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Biochemistry

Apolipoprotein B mRNA editing is associated with UV crosslinking of proteins to the editing site

(lipoproteins/RNA recognition/RNA structure/RNA-binding proteins)

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ABSTRACT **Apolipoprotein (apo) B100 mRNA undergoes editing of C-6666 to a U residue, which generates a stop-translation codon and defines the carboxyl terminus of apoB48. To aid purification of the editing enzyme we have undertaken UV crosslinking of a ^{32}P-labeled substrate for apoB mRNA editing *in vitro* to proteins in an enterocyte editing extract. Proteins of 60 (p60) and 43 (p43) kDa, prominent among crosslinking bands, were competed for by unlabeled substrate, but not by nonspecific RNA, and did not crosslink to antisense RNA. Editing *in vitro* and UV crosslinking were inhibited by NaCl and vanadyl ribonucleoside complexes and by chemical modification of sulfhydryl, imidazolium, and guanidinium groups on the protein. The editing activity copurified predominantly with p60. To define the binding site for p60 on the substrate RNA, a series of scanning and point mutant RNAs, previously used to define nucleotides 6671–6681 as essential for editing, were used in competition studies with wild-type substrate. Results demonstrated that p60 binding is centered on nucleotides 6671–6674. We suggest that p60 contains the RNA-recognition component of the apoB mRNA-editing enzyme.**

A discrete editing of C to U at nucleotide 6666 of mammalian apolipoprotein (apo) B100 mRNA converts glutamine codon 2153 to a stop-translation codon, which generates the carboxyl terminus of apoB48 (1–3). Editing *in vitro* of synthetic apoB RNA has no nucleotide or ion cofactor needs, does not require an RNA component other than the substrate, and does not involve an excision-replacement reaction with cleavage of the phosphodiester backbone of the target RNA (4–9). The sequence requirements for editing have been defined by identification of a second editing site in apoB mRNA and by the study of deletion, scanning, and point mutations in transfected cells and *in vitro* (10–13). The minimal sequence requirements for editing have been localized to a completely conserved 26-nucleotide segment between nucleotides 6662 and 6687 (11). An 11-nucleotide segment between nucleotides 6671 and 6681, downstream of the editing site, has been identified in which most mutations profoundly reduce or abolish editing, and it has been proposed that this is the enzyme binding site (13, 14). The most likely mechanism to explain this editing is a site-specific cytidine deamination. In the present study, UV crosslinking was undertaken as an adjunct to purification and further characterization of the apoB mRNA editing enzyme.

MATERIALS AND METHODS

RNA Synthesis. The DNA templates and methods used to prepare unlabeled RNA substrate and mutant substrate RNA have been described (6, 13). ^{32}P-labeled RNA was transcribed in the same way except that reaction mixtures contained 80 μCi of [α-^{32}P]UTP (3000 Ci/mmol; 1 Ci = 37 GBq), and the UTP concentration was reduced from 400 μM to 8 μM.

Intestinal Extracts, Conversion Assay, and Primer Extension Analysis. Rat enterocyte S100 extract was prepared and conversion assay and quantitative primer extension analysis were performed as described (4, 6), except that a 208-base (nucleotides 6510–6717) rat apoB RNA substrate was used (15). It is edited 2- to 3-fold better than the human substrate (N.N., unpublished data; ref. 7).

Partial Purification. Ammonium sulfate-precipitated (50% saturation) editing activity from S100 extract was resuspended and dialyzed against buffer A [20 mM Hepes/0.2 mM EDTA/1 mM 2-mercaptoethanol/20% (vol/vol) glycerol, pH 8.0] and applied to a 2.5 m × 8 cm DEAE-cellulose column (Whatman) at a flow rate of 0.5 ml/min and eluted with 100 mM KCl in buffer A. Active fractions were pooled and applied to a 5-ml Hitrap-heparin column (heparin–Sepharose matrix, Pharmacia) in buffer A at a flow rate of 0.5 ml/min, using the fast protein liquid chromatography system (FPLC) (Pharmacia), and eluted with a 0–700 mM NaCl gradient. Pooled active fractions were concentrated in a Centricon-30 (Amicon) to 500 μl and applied to a Superose-12 column (Pharmacia) in buffer A containing 150 mM NaCl on an FPLC system. The purification was around 10,000-fold with 3–5% yield. The most pure fraction contained several Coomassie blue-staining bands on SDS/PAGE.

