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Tissue-Specific Differences in the Role of RNA 3′ of the Apolipoprotein B mRNA Mooring Sequence in Editosome Assembly

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Site-specific editing of apolipoprotein B (apoB) mRNA by the cytidine deaminase, APOBEC-1 is proposed to require interactions of auxiliary protein(s) with an eleven nucleotide element, the mooring sequence, located 3′ of the C → U editing site. An analysis of the RNA sequence dependence for protein-RNA interactions and editosome assembly in rat liver and the small intestine demonstrated that the mooring sequence was a minimal requirement for these interactions. Sequences 3′ of the mooring sequence either interacted with 66 kDa and 44 kDa proteins or enhanced the interactions of these proteins with the mooring sequence. The data also suggested tissue-specific differences in the relative importance of the 3′ cis-acting 'enhancer' elements in the efficiency or stability of editosome assembly. We propose that the previously demonstrated differences in apoB mRNA editing efficiency and its regulation in liver and intestine may in part be due to differences in auxiliary protein interactions with apoB mRNA 3′ of the mooring sequence.

Apolipoprotein B (apoB) mRNA editing involves the site specific deamination of a cytidine at nucleotide 6666 (1,2). This converts a glutamine codon (CAA) to an in-frame translation stop codon (UAA) enabling the translation of a truncated protein, apoB48 instead of the full length protein, apoB100. These protein variants have different physiological effects on lipoprotein assembly, secretion and uptake by peripheral cells. Only very low density lipoproteins (VLDL) containing B100 are converted to low density lipoproteins (LDL), an atherogenic risk factor.

The apoB RNA editing site is a tripartite arrangement of cis-acting elements consisting of an enhancer element 5′ of C6666 and a spacer element and mooring sequence 3′ of C6666 (3–5). The 'mooring sequence' is necessary and sufficient for editing when placed in an appropriate context of flanking RNA sequence and background of cellular protein factors (6–9).

The cytidine deaminase responsible for apoB mRNA editing, APOBEC-1 (10–13) cannot edit RNA without yet-to-be fully characterized proteins referred to collectively as auxiliary proteins (5, 6, 9, 10–16). To date, candidate auxiliary proteins include heterogeneous ribonucleoproteins (hnRNP) C and an hnRNP A/B homolog ABBP-1 (17, 18), mooring sequence-selective RNA binding proteins of 100 kDa, 66 kDa and 55 kDa (7, 9, 15, 19–21), general RNA binding proteins 40-44 kDa (7, 9, 15, 19, 20, 22) and a protein complex, AUX240, identified by a 240 kDa antigen (15, 23).

In contrast to the well defined requirement for RNA secondary structure in A to I editing (24), RNA secondary structure does not appear to be necessary for apoB RNA editing site recognition. Secondary structures have been predicted in the vicinity C6666 (5, 26, 27) but these are potentially unstable due to the AT-richness (78%) of the region. Secondary structure mapping of the region with single and double strand-specific RNases have yielded ambiguous results (27). In fact, strong secondary structures placed proximal to the apoB RNA editing site inhibited editing in cells transfected with apoB RNA constructs in which splice junctions had been inserted within 200 nt of C6666 (25). *In vitro* editing was also inhibited by mutations that improved base-paring and thereby stabilized secondary structure surrounding the non-base paired bubble containing C6666 {Backus, J., Ph.D. thesis, University of Rochester}. Furthermore, mapping studies have so far only been performed on naked RNA,

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leaving open the possibility that secondary structure might be important but that it is selected and/or stabilized through RNA-protein interactions within the editosome (26).

Only three other mRNAs have been shown to support mooring sequence dependent C → U editing (28–30). The dearth of mammalian mRNAs that undergo C → U editing is a paradox given that the human, mouse and rat sequence databases contain numerous cDNA sequences with regions of high identity with the apoB mRNA mooring sequence (unpublished computational analysis, M. F. Forsythe, D. Landsman, J. W. Backus, and H. C. Smith). The mooring sequence alone therefore is not a good predictor of editing of other mRNAs despite the apparent sufficiency of the tripartite motif in determining site-specific editing in the context of apoB mRNA.

