with solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 7.3) and incubated for 15 min at 37°C in 0.9% NaCl solution equilibrated with oxygen. Solution A was replaced with solution B (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.5 mM EDTA, 0.25 mM DTT, pH 7.3) and the incubation continued for 10 min. After careful manipulation of the gut solution B was collected containing most of the mucosal cells. As judged by light microscopy more than 90% of the cells were epithelial. Cells were washed twice with phosphate-buffered saline, at 4°C as all subsequent procedures. Cytosolic S100 extract was prepared according to the method of Dignam (32). Briefly, cells were incubated in four times packed cell volume of Dignam buffer A (10 mM HEPES, 1.5 mM MgCl, 10 mM KCl, 0.25 mM DTT, pH 7.9) for 15 min and homogenised with 25 strokes in a Dounce homogeniser with the tight fitting pestel. The homogenate was checked microscopically for cell lysis and centrifuged for 10 min at 2000 rpm in a Sorvall RT 6000 centrifuge. The cloudy supernatant was adjusted to 50 mM KCl and centrifuged at 100,000 g for 1 h in a Beckmann 70 Ti rotor. The supernatant was dialysed for 8–10 hours against Dignam buffer D (20 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 20% glycerol) and stored at −20°C. The protein concentration of the extract was between 10–25 mg/ml. All solutions contained the following protease inhibitors at a concentration of 10 μg/ml: leupeptin, papain, pepstatin, aprotinin, benzamidine, phenylmethyl-sulphonyl fluoride.

Synthetic Apo B RNA transcripts

Two complementary 55mer oligonucleotides made on a Milligen 7500 DNA synthesiser spanning the edited site of apo B from nucleotide 6649 to 6703 were annealed and cloned into the HindIII–AccI site of the plasmid pBluescript KS generating plasmid pBS55 (25). Plasmid DNA was linearised with HindIII and transcribed using T3 RNA polymerase (Boehringer Mannheim) under standard conditions. The transcription reaction was treated with RNase-free DNaseI, phenol/chloroform extracted and purified on Sephadex G50 spin columns (Pharmacia). The RNA was ethanol precipitated, redissolved in distilled water and its concentration was estimated by optical density at 260 nm. Usually the RNA preparations had a 260:280 ratio of around or above 2. This transcription generates an RNA of 102 nucleotides and therefore the molecular weight was assumed to be 35,000. The molarity of the RNA was calculated and appropriate dilutions were made.

As an alternative substrate for the apo B mRNA editing enzyme RNA was transcribed with T7 RNA polymerase (Boehringer Mannheim) from plasmid pRSA13 containing a 448 bp RsaI fragment from human apo B cDNA spanning the edited site from nucleotide 6413 to 6860 (25). This transcription reaction generates an RNA of 498 nucleotides with a calculated molecular weight of 172,000.

In vitro editing reaction

Enterocyte S100 extract was incubated in 10 mM Hepes (pH 7.9), 50 mM KCl, 50 mM EDTA, 10 % glycerol, 0.25 mM DTT, 10 units RNase inhibitor (RNAguard, Pharmacia) with the synthetic RNA in a final volume of 20 μl. Usually 10–30 μg protein was used per assay. Under standard conditions the incubation was performed for 1 h at 30°C with 2 nM RNA substrate. The RNA was recovered by ethanol precipitation after proteinase K digestion and phenol/chloroform extraction.

Primer extension analysis for editing of apo B mRNA

In vitro editing of cytidine at position 6666 to uridine was assayed by primer extension on the synthetic RNA substrate in the presence of ddGTP as described previously (25) except that the priming oligonucleotide was labelled to a specific activity of at least 1×10^9 dpm/μg. This generates an extension product of 42 nucleotides for RNA that contains the original C at position 6666 or 53 nucleotides when this C is edited to U. The extension products were separated on a 8.5% polyacrylamide, 7 M urea gel. The gels were analysed by autoradiography. Quantitation of editing was performed by liquid scintillation counting of the excised extension products on a Packard TriCarb 22000 CA liquid scintillation counter with a quench programm with external standard. In pilot experiments recovery of labelled oligonucleotides between 100 and 400000 cpm was quantitative. For quantitation of *in vitro* editing assays were performed in triplicates each. Enzymatic activity was expressed as specific activity in terms of edited RNA per h per μg protein.

Glycerol gradient sedimentation analysis

0.2 ml of rat intestinal extract was diluted twofold with 20 mM Hepes, 50 mM KCl and 50 mM EDTA, layered on top of a linear 10 ml gradient containing 15%-35% glycerol in 20 mM Hepes (pH 7.9), 1 mM $MgCl_2$, 100 mM KCl and 0.2 mM EDTA and centrifuged in a Beckmann SW 41 Ti rotor at 100,000 g for 8 h at 4°C. Gradients were fractionated from the bottom into twelve aliquots of 0.8 ml and 10 μl were assayed for *in vitro* editing activity. Calibration of the gradients was performed in parallel runs with apoferritin (18S, 440 kDa) and catalase (11S, 240 kDa) as sedimentation markers. To investigate whether the editing enzyme assembles on the RNA substrate into a higher molecular weight complex 0.2 ml extract was incubated in a final volume of 0.4 ml under conditions for *in vitro* editing (20 mM Hepes, pH 8.0, 50 mM KCl, 50 mM EDTA) in the presence of 5 nM RNA substrate for 30 min at 30°C and then subjected to glycerol gradient sedimentation analysis as described above. The individual fractions were assayed for activity of the editing enzyme as outlined above. In order to assay for the distribution of the RNA substrate RNA was extracted from 200 μl of each fraction by phenol/chloroform extraction and ethanol precipitation and analysed by primer extension analysis as described above.