UV-Crosslinking Studies. Conversion reactions were set up with 1 × 10^6 dpm (5 ng) of ^{32}P-labeled substrate RNA and unlabeled competitor RNA as indicated, and the mixtures were incubated at 30°C for 20 min and transferred to 96-well Microtitre plates (Sterilin, Teddington, U.K.). Reaction mixtures were exposed in a Stratagene UV Stratalinker, while on ice, to 10 min of UV irradiation (254 nm) 1.5 cm from the UV source; transferred into Eppendorf tubes with RNase A at 0.5 mg/ml for 15 min at 37°C, and subjected to SDS/PAGE. The gel was fixed, dried, and exposed to Kodak X-Omat XAR film at −70°C. UV crosslinking was quantitated as previously described (6). An external standard of 25 nCi of [^{14}C]methylated carbonic anhydrase SDS/PAGE molecular weight marker (Amersham, specific activity 10–50 μCi/mg of protein) was added prior to UV crosslinking, and crosslinking bands were normalized to this. Percent competition was relative to competition without competitor.

Inhibitor Studies. Conversion or UV-crosslinking assays were set up with NaCl, vanadyl ribonucleoside complexes (VRC), *N*-ethylmaleimide (NEM), diethyl pyrocarbonate (DEPC), or phenylglyoxal (PG) at 30°C for 10 min in buffer A. Then conversion or UV-crosslinking reactions were performed. When PG or DEPC or NEM was used arginine,

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Abbreviations: apo, apolipoprotein; DEPC, diethyl pyrocarbonate; FPLC, fast protein liquid chromatography; HIV, human immunodeficiency virus; NEM, *N*-ethylmaleimide; PG, phenylglyoxal; VRC, vanadyl ribonucleoside complexes.

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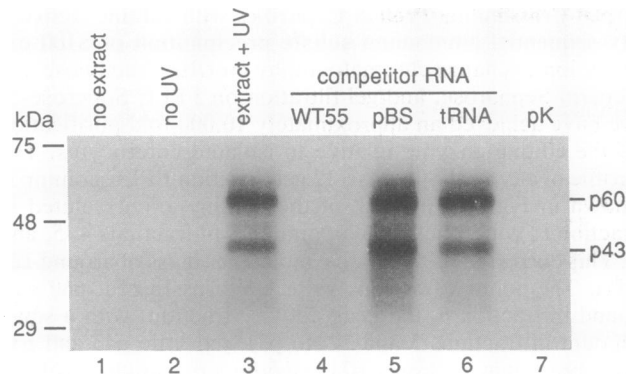

FIG. 1. SDS/PAGE analysis of UV-crosslinked samples. ^{32}P-labeled wild-type 55-nucleotide substrate RNA (WT55) was incubated with editing extract and exposed to UV irradiation, followed by RNase A digestion (lane 3). Lane 1 RNA was incubated with buffer A in place of editing extract. Lane 2 contains a sample digested with RNase A without prior UV treatment. Competition with 1 μg (200-fold molar excess) of unlabeled substrate RNA is shown: WT55 substrate (lane 4), Bluescript plasmid polylinker (pBS) (lane 5), or tRNA (lane 6). Lane 7 contains a sample digested with proteinase K (pK) prior to crosslinking and RNase A digestion. Crosslinked proteins p60 and p43 are indicated.

histidine, or cysteine (50 mM, pH 8.0), respectively, were added to the reactions to inactivate the inhibitor. These amino acids had no effect on editing or UV crosslinking in control reactions.