A partial explanation may be that APOBEC-1 and/or the same complement of auxiliary proteins are not expressed in all cell types (10–12, 15). However, even among tissues that support editing of apoB RNA, there are tissue specific differences in the ability to support additional site editing when APOBEC-1 is overexpressed (16). These differences have been attributed in part to multiple occurrences of the RNA motif UGAU in the RNA sequence flanking the tripartite motif which resemble the 5′ end of the mooring sequence have the effect of enhancing editing efficiency.

Given that editosome assembly is a requirement for apoB RNA to edit, we have evaluated the role of cis-acting RNA sequence 3′ of the mooring sequence in regulating the efficiency of editosome assembly. We demonstrate by RNA competition analysis and native gel electrophoresis that RNAs containing 3′ flanking sequence native to the editing site in addition to the tripartite motif are more efficient in editosome assembly than RNAs that contain the tripartite motif alone or bulk RNA as sequence 3′ flanking the motif. Ultraviolet light induced cross-linking of extract proteins to various mutant apoB RNA constructs revealed tissue-specific differences in the importance of 3′ flanking sequence for p66 and p44 interactions with apoB RNA.

MATERIALS AND METHODS

Extract preparation. Rat livers from male Sprague-Dawley rats (300–400 g, Charles Rivers) were perfused *in situ* with 30 ml of ice cold homogenization buffer (0.33 M sucrose, 50 mM Tris pH = 8.0, 5 mM M_gCl_2) containing 0.5 μg each aprotinin and leupeptin per ml and 1 mM phenylmethylsulfonyl fluoride (PMSF). Livers were subsequently homogenized in an equal volume (w/v) of homogenization buffer with a Teflon-to-glass homogenizer. Nuclei were cleared from the cytosol (3,500 × g, 10 min) and the S100 fraction of the cytosol was obtained by ultracentrifugation (100,000 × g, 60 min).

Rat enterocytes were prepared as described by Weiser (31) with the following modifications: (1) the initial intestinal wash contained 0.5 μg/ml each leupeptin and aprotinin, 1 mM PMSF, 0.05 mM vanadium ribonucleoside complex as RNase inhibitor, and 20 units/ml soybean trypsin inhibitor and (2) enterocyte release was accomplished with a single 30 min incubation at 37°C. An S100 extract was prepared by the method of Dignam *et al.* (32) and was subsequently dialyzed against buffer D {20 mM Hepes pH 7.9, 20% glycerol (v/v), 100 mM KCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 mM dithiothreitol (DTT)} for 4 h at 7°C.

Assembly of editosomes. Fifty μl editing reactions containing 80 μg of extract protein and 20 fmols of [α-^{32}P] ATP labeled RNA substrate were carried out for one hour at 30°C as described previously (7, 20). One-fifth of each reaction was resolved on 4.5% acrylamide 'native' gels and autoradiographed as previously described (7, 20). For competition analysis, the indicated molar excess of [^{3}H]-ATP labeled RNA was added simultaneously with 20 fmoles of ^{32}P-labeled wild type RNA followed by addition of extract on ice prior to incubation at 30°C (7, 15).

Cross-linking. Editosome assembly reactions containing ^{32}P-labeled apoB RNA were cross-linked as described previously in quartz cuvettes on ice using 254 nm ultraviolet light (7, 20). Samples were digested with 20 units each of RNase A (Sigma Chemical Co., MO) and T1 (Boehringer Mannheim, Germany) for 30 min at 37°C prior to acetone precipitation. Cross-linked proteins were resolved on 10.5% SDS polyacrylamide gels and autoradiographed.

Miscellaneous. Wild type apoB mRNAs of varying length and mutant apoB RNAs were constructed and transcribed *in vitro* as described previously (3, 4, 8). The effects of various mutations of editing efficiency were quantified by the poisoned primer extension assay and reported previously (3, 4, 8). Editosome assembly and cross-linked proteins were quantified by scintillation counting of excised gel bands. All reagents were ultrapure grade and all solutions were treated with diethyl pyrocarbonate and autoclaved.

