Apolipoprotein-B mRNA

The editing of apolipoprotein-B (apoB) mRNA involves the deamination of cytidine at nucleotide 6666 to uridine. The catalytic subunit of the editing enzyme, apobec-1, is a cytidine deaminase that requires other unidentified proteins to edit apoB mRNA in vitro. We partially purified an activity from baboon kidney that functionally complements apobec-1. The complementing activity was protease-sensitive and micrococcal nuclease-resistant, had a native molecular mass of 65 ± 10 kDa on size exclusion chromatography, and sedimented at 4.5 S in glycerol gradients. Purified recombinant His6-tagged apobec-1 immobilized on beads depleted >90% of the complementing activity from partially purified extracts. These beads edited apoB mRNA in vitro in the absence of exogenous apobec-1 or complementing activity. A functional holoenzyme containing apobec-1 and the complementing activity was eluted from the apobec-1-affinity resin using 0.5 M imidazole, whereas buffer containing 0.4 M KCl eluted only the complementing activity. The carboxyl-terminal 59 amino acids of apobec-1 were not required for interaction with the complementing activity in vitro. Our results demonstrate that the complementing protein interacts directly with apobec-1 in the absence of apoB mRNA.

Apobec-1 Interacts with a 65-kDa Complementing Protein to Edit Apolipoprotein-B mRNA in Vitro*

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Anuradha Mehta, Subhas Banerjee, and Donna M. Driscoll‡

From the Department of Cell Biology, Cleveland Clinic Foundation, Cleveland, Ohio 44195

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‡ Established Investigator of the American Heart Association. To whom correspondence should be addressed: Dept. of Cell Biology, Cleveland Clinic, 9500 Euclid Ave., NC-10, Cleveland OH 44195. Tel.: 216-445-9758; Fax: 216-444-9404.

† The abbreviations used are: apoB, apolipoprotein-B; apobec-1, apoB mRNA editing enzyme catalytic polypeptide 1; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NTA, nitrilotriacetic acid.

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pressed as a His$_6$-tagged protein in the E. coli strain, M15[pREP4]. A KpnI fragment of the apobec-1 cDNA was subcloned into pQE32 to generate the deletion mutant, apobec-1/Kpn, which lacks amino acids 171–229. Bacterial cultures grown to an A$_{600}$ of 0.7 were induced with 2 mM IPTG for 3 h. Cells were harvested and sonicated in 5 ml of binding buffer (10 mM Hepes, pH 7.9, 0.5 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 10 mM imidazole) per g of cells. All buffers also contained 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, and 50 μM/ml antipain. After centrifugation at 10,000 × g for 30 min, the supernatant was incubated with Ni-NTA resin (Qiagen) at 4°C for 2 h. The resin was packed into a column and washed overnight in wash buffer (20 mM Hepes, pH 7.9, 100 mM NaCl, 20% glycerol, 10 mM imidazole). Proteins were eluted with a linear gradient of 50 to 500 mM imidazole in wash buffer. Fractions containing apobec-1 were dialyzed against buffer A (20 mM Hepes, pH 7.9, 100 mM KCl, 20% glycerol, 5 mM β-mercaptoethanol), and concentrated using Centriprep concentrators (Amicon).

**Analysis of Recombinant Apobec-1**—Approximately 4 mg of protein were obtained from 1 liter of bacterial culture, of which 80% was apobec-1, based on SDS-polyacrylamide gel electrophoresis, Coomassie Blue staining, and Western blot analysis. In addition to the 28-kDa apobec-1 and 56-kDa protein, a form of apobec-1 varying in size was described that often co-eluted between preparations and was absent in extracts of bacteria transformed with the expression vector. In Western blot analysis, the purified 28-kDa apobec-1, but not the 56-kDa protein, reacted with the rabbit anti-apobec-1 peptide antibody. Based on V8 protease mapping (23), the peptide maps of the 56- and 28-kDa proteins were identical within a range of 23%, but the peptide maps of the 28-kDa apobec-1, but not the 56-kDa protein, reacted with the Staphylococcus aureus V8 protease. The results were consistent with the presence of a dimer. A few bacterial proteins also copurified with apobec-1. These minor contaminants were obtained when proteins were purified from IPTG-induced M15[pREP4] bacteria that were transformed with the vector only. The proteins from vector-transformed bacteria served as a negative control in the protein interaction experiments.