RESULTS

UV Crosslinking of Proteins to RNA Substrate. A synthetic apoB RNA substrate containing 55 nucleotides that spans the apoB mRNA editing site undergoes efficient editing *in vitro* (4, 6). Here we have used UV crosslinking to ^{32}P-labeled 55-base RNA substrate to identify proteins that bind specifically to the substrate. In crude S100 extract derived from rat small intestinal enterocytes, UV crosslinking was unsatisfactory. We have therefore examined crosslinking in extracts after partial purification by ammonium sulfate precipitation and DEAE-cellulose and heparin–Sepharose chromatography as described. Prominent UV-crosslinking proteins of 60 (p60) and 43 (p43) kDa were observed. Although these protein bands were a constant feature of all preparations, their intensity varied from one purification to another, and sometimes p71 or other less constant bands, such as p39, were seen (Figs. 1–4). Crosslinking of p60 was fully blocked by competition with 100–500 ng (20- to 100-fold molar excess) of unlabeled apoB RNA substrate (Figs. 1 and 4). Most of p71 crosslinking was also blocked by competition with these concentrations of substrate RNA (Fig. 4). p43 and p39 crosslinking was almost completely blocked by competition with 1 μg (200-fold molar excess) of RNA substrate (Figs. 1 and 4). Rat 208 nucleotide substrate RNA showed an identical UV-crosslinking pattern (results not shown). The apoB RNA substrate contains 40 bases of Bluescript plasmid polylinker. No competition was observed with RNA transcribed from the sense strand of the Bluescript polylinker or with tRNA. Pretreatment of the editing extract with proteinase K completely disrupted UV crosslinking. Crosslinking was not observed with antisense substrate RNA (results not shown).

Inhibitors of Editing Disrupt UV Crosslinking. Editing of the synthetic apoB substrate RNA is inhibited by NaCl concentrations above 150 mM (Fig. 2*A*). UV crosslinking of p60 and p71 was similarly inhibited, with a profile that followed the inhibition of the editing activity. UV crosslinking of p43 was inhibited with NaCl, but at much higher concentrations (Fig. 2*A*). VRC (unpublished observations; ref. 14), NEM, DEPC, and PG inhibit editing (Fig. 2 *B–E*, respectively) (6). The UV crosslinking of p60 was selectively disrupted by NEM in a concentration-dependent fashion that paralleled disappearance of editing activity (Fig. 2*C*). The crosslinking of all of the crosslinking proteins was disrupted by the other inhibitory reagents in a concentration-dependent