RESULTS

To evaluate how the length and sequence of RNA flanking the tripartite motif may affect editosome assembly, RNA excess competition was performed using varying lengths of apoB RNA as competitor under editosome assembly conditions. Editosome assembly was evaluated on ^{32}P-labeled apoB RNA, 498 nt in length (448 nt of apoB RNA, nt 6413–6860 plus 50 nt of flanking polylinker) by native gel electrophoresis and autoradiography. Without competition, extracts efficiently assembled editosomes ('B' complexes, Fig. 1, second lane) on apoB RNA within one hour of incubation as evident by the near complete conversion of input ^{32}P-labeled RNA ('A' at t_0, first lane) into B complexes. The same RNA but unlabelled, effectively competed for proteins in the extract such that a 10-fold molar excess of unlabelled competitor virtually eliminated radiolabeled B complexes (fourth lane). ApoB RNA 55 nt long (nts 6649–6703) assembled B complexes with similar kinetics as apoB RNA 498 nt long (20) but supported only 50% as much editing as apoB RNA that was 498 nt long (6, 35). As competitor, the shorter RNA was less effective than apoB RNA 498 nts long (lanes six through eight). A hundred-fold molar excess of unlabelled 55 nt apoB RNA was required to achieve a similar level of competition as that observed with only a 10-fold molar excess of 498 nt apoB RNA competitor. Taken together with data on editing efficiency, these findings are consistent with the possibil-

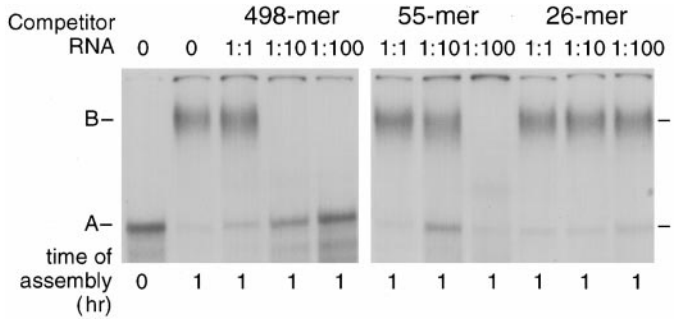

FIG. 1. Competition analysis of editosome assembly. Editosomes were assembled on radiolabeled apoB RNA 498 nt long for one hour either without or with excess unlabeled RNA competitor and resolved by native gel electrophoresis. The length of the unlabeled competitor apoB RNA is indicated at the top of the panels. The molar excess of competitor RNA added at t = 0 is indicated at the top of each lane. B corresponds to the migration of the editosome and A indicates the migration of input RNA.

ity the editosomes assembled on 55 nt of apoB are less stable than those assembled on longer RNAs.

ApoB RNA 26 nt long (6662–6687) could not compete with ^{32}P-labeled apoB RNA 498 nt long for the proteins that form editosomes (Fig. 1, lanes nine through eleven). These findings are consistent with previous data demonstrating that apoB mRNA sequence 498 nts long encompassing C6666 were edited efficiently in cells and *in vitro* but that only very low levels of editing were observed with 26 nt of apoB RNA sequence (6, 8, 20, 33–35). The competition analyses suggested therefore that apoB RNA sequence outside of the tripartite motif was important for the efficiency with which editosomes assembly and/or their stability.

The role of the flanking RNA sequence in editosome assembly was further evaluated on apoB mutant and chimeric constructs. Antisense apoB RNA, from a region of apoB RNA (7336–7678) that lacked the proposed enhancer motifs UGAG, cannot assemble B complexes (Fig. 2, lanes three and four). The same size of apo B RNA (350 nt) mutagenized to contain a functional tripartite editing motif (3) demonstrated the capacity to support editosome assembly (lanes five and six). Similarly, editosome assembly was supported by HalbΔ17E21, a 700 nt albumin-apoB chimera (lanes seven and eight) in which 39 nt of apoB RNA containing the tripartite motif had been centrally inserted (35). Despite the presence of a tripartite motif and 170–350 nts of bulk RNA 5′ and 3′ of the editing site, US6434 wt and HalbΔ17E21 were less effective substrates for editosome assembly compared to wild type apoB RNA. These data corroborate earlier findings suggesting that apoB RNA which naturally flanks the tripartite motif has a unique capability of supporting high editing efficiency (35).

Mutations that change the position of the mooring sequence (3) such that it lies further C6666 (RNA construct apoB +A4) or closer (RNA construct apoB −AT) to C6666 did not diminish the yield of editosomes assembly relative to wild type RNA (Fig. 2, compare B complexes in the tenth and twelfth lanes to that in the second lane). Interestingly, mutation of the 3′ end of the mooring sequence from AT-rich sequence to GC-rich sequence (RNA construct apoB ΔGGCG) markedly inhibited editing activity (3) but did not alter the ability of this RNA to assemble editosomes (lanes thirteen and fourteen). Taken together the data suggest that the UGAU sequence that makes up the 5′ end of the mooring sequence (and the enhancer of the tripartite motif) and the apoB RNA 3′ flanking sequence that naturally flanks the tripartite motif play important roles in the assembly of complexes and/or their stability. Whether these complexes are active in editing or not appears to be determined by other criteria.