In control gradients RNA substrate was sedimented under identical conditions in the absence of rat enterocyte cytosolic extract. As substrate RNA in these experiments the 498 nucleotide RNA transcribed from pRSA13 was used.

Micrococcal nuclease treatment of extract

About 20–30 μg protein of rat intestinal cytosolic extract in a final volume of 10 μl was incubated for 15 min at 30°C in the presence of 30 units micrococcal nuclease (Pharmacia or Boehringer Mannheim) with or without 2 mM CaCl. In order to inactivate micrococcal nuclease EDTA at 100 mM was included to chelate calcium. After the preincubation period EDTA was added to all assays and subsequently the editing reaction was performed in a final volume of 20 μl, precisely as described above, in the presence of 50 mM EDTA.

RNase A treatment of extract

200 μl extract was incubated with 2 mg of polyacrylamide beads coupled with RNase A (RNase A Enzygel, Boehringer Mannheim, 100 units according to Kunitz per gram beads) that had been extensively prewashed with Dignam buffer D. At various timepoints the supernatant was tested for editing activity. To specifically inactivate the RNase A in control experiments 25 μl of human placental RNase inhibitor (RNAguard, Pharmacia, 40 units/μl) was included during the incubation with the beaded RNase A. In initial experiments the amount of RNase A sufficient to abolish editing was titrated. Centrifugation completely removed the beaded RNase A from the extract as shown by the unaffected primer extension analysis of the RNA substrate and also in separate control experiments with radiolabelled RNA substrate.

Oligonucleotide directed cleavage with RNase H

The following overlapping oligonucleotides spanning the editing site in the apo B mRNA from position 6651 to 6703 were made on a MilliGen 7500 DNA synthesiser and purified on acrylamide gels:

I. CAG ACA TAT ATG ATA CAA (6651 to 6668)
II. CAA TTT GAT CAG TAT ATT (6666 to 6683)
III. CAG TAT ATT AAA GAT AGT (6675 to 6692)
IV. AAA GAT AGT TAT GAT TTA (6684 to 6701)
V. CTT CTT CTT CTT CTT unspecific control oligonucleotide
VI. TAT ATG ATA CAA TTT GAT CAG T (6657 to 6678)
VII. TAT ATG ATA CAA TTT GAT C (6657 to 6675)
VIII. TAT ATG ATA CAA TTT G (6657 to 6672)
IX. CAA TTT GAT CAG TAT ATT AAA GAT AGT TAT GAT TTA CA (6666 to 6701)

Oligonucleotides were tested for an effect on *in vitro* editing at 0.1 μg/μl final concentration (16 μM). To test for oligonucleotide directed cleavage with RNase H extract was preincubated for 30 min at 30°C with oligonucleotides at 0.1 μg/μl and 0.1 units RNase H/μl (Boehringer Mannheim) in the presence of 5 mM MgCl and 50 μM ATP as described (33). *In vitro* editing was performed for 1 h as described above except that EDTA was completely omitted from the reaction. To avoid interference of the oligonucleotides with the subsequent primer extension assay the samples were digested for 20 min with 20 units RNase-free DNaseI (Boehringer Mannheim) prior to proteinase K digestion and phenol extraction. As assessed in separate experiments the primer extension assay was unaffected.

Partial purification of the editing enzyme

Chromatography on DEAE-Sepharose. 100 ml of rat intestinal extract (10 mg protein per ml) in 20 mM Hepes, 50 mM KCl and 1 mM EDTA, pH 7.3, was applied to DEAE-Sepharose (Pharmacia), column size 8 cm×2.5 cm, at a flow rate of 1 ml per min. Bound protein was eluted with a linear salt gradient (50–400 mM KCl), fractions of 5 ml were collected, dialysed against 20 mM Hepes, 50 mM KCL, 1 mM EDTA, 20% glycerol and assayed for *in vitro* editing.

Chromatography on heparin-agarose. Active fractions after chromatography on DEAE-Sepharose were pooled and applied to heparin-agarose (Sigma), column size 6 cm×1.5 cm, in 20 mM Hepes, 50 mM KCl, 1 mM EDTA, 20 percent glycerol, pH 7.3 at a flow rate of 0.5 ml/min. Bound protein was step-eluted by raising the KCl concentration to 300 mM and after dialysis tested for editing activity.

CsCl buoyant density ultracentrifugation

CsCl buoyant density ultracentrifugation was performed as described (29). 5 ml of by chromatography on DEAE-Sepharose and heparin-agarose partially purified editing enzyme was adjusted to 15 mM MgCl and CsCl was added to a final density of 1.45 g/ml. This was overlaid by 1 ml of CsCl solution with a density of 1.45 g/ml and with another 5 ml of a density of 1.33 g/ml and centrifuged to equilibrium in a Beckmann 70.1 Ti rotor at 200,000 g for 36 h at 4°C. Fractions of 0.5 ml were harvested from the bottom and their density determined by weighing and also by measuring the refractive index. After extensive dialysis against Dignam D buffer with several buffer changes fractions were assayed for editing activity and protein concentration.