**Western Blot Analysis—**Antibodies were generated against a synthetic peptide corresponding to amino acids 10–21 of rat apobec-1 (16). The peptide was synthesized using the multiple antigen peptide method (24), and injected into rabbits using standard procedures (25). For Western blot analysis, proteins were resolved on SDS-polyacrylamide gel electrophoresis, 12% gels, and transferred to polyvinylidene difluoride membranes (25). Membranes were incubated with the primary antibody (1:2000) and an affinity-purified goat anti-rabbit peroxidase-conjugated antibody (Boehringer Mannheim). Proteins were detected using ECL reagents (Amersham Corp.).

**Purification of Complementing Activity—**Whole cell extracts were prepared from baboon kidney as described previously (11). After centrifugation at 100,000 × g, the supernatant was fractionated into a 14-kilobase pair apoB mRNA (Fig. 1). Relative to the levels of apoB mRNA in the liver (100%), the levels of apoB mRNA were 50% in the small intestine, 5% in the spleen, and 1.3% in the stomach. No hybridizable apoB mRNA was detected in other baboon tissues, including kidney (27). Northern blot analysis of baboon tissues detected a 14-kilobase pair apoB mRNA (Fig. 1). Relative to the levels of apoB mRNA in the liver (100%), the levels of apoB mRNA were 50% in the small intestine, 5% in the spleen, and 1.3% in the stomach. No hybridizable apoB mRNA was detected in other baboon tissues, including kidney (Fig. 1). We were also unable to detect apoB mRNA in baboon kidney using the more sensitive approach of reverse transcription–polymerase chain reaction (data not shown). Based on these results, we chose to characterize the complementing activity in kidney, a tissue that lacks detectable editing activity and apoB mRNA.

**Baboon Kidney Extracts Complement Recombinant Apobec-1**—Rat apobec-1 was expressed in bacteria as a His$_6$-tagged protein, and the protein was purified to 80% homogeneity on Ni-NTA resin under native conditions. Recombinant apobec-1 lacked intrinsic editing activity in the standard *in vitro* editing assay and when tested over a wide range of concentrations and incubation times (data not shown). Editing activity was restored when apobec-1 was complemented with the 15–30% ammonium sulfate fraction of baboon kidney extract (Fig. 2). Partial Purification of the Complementing Activity, a 65-kDa Protein—The complementing activity in kidney extracts was sensitive to pretreatment with proteinase K, but not to micrococcal nuclease (data not shown). These results suggest that the complementing activity in kidney extracts was sensitive to pretreatment with proteinase K, but not to micrococcal nuclease (data not shown). These results suggest that the activity of a protein which does not require a free RNA component to complement apobec-1. To partially purify this activity, whole cell extracts of baboon kidney were fractionated with 15–30% ammonium sulfate, which precipitated 80% of the complementing activity but only 6% of the total protein. This

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**Apobec-1 Interacts with Complementing Activity in Vitro**

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**RESULTS**

**Expression of ApoB mRNA in Baboon Tissues**—We previously demonstrated that the complementing activity is widely expressed in baboon tissues, whereas the expression of the editing enzyme is restricted to the small intestine (20). In humans, apoB mRNA is expressed in a variety of human tissues, including kidney (27). Northern blot analysis of baboon tissues detected a 14-kilobase pair apoB mRNA (Fig. 1). Relative to the levels of apoB mRNA in the liver (100%), the levels of apoB mRNA were 50% in the small intestine, 5% in the spleen, and 1.3% in the stomach. No hybridizable apoB mRNA was detected in other baboon tissues, including kidney (Fig. 1). We were also unable to detect apoB mRNA in baboon kidney using the more sensitive approach of reverse transcription–polymerase chain reaction (data not shown). Based on these results, we chose to characterize the complementing activity in kidney, a tissue that lacks detectable editing activity and apoB mRNA.

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**Fig. 1. Expression of apoB mRNA in baboon tissues.** Total RNAs (20 μg) from baboon tissues were analyzed by Northern blotting using a 32P-labeled baboon apoB100 cDNA as the probe, as described under "Materials and Methods." The position of the 14-kilobase pair apoB mRNA is indicated on the right.
fraction contained a large number of high molecular weight proteins, and an additional 6-fold purification was achieved by chromatography on Sephacryl S300. The complementing activity eluted as a broad peak in a volume corresponding to an average size of $65 \pm 10$ kDa (Fig. 2). In glycerol density gradient centrifugation, the activity sedimented as a single peak around 4.5S (data not shown). This corresponds to a molecular mass of 65 kDa, assuming that the activity is a globular protein. When the fraction from the gel filtration column was chromatographed on Q Sepharose, the complementing activity eluted in a single peak between 150 and 200 mM KCl.