Proteins of 66 kDa and 44 kDa cross-linked to apoB RNA early during the assembly of editosomes and were integral components of native gel 'B' complexes (7, 15, 20). We evaluated the role of the mooring sequence and RNA sequence 3′ of the mooring sequence in editosome assembly at the level of ultraviolet light induced (UV) cross-linking of p66 and p44 in liver and small intestinal cell extracts. As described previously (7) the yield of cross-linked p66 was 15-fold higher than that of p44 in hepatic extracts while p66 cross-linking in intestinal extracts was only 5-fold higher than that of p44 (Fig. 3, compare lane one in the upper and lower panels).

Scrambling the 11 nt mooring sequence in the context of an otherwise wild type 448 nt apoB RNA (RNA construct MI) markedly inhibited editing (4), reduced hepatic p66 cross-linking by 50% and virtually elimi-

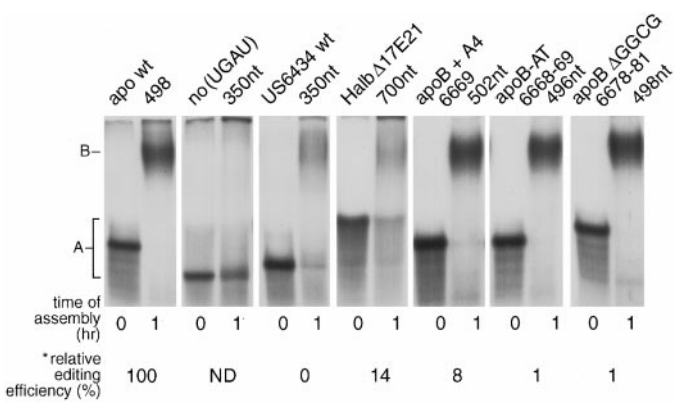

FIG. 2. Sequence requirements for editosome assembly. Editosomes were assembled on radiolabeled wild type or mutant apoB RNAs. For each RNA, autoradiographs show complexes before the incubation (0 h) or after a one hour assembly reaction (1 h). The type of RNA used for each assembly reaction is given at the top of the lanes. The relative editing efficiency at C6666 ('C66') were determined under *in vitro* editing conditions in rat intestinal cell extracts as described previously (3, 35) and are indicated below each lane. B corresponds to the migration of the editosome and A indicates the migration of input RNA.

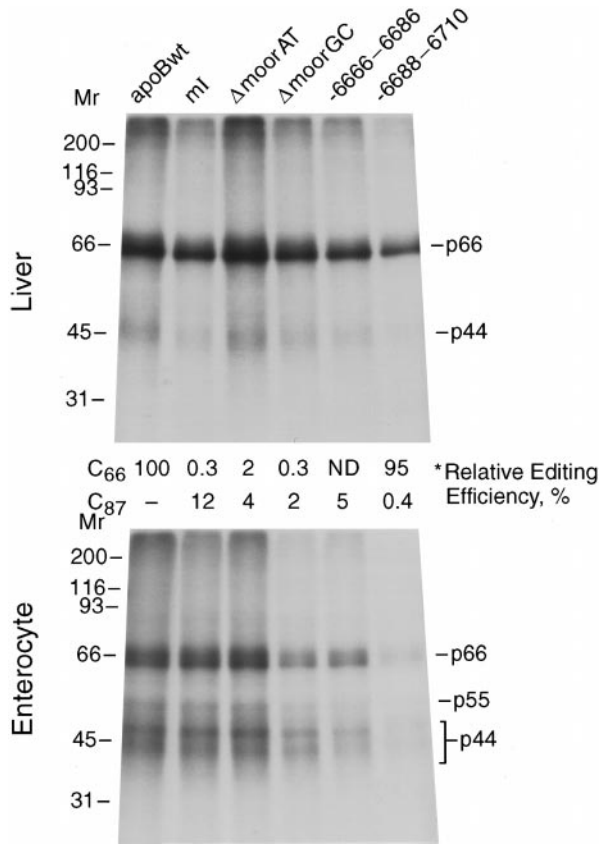

FIG. 3. Evidence for multiple sites for p66 and p44 interaction with ApoB RNA. Wild type apoB RNA and mutations thereof were radiolabeled and subjected to editosome assembly in liver (upper panel) or small intestinal cell (lower panel) extracts and UV cross-linking. The autoradiographs of P.A.G.E. resolved proteins are labeled at the top of each lane for the radiolabeled RNA that was used in the assembly reaction. The migration of molecular mass markers is indicated to the left and the migration of p66 and p44 is indicated to the right. The relative editing efficiency at C66 and at an inserted cytidine at nt 6687 ('C87') were determined previously (3, 35).