RESULTS

Biochemical characterisation of the apo B mRNA editing activity

To study the biochemical properties of the apo B editing activity a quantitative primer extension assay for *in vitro* editing was established. Rat enterocyte cytosolic extract proved to be a good source for editing activity *in vitro*. The synthetic RNA template of the *in vitro* editing reaction was extracted and reverse transcribed in the presence of ddGTP with a priming oligonucleotide ending 8 basepairs downstream of the edited C of the apo B mRNA as described (25). An extension product of 42 basepairs is generated for RNA that is not changed, but edited RNA results in a 53 basepair extension product. After separation of these two extension products on a polyacrylamide sequencing gel and subsequent autoradiography, edited versus not edited RNA was quantitated by liquid scintillation counting of the excised extension products.

In vitro editing in rat intestinal cytosolic extract was demonstrated within 5 min and increases linearly within 1 hour (Fig.1). The editing reaction has a temperature optimum around 30°C; above 55°C no editing can be detected (Fig.2). Independent of the buffering substances used, the pH-optimum for the editing reaction was 8.5 (Fig.2). *In vitro* editing increases linearly with protein concentration (Fig.2) and is linear in time up to 2 hours at low (0.5 nM) as well as high (10 nM) concentrations of RNA substrate (Fig.2). Subsequently all reactions were performed for 1 h at 30°C at a pH of 8.5 with 20–30 μg protein per assay. Under these conditions between 8–12% of the RNA substrate at low concentrations (0.5 nM)

and 1.0–1.5% of the RNA substrate at high concentrations (5 nM) was edited by 20–30 μg protein of the cytosolic extract, depending on the quality of the extract preparation used.

To further optimise the *in vitro* editing reaction nucleotides as potential cofactors were tested for an effect on editing. Supplementation of extracts with uridine, adenosine, cytidine or guanosine 5′ mono-,di- or triphosphate did not stimulate editing activity. Depletion of residual triphosphates as potential energy sources by pretreating the extracts with alkaline phosphatase or apyrase had no effect on the editing activity. This is consistent with the editing activity not being sensitive to exhaustive dialysis, which removes low molecular weight components.

Previously, we have shown, that EDTA enhances editing activity in McArdle Rh7777 cytosolic extract (25). The editing activity in rat enterocyte cytosolic extract was also enhanced by EDTA, but not dependent on its presence. Therefore, EDTA at 50 mM was normally included in the editing reaction. In order to investigate this EDTA effect divalent cations were tested in the editing reaction. Iron, nickel, cobalt, zinc, copper and cadmium at a concentration of 2 mM all inhibited editing by more than 80%. Calcium and magnesium at a concentration of 2 mM increased the editing reaction by about 20%. An absolute requirement for these cations in the editing reaction, however, does not exist, as shown by editing activity in the presence of 50 mM EDTA.

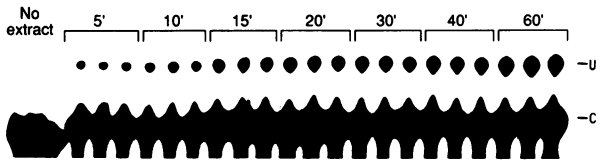

Figure 1. Time course of editing activity in rat enterocyte S100 extract. 30 μg protein of rat enterocyte S100 extract was incubated with 2 nM RNA substrate in 20 mM Hepes, 50 mM KCl, 50 mM EDTA, 10% glycerol, pH 8.0, in a final volume of 20 μl for the indicated period of time. RNA was extracted and assayed for editing by primer extension analysis, an 8 h autoradiograph is shown.

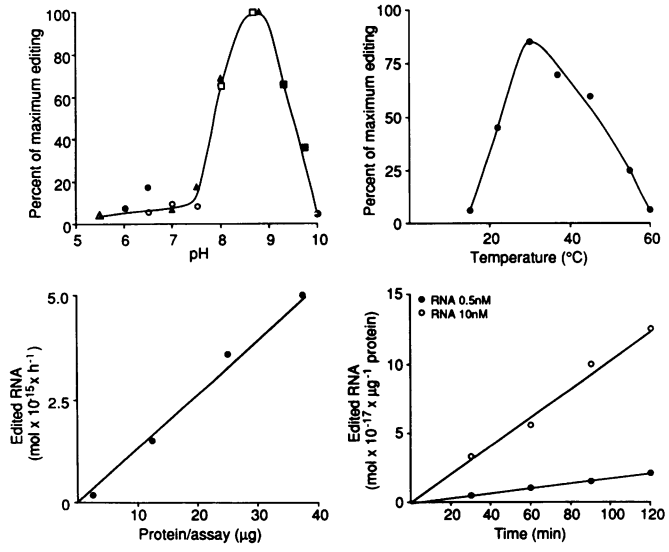

Figure 2. Optimisation of editing activity in rat enterocyte S100 extract. Under standard conditions the influence of pH (top left), temperature (top right), protein concentration (bottom left) and time at 0.5 and 10 nM RNA substrate concentration (bottom right) was studied. Editing assays were performed in triplicates and quantitated by liquid scintillation counting of the excised extension products after polyacrylamide gel electrophoresis. The pH optimum was shown to be independent of the buffering substance used: △ acetate, ● bis-Tris, ○ MOPS, ▲ HEPES, □ EPPS, ■ CHES, ⊕ CAPS.