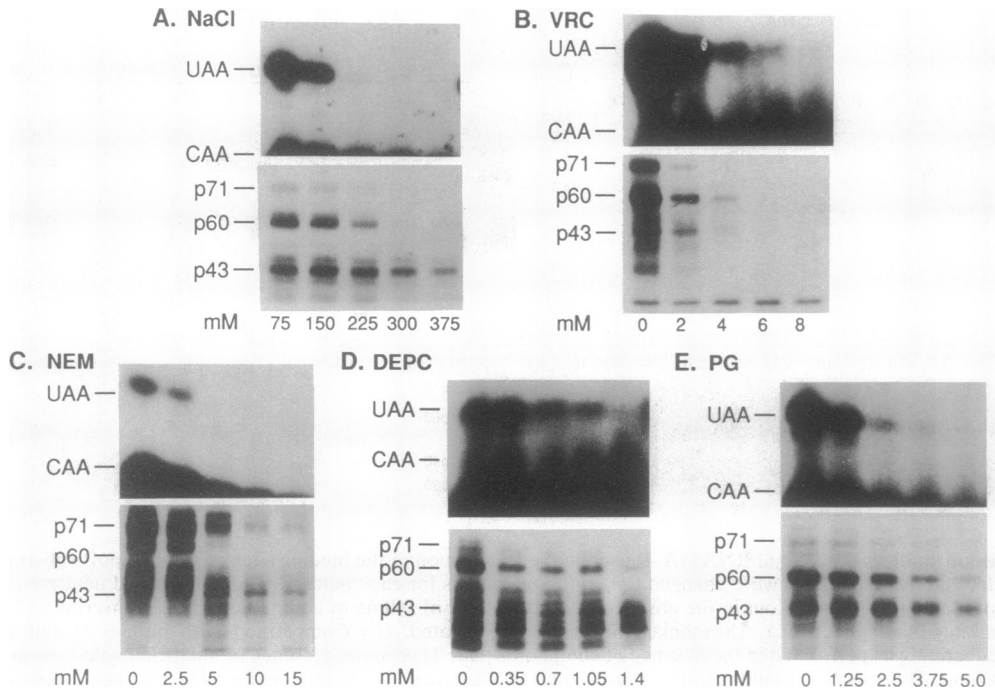

FIG. 2. Inhibitors of editing and UV crosslinking. Conversion or UV-crosslinking assay reaction mixtures were supplemented with inhibitors as indicated and described in *Materials and Methods*. CAA and UAA products of primer extension and p71, p60, and p43 crosslinking bands are indicated.

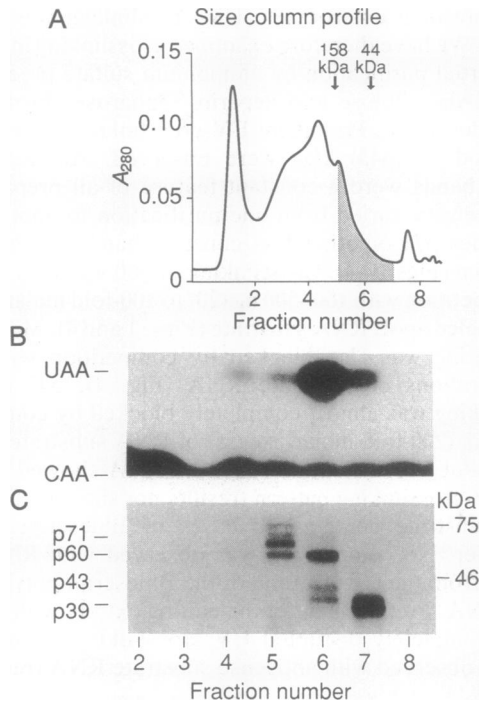

FIG. 3. Partial purification of the editing enzyme and p60. (*A*) A_{280} protein profile of the Superose-12 chromatography, with the positions of the 158- and 44-kDa size markers shown. The shaded area contains the editing enzyme. (*B*) CAA and UAA products of primer extension. (*C*) UV crosslinking of p71, p60, p43, and p39.

fashion that by and large followed inhibition of editing (Fig. 2 *B*, *D*, and *E*).

p60 Crosslinking Protein Copurifies with Editing Activity. By sequential ammonium sulfate precipitation of S100 extract, ion-exchange chromatography on DEAE-cellulose and heparin-Sepharose, and gel filtration on FPLC Superose-12 we have achieved an approximately 10,000-fold purification of the editing enzyme relative to isolated enterocytes. The profile of a typical Superose-12 gel filtration FPLC column is shown in Fig. 3. The bulk of the editing activity eluted in fraction 6, with much smaller amounts in fractions 4, 5, and 7. This corresponds to a native molecular mass of around 120 kDa. The column fractions were UV crosslinked. p60 was found in fraction 6, the peak activity fraction, with a small amount in fractions 5 and 7. No p71 and little p43 and p39 were present in fraction 6. p71 and other crosslinking proteins were present in fraction 5. p39 and a prominent protein of 35 kDa were present in fraction 7. Thus, predominantly p60 copurifies with the apoB mRNA editing enzyme.