nated p44 cross-linking (Fig. 3, upper panel, second lane). Interestingly, there was no quantifiable reduction in the yields of intestinal cell p66 and p44 cross-linking to this construct (lower panel, second lane).

Mutation of the eleven nt mooring sequence to all ATs or GCs (RNA constructs Δmoor AT and Δmoor GC respectively) virtually eliminated editing activity (4). In contrast, cross-linking of hepatic and intestinal p66 and p44 to Δmoor AT was equal or better than that observed with wild type apoB RNA. In contrast, RNA construct Δmoor GC cross-linked less efficiently than wild type or Δmoor AT RNAs. The yield of hepatic p66 and p44 on Δmoor GC RNA were reduced by 50% relative to wild type apoB RNA (Fig. 3, fourth lane). The yield of p66 and p44 cross-linking to Δmoor GC in intestinal cell extracts was only 30% of that seen on wild type apoB RNA. Taken together the data suggest that although the mooring sequence is required for editing, it may not be the sole site for p66 and p44 interactions. The data also suggest that AT-rich RNA sequence is important of high yields of p66 and p44 cross-linking.

The most ready explanation for these findings is that the UGAU motifs in the flanking RNA sequence 3′ of the mooring sequence not only play an important role in editing efficiency (4, 16, 35) but may also interact with p66 and p44. To evaluate this possibility, C6666, the spacer element and the mooring sequence of the tripartite motif were deleted from the 498 nt apoB sequence (RNA construct −6666–6686) and cross-linking activity analyzed. Hepatic p66 and p44 cross-linking yields on −6666–6686 RNA were reduced to 40% of that observed on wild type apoB RNA (Fig. 3, upper panel, fifth lane). Tissue specific difference were once again observed in the reduced yield of intestinal p66 and p44 cross-linking to −6666–6686 RNA to 20% of that observed on wild type apoB RNA (Fig. 3, lower panel, fifth lane). The data demonstrated the importance of the mooring sequence in supporting cross-linking but also suggested that cross-linking of p66 and p44 could take place with lower yields in the absence of the mooring sequence.

A downstream RNA sequence (6695–6706) referred to as the 'cryptic' mooring sequence has been shown to support editing under the experimental condition where a cytidine was inserted 5′ of the site (4) and to be important for 'promiscuous' editing of cytidines when APOBEC-1 is overexpressed (16). The cryptic mooring sequence is therefore a candidate site for p66 and p44 interaction. To evaluate this possibility, the RNA construct −6688–6710 was subjected to *in vitro* editosome assembly and UV cross-linking. Deletion of this sequence markedly inhibited p66 and p44 cross-linking in both tissue types despite the fact that the tripartite motif surrounding C6666 was intact (Fig. 3, upper and lower panels, sixth lane). Cross-linking yields of hepatic p44 and intestinal p66 and p44 were virtually eliminated on −6688–6710 RNA whereas the yield of hepatic p66 was reduced to 15% of that observed on wild type apoB RNA.

DISCUSSION

In this report we demonstrate three novel characteristics of the cis-acting RNA sequence requirements of editosome assembly. First, RNA sequences flanking the tripartite motif are important for efficient editosome assembly and/or their stability. Second, there are multiple sites of protein interaction in the vicinity of the editing site. Sequences within the 3′ flanking region and in particular UGAU motifs such as that within the cryptic mooring sequence are involved in UV cross-linking of 66 kDa and 44 kDa proteins. Third, tissue-specific differences in editing efficiency are possibly associated with differences in the requirement of

the mooring sequence and 3′ flanking sequences for p66 and p44 UV cross-linking activity.