After optimisation of conditions for measurement of initial velocity V_0 of apo B mRNA editing in rat enterocyte S100 extract we studied the dependence of enzyme activity on substrate concentration. Formation of edited RNA increased with RNA substrate concentrations. Saturation was reached at around 5 nM (Fig.3). The double reciprocal plot according to Lineweaver-Burk gave a straight line (Fig.3). The regression analysis of three independent experiments of $^1/_v$ on $^1/_s$ resulted in correlation coefficients of 0.993, 0.993 and 0.997 with a mean value for the apparent K_m of 2.19 ± 1.66(SD)nM and a mean value for Vmax of 1.2 ± 0.346(SD) 10^{-16}mol h^{-1} μg^{-1}protein.

Nearly identical kinetic parameters were obtained when instead of the 102 nucleotide RNA substrate transcribed from pBS55 a 498 nucleotide RNA substrate transcribed from pRSA13 was used. Especially, linearity of *in vitro* editing within 1 h and substrate saturation around 5 nM was also demonstrated with this longer RNA substrate.

By heating extracts to 60°C for 5 min or by treating with proteinase K (25) editing activity was destroyed. Editing was also abolished by 2 mM of either N-ethylmaleimide, p-chloromercurybenzoate, phenylglyoxal or diethylpyrocarbonate, substances known to derivatise the sulphydryl-groups of cysteine, the guanidinium-group of arginine and the imidazolium-group of histidines. Therefore, the editing activity must be or contain a protein.

The editing enzyme does not assemble into a high molecular weight complex on the RNA substrate

When rat enterocyte cytosolic extract was subjected to glycerol gradient sedimentation, the editing enzyme sedimented around 11 S. Sedimentation analysis was used to investigate whether the apo B mRNA enzyme assembles into a higher order complex on its RNA substrate, as has been demonstrated for the assembly of splicing and polyadenylation specific complexes. In order to resemble most closely the natural substrate of the editing enzyme in these experiments the 498 nucleotide RNA substrate was used.

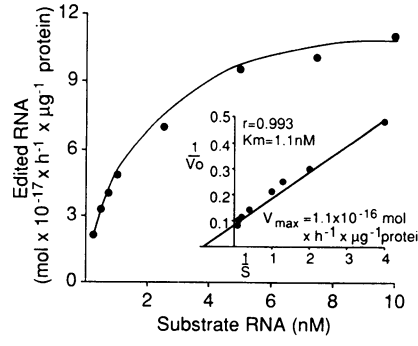

Figure 3. Substrate dependence of editing activity in rat enterocyte S100 extract. Editing assays were performed under optimised conditions for 1 h with increasing concentrations of RNA substrate. Edited RNA was quantitated by liquid scintillation counting of the excised extension products. Regression analysis of $^1/_s$ on $^1/_v$ was performed according to Lineweaver-Burk. Results of a single experiment in triplicate assays are shown.

After a preincubation period of 30 min under conditions of *in vitro* editing with 5 nM of RNA substrate glycerol gradient sedimentation analysis was performed and the distribution of the editing enzyme and the RNA substrate on the gradient was analysed. A typical experiment is shown in figure 4. The editing enzyme did not show a mobility shift after preincubation with the 498 nucleotide RNA substrate compared with a gradient under identical conditions where rat enterocyte cytosolic extract was sedimented without preincubation with the RNA substrate. In both cases the editing enzyme sedimented around 11 S (Fig. 4 a and b). The distribution of the RNA substrate on the gradients was analysed by primer extension analysis after phenol/chloroform extraction and ethanol precipitation. The RNA substrate sedimented in a broad peak around 11 S and within this peak edited and unedited RNA distributed equally (Fig.4 c). On control gradients, where RNA was sedimented without extract, the RNA sedimented in a similar range (Fig. 4 d). Both the editing enzyme and the RNA substrate sedimented well above the marker for 18 S, apoferritin with a molecular weight of 440 kDa. Similar results were obtained when the 102 nucleotide RNA was used as substrate and also under different centrifugation conditions.

Editing activity in S100 extract is abolished by micrococcal nuclease and RNase A, but resistant to RNase H