**Characterization of the Complementing Activity**—The 15–30% ammonium sulfate fraction was used to characterize the complementing activity in vitro. Maximum editing was achieved with 20 $\mu$g of partially purified kidney extract and 1 $\mu$g of apobec-1, and an excess of either complementing activity (Fig. 3A) or apobec-1 (data not shown) inhibited editing. After the addition of complementing activity to apobec-1 in the in vitro editing assay, editing was detected within 5 min and increased for 3 h (Fig. 3B). Preincubation of apobec-1 or the complementing activity with each other or with the synthetic apoB mRNA did not change the kinetics of editing (data not shown). The optimal KCl concentration for the editing activity of apobec-1 and the complementing protein was between 90 and 125 mM, with a pH optimum of 8.0. The complementing activity was heat-sensitive, with 50% of the activity lost after incubation at 40 °C for 10 min prior to addition to the in vitro editing assay.

The activity of the reconstituted enzyme was completely inhibited by the addition of basic and acidic proteins, including lysozyme and bovine serum albumin, but not by the addition of total cellular protein from COS cells or reticulocyte lysate (Table I). Moderate concentrations of EDTA, EGTA, monovalent cations, or divalent cations had no effect on apobec-1 and the complementing activity (Table I). In contrast to the general characteristics exhibited by most nucleic acid binding proteins, the editing activity of apobec-1 and the complementing protein was not affected by the addition of heparin, tRNA, single-stranded DNA, double-stranded RNA, or ribohomopolymers (Table I). Although it has been reported that the activity of apobec-1 is inhibited by poly(U) (28), we found that even a 10,000-fold molar excess of poly(U) did not inhibit editing in our system (data not shown).

**Complementing Activity Interacts with Apobec-1**—To test whether the complementing activity and apobec-1 can interact

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**FIG. 2. Sephacryl S300 chromatography of the complementing activity.** The 15–30% ammonium sulfate fraction of a baboon kidney extract was chromatographed on Sephacryl S300. The starting material is shown in the second lane. Column fractions (10 $\mu$l) were assayed for their ability to complement purified His$_6$-tagged apobec-1 in an in vitro editing assay. The samples were analyzed by primer extension, and the positions of the products from the unedited (CAA) and edited (UAA) RNAs are indicated. The results are expressed as percent editing (% UAA/UAA + CAA). The relative molecular size of the peak complementing activity was determined by comparison to a standard curve as described under "Materials and Methods."

**FIG. 3. Characterization of the complementing activity and apobec-1. A,** editing assays (60 $\mu$l) were performed with 1 $\mu$g of purified His$_6$-tagged apobec-1 and increasing amounts of complementing activity (15–30% ammonium sulfate fraction of a baboon kidney extract) as indicated. The samples were analyzed as described in Fig. 2. B, in vitro editing assays (20 $\mu$l) containing partially purified complementing activity (20 $\mu$g) and purified His$_6$-tagged apobec-1 (1 $\mu$g) were incubated for 5 min to 8 h as indicated.
Apopec-1 Interacts with Complementing Activity in Vitro

Table I

| Reagent                        | Concentration |
|--------------------------------|---------------|
| 100% Inhibition                |               |
| Bovine serum albumin           | 1 mg/ml       |
| Lysozyme                       | 1 mg/ml       |
| Polyethylene glycol            | 10%           |
| Glycerol                       | 40%           |
| Ammonium acetate               | 0.2 M         |
| EDTA                           | 25 mM         |
| Moderate inhibition            |               |
| EGTA                           | 10 mM         |
| Polyethylene glycol            | 5%            |
| COS cell protein               | 1.5 mg/ml     |
| Reticulocyte lysate protein    | 1.5 mg/ml     |
| ZnCl₂                          | 10-50 μM      |
| Heparin                        | 15-30 μg/ml   |
| Spermidine                     | 0.5 mg/ml     |
| DTABb                          | 0.5 mM        |
| Poly(A)poly(U)                 | 100 ng        |
| Ribohomopolymers (poly(A), poly(C), poly(G), poly(U)) | 500 ng |
| Total RNA from COS cell        | 500 ng        |
| Double-stranded RNA            | 100 ng        |
| tRNA                           | 250 ng        |
| Single-stranded DNA            | 100 ng        |

a DTAB, dodecyltrimethylammonium bromide.