The mooring sequence hypothesis is a mechanistic model for apoB RNA editing in which RNA sequence 3′ and in the immediate vicinity of C6666 is required to position or 'moor' proteins involved in editing through protein-RNA and protein-protein interactions (3, 6, 26). The catalytic subunit APOBEC-1 has a low affinity for AU-rich sequence within the 3′ end of the mooring sequence (36–38) and may accommodate this RNA segment at the interface of APOBEC-1 dimers (39). ApoB RNA editing and editing site specificity require auxiliary proteins, presumed to form RNA recognition complexes upon which functional editing complexes can be assembled (6, 7). To date several RNA binding proteins have been identified as candidate auxiliary proteins (7, 9, 15, 17–22). Depending on the cellular source, these proteins have been described as having the capacity to selectively UV cross-linking to apoB RNA. In this regard, p44 interactions at the editing site have been proposed to uniquely involve the AU-rich spacer element and 5′ end of the mooring sequence (AAUUUG, nts 6667–6672) (27). Our data corroborate the AU-rich requirement for p44 binding but suggest other interactions of p44 and p66 within the 3′ flanking sequence are also possible and are important for efficient and/or stable association of these proteins with the tripartite motif.

Earlier work supported the role of sequences flanking the tripartite motif as enhancers of editing activity (16, 17, 35). The length and sequence of RNA flanking the tripartite motif can affect the efficiency of RNA editing through what has become known as the 'bulk' RNA effect (6, 26, 33, 35, 41). Analysis of the bulk sequence has shown that apoB mRNA (particularly 3′ of C6666) is unlike other cellular mRNAs in that it contains a disproportionate number of UGAU mooring-sequence-like motifs (eleven repeats) in the 500 nts flanking C6666 (26). The finding that a cytidine at nucleotide position 6802 (42) and a cytidine substituted by site directed mutagenesis at position 6687 (4) were edited suggested that factors involved in editing were associated with other UGAU motifs as well as with the mooring sequence. Furthermore, the presence or absence of a cytidine 5′ of the mooring sequence did not affect editosome assembly or protein cross-linking (7). Taken together with the findings presented here, the data suggest that B complex formation and p66/p44 cross-linking are characteristic of apoB RNA from the region of the editing site but do not require a functional editing site.

Our data suggested an important role of sequence 6688–6610 in p66 and p44 cross-linking to apoB RNA. This region supported p66 and p44 cross-linking when the mooring sequence was mutated (RNA constructs MI and Δmoor AT) or deleted (RNA construct −6666–6686) but demonstrated the most marked suppression of cross-linking to the mooring when deleted (RNA construct −6688–6710). The data suggested binding of p66 and p44 at one or more sites outside of the tripartite motif with a preference for AT-rich sequence in the region of the tripartite motif. These findings support a proposal from earlier studies in which APOBEC-1 had been overexpressed in transgenic animals (43, 44) or transfected cells (16, 45) that editing of additional cytidines, some as far as fifty nucleotides away from the mooring sequence, may be facilitated by multiple UGAU motifs in apoB flanking RNA sequence (45). In this regard the cryptic mooring sequence within nt 6688–6710 has been shown to play a cell type specific role in promiscuous editing site selection (16). The current findings suggest that promiscuous site editing may arise from APOBEC-1 interactions with auxiliary proteins bound to UGAU sites outside of the tripartite motif. We cannot rule out the possibility that the enhancing effect of the 3′ flanking sequence is the result of an aggregate macromolecular complex, held together by interactions between multiple complexes of UGAU-p66/p44, to which APOBEC-1 binds.

The tissue-specific differences in the requirement for flanking sequence in p66 and p44 UV cross-linking are consistent with biochemical evidence suggesting that these proteins were identifiable in 11S complexes in small intestinal cell extracts and 60S complexes in hepatic cell extracts (7) and that although each tissue extract assembled 27S editosomes *in vitro*, they did so with different kinetics and the resultant complexes differed in their stability (6–8). ApoB 5′ flanking sequence beyond nt 6661–6665 (the enhancer of the tripartite motif) has also been shown to stimulate editing efficiency in rabbit liver extracts (40) but did not show this effect in rat liver or intestinal extracts (35). Auxiliary proteins may therefore also demonstrate species specific differences in their interactions with UGAU motifs.

In summary, the data presented in this report suggest that apoB RNA in the vicinity of the editing site is coordinated as a unique ribonucleoprotein complex and that this higher-order structure may be an important determinant in the mechanism of apoB RNA editing and its regulation.

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