RNA molecules are known to be involved in many RNA processing events and have recently been shown to participate in RNA editing in kinetoplastid protozoa (10–12). Therefore, we have addressed the issue as to whether the apo B mRNA editing enzyme contains an RNA component. To test for a requirement for RNA, the editing extract was treated with micrococcal nuclease. The use of micrococcal nuclease to demonstrate an RNA requirement is well established. It has been applied to demonstrate the involvement of RNA in pre-mRNA splicing (27), histone pre-mRNA maturation (34) and pre-ribosomal RNA processing (35). High concentrations of micrococcal nuclease are generally required to disrupt a ribonucleoprotein particle (27,34–36). Therefore, extract was pretreated with increasing concentrations of micrococcal nuclease. The activity of micrococcal nuclease has an absolute requirement for calcium. By chelating calcium with EGTA or EDTA the micrococcal nuclease is completely inactivated. In our normal editing reaction 50 mM EDTA is present. This is sufficient to chelate the 2 mM calcium used in the pretreatment phase. Therefore, our normal editing mixture is adequate to inactivate micrococcal nuclease. With increasing concentrations of micrococcal nuclease the editing activity was progressively inhibited (Fig.5). Inhibition was apparent with concentrations of micrococcal nuclease as low as 60 U/ml, complete inhibition normally required about 3000 U/ml. In order to rule out the phenomenon of 'substrate masking', as described for the artifactual nuclease sensitivity of chloroplast t-RNA processing (37), micrococcal nuclease treated extract was supplemented with yeast total RNA up to 0.5 mg/ml as competitor for the inactivated micrococcal nuclease and also with poly(A) to 0.5 mg/ml, as recommended (38), but this did not restore activity (results not shown). Micrococcal nuclease inactivated extract could also not be reactivated by adding back heat inactivated S100 extract or total intestinal RNA prepared from S100 extract after proteinase K digestion in the presence of 1% SDS (results not shown).

If the editing activity contains an RNA species, as suggested by its sensitivity to micrococcal nuclease and resistance to RNase-free DNaseI, the editing activity should also be affected by pretreatment with RNase A. To circumvent problems of inactivation RNase A coupled to polyacrylamide beads was used which can be removed by centrifugation. Titration of RNase A showed again a dose-dependent inactivation of editing activity. At about 1 Kunitz unit RNase A per ml editing activity was nearly completely abolished within 10 minutes. The specificity of this inactivation was tested using a specific proteinaceous inhibitor for RNase A from human placenta. While within 10 min editing

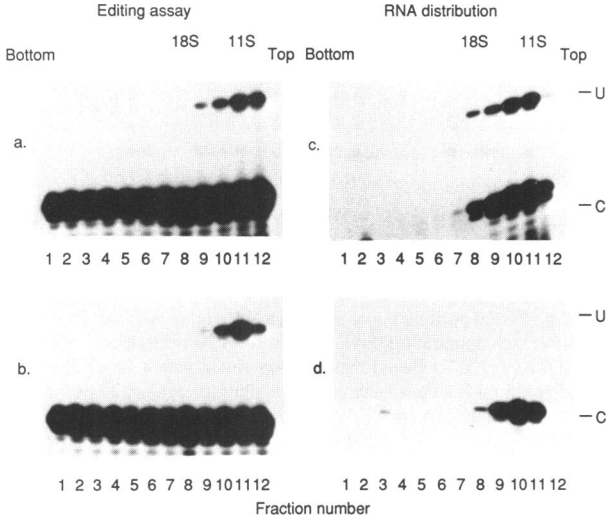

Figure 4. Glycerol gradient sedimentation analysis of editing activity in rat enterocyte S100 extract. After a preincubation period of 30 min under conditions for *in vitro* editing with 5 nM of the 498 nucleotide RNA substrate 400 µl of rat enterocyte cytosolic extract was subjected to glycerol gradient sedimentation analysis (15%-35%) at 100,000 g for 8h. Gradients were harvested from the bottom into 12 fractions of 0.8 ml. 10 µl of each fraction was analysed for *in vitro* editing activity (4 a) and RNA from 200 µl of each fraction was extracted by phenol/chloroform and ethanol precipitation and analysed by primer extension assay (4 c). In a control gradient 400 µl of rat enterocyte cytosolic extract was sedimented without preincubation with the RNA substrate and the fractions of the gradient were analysed for in vitro editing activity (4 b). In a further control gradient 400 µl buffer containing 5 nM RNA substrate was sedimented and the distribution of the RNA was assayed by primer extension analysis (4 d). All gradients represent a parallel run.

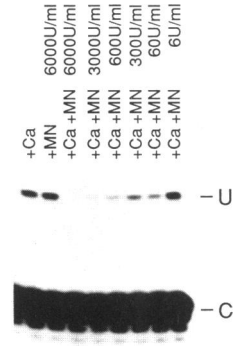

Figure 5. Micrococcal nuclease sensitivity of editing activity in rat enterocyte S100 extract. 30 µg of S100 extract in 10 µl Dignam buffer D was incubated for 15 min at 30°C in the presence of 2 mM CaCl alone, 60 units micrococcal nuclease alone, or 2 mM $CaCl_2$ plus increasing concentrations of micrococcal nuclease, as indicated. Subsequently, *in vitro* editing reactions were performed under standard conditions. Editing was assayed by primer extension analysis, a 5h autoradiograph is shown.

activity was progressively inhibited by the beaded RNase A, addition of the RNase A inhibitor completely prevented this inhibition (Fig.6).

Next we tested the hypothesis that the observed nuclease sensitivity of the editing enzyme reflects the contribution of an RNA species base-pairing with the apo B mRNA. This would resemble the editing mechanism as observed in kinetoplastid protozoa, where guide RNAs are considered to basepair with the edited region (10−12). RNase H selectively cleaves the RNA strand of a RNA-DNA heteroduplex. Presumptive guide RNAs should be cleaved when S100 extract is incubated with RNase H together with oligonucleotides spanning the edited region of apo B mRNA.