UV Crosslinking to Mutant Substrates. The 55-base apoB substrate RNA was used as a template to prepare a series of scanning mutants in which 6 bases were changed to their complement (Fig. 4*A*; Table 1) (13). To localize the binding sites of the UV-crosslinking proteins, mutant RNAs were evaluated for their ability to compete with wild-type 55-base human RNA substrate for crosslinking. We used 500 ng (100-fold molar excess) of unlabeled wild-type or of scanning mutant RNA (Fig. 4*B*). Mutant D, which is not edited, did not compete compared to wild-type or the other scanning mutants RNA with respect to p60. In contrast, mutant E, the contiguous mutant downstream from mutant D, which also does not edit, was almost as effective a competitor as wild-type RNA. p43 was not effectively competed for by the wild type or by mutants A, B, C, D, and G. Mutants E, F, H, and I were apparently effective competitors for p43 as well as p60.

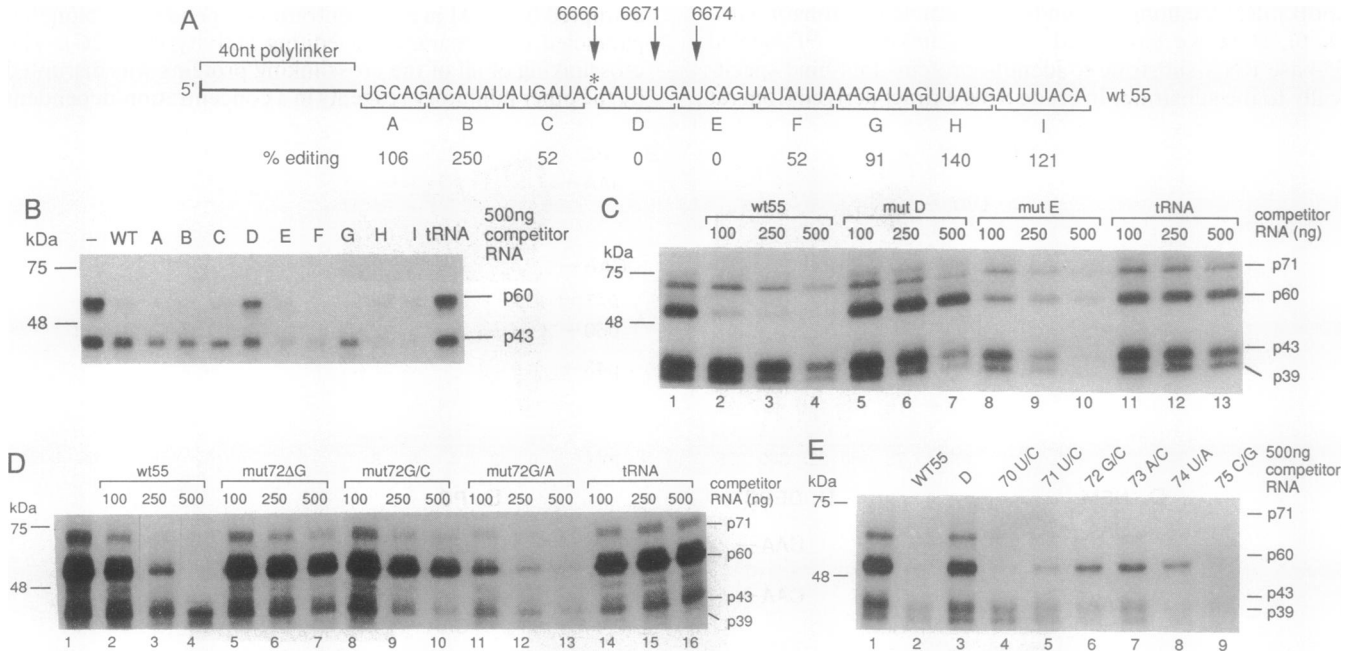

FIG. 4. Competition studies. (*A*) Mutant RNAs (A–I) used in the localization of the binding sites of p60 on apoB substrate apoB RNA (13). The bracketed nucleotide (nt) sequences were changed to their complements for each mutant. C-6666 remained unaltered and is indicated by an asterisk. (*B*) Crosslinking was carried out in the absence (−) or presence of 500 ng of competitor RNA: transcript 55-base RNA substrate (WT), scanning mutants A–I, and tRNA. Crosslinked proteins are indicated. (*C*) Competition with mutant D and E RNA at various concentrations. Incubation was performed in the absence of competitor (lane 1) or in the presence of unlabeled wild-type apoB substrate RNA (lanes 2–4), mutant (mut) D (lanes 5–7), mutant E (lanes 8–10), and tRNA (lanes 11–13). (*D*) Competition with point mutants of position 6672. Incubation was performed in the absence (lane 1) or presence of unlabeled apoB substrate RNA (wt55) (lanes 2–4), G-6672 deletion mutant (mut72ΔG; lanes 5–7), G-6672 → C substitution mutant (mut72G/C; lanes 8–10), G-6672 → A substitution mutant (mut72G/A; lanes 11–13), and tRNA (lanes 14–16). (*E*) Precise localization of p60 recognition site. Incubation was performed in the absence (lane 1) or presence (lanes 2–9) of competitor RNA as indicated. Mutant RNAs are named as in *D*.

Table 1. Localization of p60 binding site

Competitor RNA	% editing* compared with wild type	% competition compared with no competitor
None		—
wt55	100	88
Mutant D	0	23
6670U/C	50	81
6671U/C	20	73
6672ΔG	0	ND
6672G/C	0	62
6672G/A	60	ND