Several overlapping oligonucleotides were tested without and with RNase H for an effect on editing activity *in vitro*. Despite a variety of conditions tested none of the oligonucleotides reproducibly affected the *in vitro* editing reaction on its own or after preincubation together with RNase H (results not shown). These results made it unlikely that the nuclease sensitivity of the editing enzyme was due to an interaction of the editing enzyme with the RNA substrate by base pairing, for example via guide RNAs.

Partially purified editing enzyme is resistant to nucleases but inhibited by nuclease-inactivated crude extract

In order to further investigate whether the editing enzyme contains an integral RNA component, partially purified editing enzyme was examined for nuclease sensitivity. After chromatography on DEAE-Sepharose the editing activity was purified about 4-fold over S100 extract as assessed by measuring the specific activity as outlined above. It still exhibited nuclease sensitivity in the same way as crude extract (Fig.7A and B). However, after further purification on heparin agarose, leading to a 20-fold increase in specific activity, the editing activity became resistant to nuclease treatment (Fig.7A and B). When this partially purified editing enzyme was supplemented with nuclease-inactivated crude S100 extract, editing activity was completely inhibited in a dose-dependent manner. (Fig.7B). Untreated, active S100 extract added back to partially purified editing activity did not inhibit *in vitro* editing (results not shown). Similarly, in crude cytosolic extract the editing enzyme was sensitive to pretreatment with

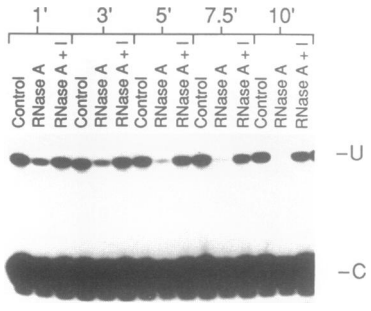

Figure 6. RNase A inactivation of editing activity in rat enterocyte S100 extract. 200 μl of rat intestinal extract containing 8 mg of protein was incubated between 1 and 10 min without any addition (control), with 2 mg of polyacrylamide beads coupled to RNAse A (specific activity 100 units/g beads) (RNase A) or with 2 mg of polyacrylamide beads coupled to RNase A in the presence of 25 μl RNase A inhibitor (RNase A + I). The supernatant at each time point was tested for editing activity. A 5 h autoradiograph of the primer extension assay is shown.

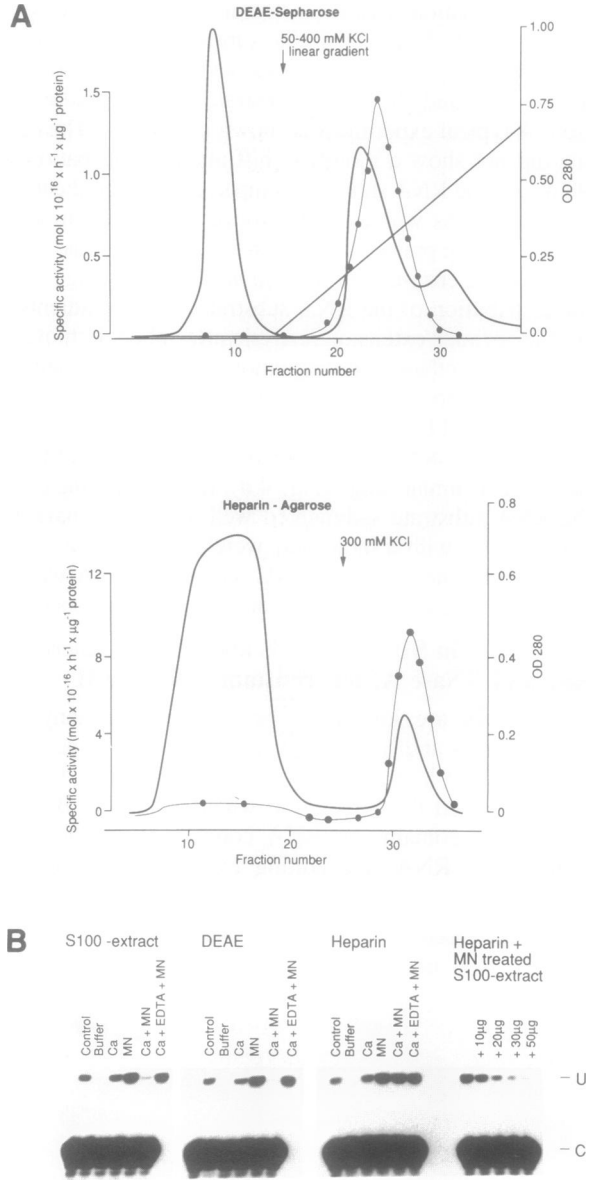

Figure 7A : Partial purification of apo B mRNA editing enzyme. Rat enterocyte S100 extract was applied to DEAE-Sepharose in 20 mM Hepes, 50 mM KCl, 1 mM EDTA, pH 7.3 . Bound material was eluted with a linear 50−400 mM KCl gradient. 5 ml fractions were collected, dialysed against 20 mM Hepes, 50 mM KCL, 1 mM EDTA, pH 7.3, and tested for *in vitro* editing activity with the RNA substrate at 2 nM. Editing was quantitated by liquid scintillation counting of the excised extension products and specific activity (mol edited RNA μg protein^{-1}h^{-1}) was calculated. Active fractions were pooled and applied in 20 mM Hepes, 50 mM KCl, 1 mM EDTA, pH 7.3, to heparin-agarose. Bound material was step-eluted with 300 mM KCL. After dialysis bound and unbound material was tested for editing activity (2 nM RNA substrate) and specific activity was calculated. **B.** Micrococcal nuclease treatment of partially purified editing enzyme. Crude S100 extract (40 μg), DEAE-Sepharose peak fraction (15 μg) and heparin-agarose eluate (3 μg) were incubated in 10 μl 20 mM Hepes, 50 mM KCl, 20% glycerol, pH 7.3, for 15 min at 30°C without any addition (first lane each), with 2 mM $CaCl_2$ (third lane each), with 30 units micrococcal nuclease (forth lane each), with 2 mM $CaCl_2$ plus 30 units micrococcal nuclease (fifth lane each) or with 2 mM $CaCl_2$ plus 100 mM EDTA plus 30 units micrococcal nuclease (sixth laneeach). *In vitro* editing reactions were performed for 1 h in a volume of 20 μl in the presence of 50 mM EDTA. By chromatography on heparin-agarose partially purified editing activity (3 μg protein) was supplemented with increasing concentrations (10 to 50 μg protein) of micrococcal nuclease inactivated crude S100 extract (15 min, 30°C, 2 mM $CaCl_2$, 3 units micrococcal nuclease/μl) prior to *in vitro* editing for 1 h under standard condition (right panel). A 3 h autoradiograph of the primer extension assay is shown.

RNase A, but after chromatography on heparin-agarose the editing enzyme was insusceptible to digestion with RNase A. Furthermore, crude extract inactivated with RNase A inhibited partially purified editing enzyme (results not shown). The nuclease sensitivity of the editing enzyme in crude extract and its resistance to nucleases after partial purification was also demonstrated when the 498 nucleotide RNA substrate was used for the *in vitro* editing reaction. Taken together, these experiments strongly indicated that the editing enzyme does not contain an RNA component and that the observed nuclease sensitivity is most likely due to the generation of inhibitors for the editing reaction by nuclease digestion. These consist most probably of small RNA molecules generated by partial nuclease digestion of endogenous RNA species (39).

Apo B mRNA editing enzyme has the density of pure protein

In order to assess more directly that the editing enzyme consists of pure protein without integral RNA component, the density of the editing enzyme was determined by CsCl buoyant density ultracentrifugation after partial purification on DEAE-Sepharose and heparin-agarose. Ribonucleoproteins usually have a density well above pure protein due to their RNA component (29). The editing enzyme, however, banded with the bulk of the protein around a density of 1.3 g/ml, the density of soluble protein (Fig.8). On CsCl buoyant density ultracentrifugation of crude S100 extract the editing enzyme banded at exact the same density and was also resistant to digestion with micrococcal nuclease or RNase A (results not shown). These results confirm that the editing enzyme is a pure protein without essential RNA component.

DISCUSSION

The present investigation and other recent results (40,41) allow important conclusions about the biochemical nature of the apo B mRNA editing activity and its relationship to other mRNA processing events. The apo B mRNA editing activity has no nucleotide cofactor requirement, demonstrates no lag period for the editing process to become active, does not assemble into a higher order complex on its RNA substrate and shows substrate saturation described by the Michaelis-Menten equation. The editing enzyme consists of pure protein and does not require an RNA component for activity. Therefore, apo B mRNA editing bears little analogy to more complex mRNA processing events such as splicing (27,28) or 3′ cleavage and polyadenylation of pre-mRNA (29,30) as these are energy consuming, require assembly of multicomponent complexes and involve small nuclear ribonucleoproteins (snRNP). In addition, our results demonstrate apo B mRNA editing to be quite distinct from editing in kinetoplastid protozoa, that uses guide RNAs for recognition of the edited site (10−12).

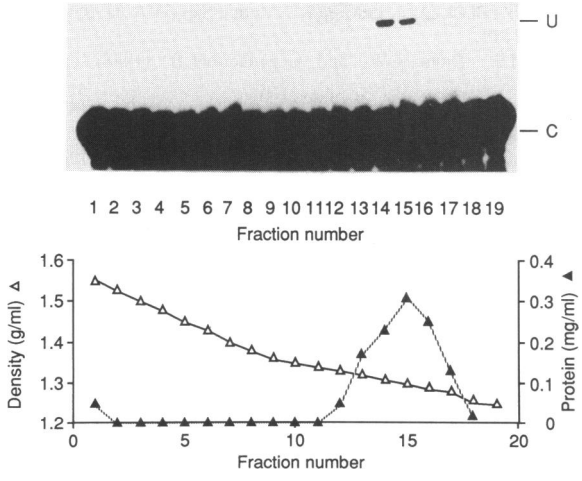

Figure 8. CsCl buoyant density ultracentrifugation of partially purified editing enzyme. 3 ml of by chromatography on DEAE-Sepharose and heparin-agarose partially purified editing enzyme was subjected to CsCl density ultracentrifugation, the gradients were harvested from the bottom in 0.5 ml fractions and their density and protein concentration was measured. After dialysis fractions were tested for editing activity. The primer extension assay and the density and protein distribution over the gradient is shown.