6673A/C	0	57
6674U/A	0	68
6675C/G	10	94

ND, not determined.
*Specificity was fully maintained in the purest editing extract.

To confirm these results, competition was performed with various amounts (100–500 ng; 20- to 100-fold molar excess) of wild-type or of mutants D and E RNA. Wild-type 55 and mutant D RNA equivalently competed for crosslinking to p71, p43, and p39 but again showed a striking difference with respect to p60 (Fig. 4*C*). Whereas 100–250 ng of wild-type RNA completely blocked crosslinking to p60, 500 ng of mutant D had little effect. In contrast, crosslinking to the four proteins p71, p60, p43, and p39 was blocked equivalently by 500 ng of mutant E RNA. Competition with mutants F, H, and I was similar to that with mutant E (results not shown). tRNA was ineffective as a competitor.

Point mutations between nucleotides 6671 and 6681 greatly reduced or abolished editing (13). We therefore examined mutants of position G-6672 (Fig. 4*D*), because this residue defines the boundary between scanning mutant E, which binds p60, and D, which does not. Deletion of the G (mutant 6672ΔG) at this position or substitution of a C (mutant 6672G/C) abolished editing, whereas substitution of an A (mutant 6672G/A) in this position only reduced editing by some 40%. These mutant RNAs were used at various concentrations (20- to 100-fold molar excess) as competitors of wild-type 55-base substrate. In the UV-crosslinking studies with wild-type 55-base RNA as the standard the most prominent result was that mutants 6672ΔG and 6672G/C were not effective as competitors for p60 crosslinking (Fig. 4*D*). p71 also showed some reduction in competition with these mutants compared with wild-type RNA. The behavior of both of these mutants with respect to p43 was similar to that of wild-type 55-base substrate. With mutant 6672G/A, which is edited with 60% of the efficiency of wild-type RNA, competition was similar to wild-type RNA for p71, p60, and p43. Thus, with respect to position 6672, editing and p60 binding parallel each other.

To further delineate the p60 binding site, point mutants of nucleotides 6670 to 6675 were examined in competition studies. The results show substantially reduced competition with mutant D and mutants at positions 6671–6674 compared with wild-type RNA (Table 1 and Fig. 4*E*). The reduction of competition with mutation of position 6670 was small, and mutation of nucleotide 6675 did not affect competition. These results demonstrate p60 binding between nucleotides 6671 and 6674.