Rat enterocyte S100 extract was demonstrated to be a good source for apo B mRNA editing *in vitro* that allowed an enzymatic characterisation of the editing activity. Editing *in vitro* was optimised for temperature, pH and protein concentration and shown to be linear in time from 5 minutes up to 2 hours. Positive nucleotide or ion cofactors were not identified. Editing is independent of energy in form of nucleoside triphospates as shown by unimpaired editing activity after treatment of extract with alkaline phosphatase. The absence of low molecular weight cofactors is further supported by the failure of dialysis or partial purification to affect editing activity. Editing activity in S100 extract shows Michaelis-Menten kinetics. These results suggest that a single enzyme mediates apo B mRNA editing *in vitro*. Consistent with the concept of a single enzyme are the recent findings that apo B mRNA editing creates a genuine uridine from cytidine without cleaving the phosphodiester backbone of the RNA (40,41). Therefore, the assumption that the editing enzyme is a site-specific cytidine deaminase, as previously proposed as the simplest explanation for the mechanism of apo B mRNA editing (25,42), is now supported by several lines of evidence (40,41).

Many mRNA processing events require assembly of a complex and it was therefore investigated whether also the apo B mRNA editing activity assembles on the RNA substrate into a higher order complex. On glycerol gradients a mobility shift of the editing enzyme after preincubation with the RNA substrate was not observed. The editing enzyme sedimented with the bulk of the protein around 11 S. It has been recently reported that edited apo B mRNA is preferentially found in multicomponent complexes around 27 S, as assessed by glycerol gradient sedimentation analysis and native gel electrophoresis of rat liver cytosolic extract (43). However, direct evidence that these so called 'editosomes' (43) contain apo B mRNA editing activity has not been provided. In our preparation of rat enterocyte cytosolic extract (i) the editing enzyme does not assemble into a higher order complex on the RNA substrate and (ii) the edited RNA substrate does not sediment with a sedimentation coefficient of 27 S. Instead, edited and unedited RNA sediment equally distributed in a broad peak around 11 S. Therefore, our results do not support the suggestion that the editing enzyme requires assembly into a higher order complex (43). This conclusion is also based on the kinetic analysis of the editing activity. If assembly into a multicomponent complex was required for editing of apo B mRNA *in vitro*, a lag period might proceed the appearance of edited RNA. *In vitro* splicing requiring assembly of the spliceosome can only be detected after a lag period of about 30 minutes (44,45). However, in our system *in vitro* edited RNA

is clearly demonstrated within 5 minutes and increases linearly from 5 minutes up to 2 hours.

A further issue addressed in this investigation was the possible involvement of an RNA component in apo B mRNA editing. To disrupt snRNPs high concentrations of nucleases are generally required (34–36). The inactivation of the editing activity in crude extract by micrococcal nuclease is dose-dependent. At very low concentrations (6 units/ml) no inhibition was observed. This is in line with another report on editing activity in baboon enterocyte S100 extract, in which also at 5 units/ml no inhibition was observed (46). However, this concentration is not sufficient to disrupt most ribonucleoproteins, as demonstrated in previous investigations (27,34–36). Since treatment with RNase A also inactivated editing activity in rat enterocyte S100 extract, the nuclease sensitivity for the apo B mRNA editing enzyme was studied further. If the nuclease sensitivity were to reflect an integral RNA component, it should be retained during purification of the editing enzyme. However, after chromatography on DEAE-Sepharose and subsequently heparin-agarose the editing enzyme became resistant to nuclease treatment. The nuclease sensitivity could be simulated by adding back nuclease inactivated crude S100 extract. Therefore, we concluded that inhibitors for *in vitro* editing are generated by nuclease digestion and cause the nuclease sensitivity. A similar phenomenon explained a dispute about RNA requirement for protein import into mitochondria. The original report failed to demonstrate the generation of inhibitory substances by RNase treatment despite of adding back nuclease treated extract to untreated, active extract (47). Others then showed inhibition by nuclease digestion of ribosomes, which themselves are not necessary for mitochondrial import of proteins (48). Similarly, our initial control experiments failed to detect the generation of inhibitors as equal amount of nuclease treated extract does not sufficiently inhibit active extract. The conclusion that the editing enzyme consists of protein alone is confirmed by the density of 1.3 g/ml on CsCl buoyant density ultracentrifugation, that of pure protein. These two features of the editing enzyme, the complete nuclease resistance in a partially purified state and the density of 1.3 g/ml, make it highly unlikely, that an RNA species contributes to the editing reaction of apo B mRNA.

The present investigation demonstrates the apo B mRNA editing activity in rat enterocyte S100 extract as an enzyme that mediates the C to U transition without low molecular weight cofactor requirement. Apo B mRNA editing is very different mechanistically from editing in kinetoplastid protozoa. It also has little relationship to basic and conserved mRNA processing events like splicing or 3'cleavage and polyadenylation as it does not require energy, assembly of a higher order complex or RNA components. These findings suggest that the apo B mRNA editing enzyme represents a separate and probably more recent evolutionary development and may not have its origin in the ancient 'RNA-world' (2–4,49).

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