DISCUSSION

RNA structure is recognized as important in sequence-specific RNA–protein interactions. RNA stem-loops provide necessary structure for the recognition of RNA by the R17 phage coat protein, the iron-response binding protein, the human immunodeficiency virus (HIV) Tat and Rev proteins,

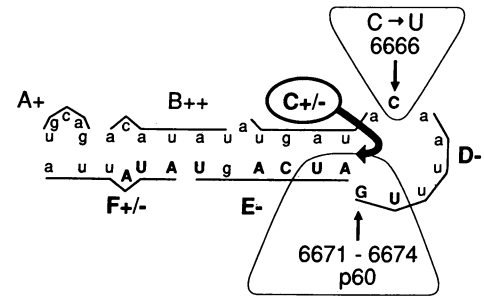

FIG. 5. The apoB mRNA editing site. A highly conserved stem-loop predicted by the computer is shown. The C → U editing site at position 6666 and p60 binding site between nucleotides 6671 and 6674 are indicated. The positions of scanning mutants A–F are indicated by the solid lines. Scanning mutants D and E abolish editing. Scanning mutant C, which would be predicted to disrupt the stem-loop shown, reduces editing to 50% (curved arrow). Mutant F also reduces editing to 50%. Bases shown in uppercase are the editing site and the positions of point or deletion point mutations that abolish editing. Lowercase letters are sites at which mutation does not affect editing. Note that G·U base pairing has been used at two positions in the predicted stem.

and for several of the proteins involved in splicing (16–20). Small extrahelical bulges and unpaired residues are also important for specific RNA–protein binding, and for the Rev, Tat, and R17 proteins specific binding occurs at the junction between single-stranded and helical RNA regions (16, 18, 19).

Although a highly conserved stem-loop is predicted at the apoB mRNA editing site, with the edited nucleotide at position 6666 within the loop (Fig. 5) (11), our previous mutagenesis study has shown that maintenance of this precise stem-loop is not necessary for editing to take place (13). Our previous study has also shown that nucleotides 6671 to 6681, which are apparently not paired and therefore are not required for stem-loop formation, are necessary for editing (13). This region of the RNA is, however, very A+U-rich (11), so that low-energy structures not predicted by the computer might be formed and act in RNA–enzyme recognition.

In the present study we demonstrate that there is an interaction between p60, the putative RNA-binding component of the apoB mRNA-editing enzyme, and its target RNA at the nucleotide sequence UGAU between positions 6671 and 6674. By analogy with other RNA-binding proteins this limited region must embody a secondary structure that mediates specific RNA–protein interaction, perhaps similar to the small extrahelical bulges present at the HIV Tat and Rev proteins, and the R17 phage coat protein binding sites (16, 18, 19). Interestingly, the HIV transactivation response RNA also undergoes a specific editing of A to I at the HIV Tat protein binding site, and this is mediated by cooperation between the Tat protein and double-stranded RNA adenosine deaminase (21).

Although each of the inhibitors used here may alone be relatively nonspecific, together they support a role for the UV-crosslinking proteins in apoB mRNA editing, and NEM was selective in disrupting p60 crosslinking, suggesting a role for sulfhydryl groups in its RNA binding. Cysteine residues are also implicated in RNA binding and iron sensing of the iron-response binding protein (16, 22). Of the other proteins that crosslink to the RNA substrate, p43 may be proteolytically derived from p60, as it has a similar peptide map (N.N. and J.S., unpublished data). Lau and colleagues (23) have previously demonstrated a 40-kDa protein that UV crosslinks to this editing site. This may be similar to our p43. The other crosslinking proteins, p71 and p39, may be general RNA-binding proteins with multiple low-specificity binding sites on the substrate, or in some other way they may be associated

with the editing enzyme. We suggest that p60 is the RNA recognition component of the apoB mRNA-editing enzyme. The complete enzyme may also have other components, as on gel filtration it has a native molecular mass of around 120 kDa (this study; ref. 5).

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