Complementing activity interacts with His₆-tagged apobec-1 in vitro. A, partially purified complementing activity (100 μg) was incubated with apobec-1-Ni-NTA or vector-Ni-NTA beads (100 μl) for 2 h. Equal amounts of the starting material and the unbound fractions (15 μg) were assayed for complementing activity in the presence of purified apobec-1. Lane 1, buffer; lane 2, starting material; lane 3, unbound to vector-Ni-NTA; lane 4, unbound to apobec-1-Ni-NTA. B, complementing activity was incubated with apobec-1-Ni-NTA beads as described above. After extensive washing, an aliquot of the beads (20 μl) was assayed directly for editing activity. Lane 1, buffer; lane 2, 15 μg of partially purified complementing activity (starting material) and 1 μg of apobec-1; lane 3, apobec-1-Ni-NTA beads (0.5 ml), and proteins were eluted with 2 mol of buffer D containing either 0.5 Mimidazole or 0.4 M KCl (0.5 ml/fraction). The imidazole-eluted fractions were pooled, and an aliquot (10 μl) was assayed alone for editing activity. Aliquots of the KCl-eluted fractions (10 μl) were assayed for editing activity in the presence or absence of apobec-1 (1 μg) as indicated.

Interaction with the Complementing Activity Does Not Require the Leucine-rich Region in Apobec-1—The carboxyl terminus of apobec-1 contains a leucine-rich region (amino acids 182–210) which is required for editing activity (16, 18, 21). To test whether this sequence is required for interaction with complementing activity, a deletion mutant that lacked amino acids 171–229 (apobec-1/Kpn) was expressed in bacteria (Fig. 5A, lane 3). Purified apobec-1/Kpn edited apoB mRNA in vitro with low efficiency, possessing only 0.2% of the activity of the wild type protein (data not shown). However, apobec-1/Kpn immobilized on nickel-NTA resin was able to deplete approximately 90% of the complementing activity in the in vitro binding assay (Fig. 5B, lane 4). To confirm that the complementing activity had bound to the truncated apobec-1 protein, the apobec-1/Kpn-Ni-NTA resin was eluted with 0.4 M KCl (Fig. 5C). The recovery of complementing activity from the apobec-1/Kpn

Fig. 4. Complementing activity interacts with His₆-tagged apobec-1 in vitro. A, partially purified complementing activity (100 μg) was incubated with apobec-1-Ni-NTA or vector-Ni-NTA beads (100 μl) for 2 h. Equal amounts of the starting material and the unbound fractions (15 μg) were assayed for complementing activity in the presence of purified apobec-1. Lane 1, buffer; lane 2, starting material; lane 3, unbound to vector-Ni-NTA; lane 4, unbound to apobec-1-Ni-NTA. B, complementing activity was incubated with apobec-1-Ni-NTA beads as described above. After extensive washing, an aliquot of the beads (20 μl) was assayed directly for editing activity. Lane 1, buffer; lane 2, 15 μg of partially purified complementing activity (starting material) and 1 μg of apobec-1; lane 3, apobec-1-Ni-NTA beads (0.5 ml), and proteins were eluted with 2 mol of buffer D containing either 0.5 M imidazole or 0.4 M KCl (0.5 ml/fraction). The imidazole-eluted fractions were pooled, and an aliquot (10 μl) was assayed alone for editing activity. Aliquots of the KCl-eluted fractions (10 μl) were assayed for editing activity in the presence or absence of apobec-1 (1 μg) as indicated.

shown). Near quantitative depletion of the complementing activity was obtained when 100 μg of protein were incubated with 1 mg of resin-bound apobec-1 (Fig. 4A, lane 4). Binding to the apobec-1-Ni-NTA resin was saturable at higher concentrations of complementing activity (data not shown). After extensive washing, the beads were assayed for editing activity in the absence of exogenous apobec-1 or complementing activity. Editing activity was detected in the apobec-1-Ni-NTA beads which had been incubated with complementing activity (Fig. 4B, lane 3), suggesting that a functional holoenzyme had been generated on the resin. No editing activity was detected in the vector-Ni-NTA beads that had been incubated with complementing activity or in apobec-1-Ni-NTA beads alone (data not shown).

In Fig. 4C, the in vitro binding assays were scaled up to perform affinity chromatography using apobec-1 as a ligand. Complementing activity was bound to apobec-1-Ni-NTA, and proteins were step-eluted with buffer containing either 0.5 M imidazole or 0.4 M KCl. The imidazole-eluted fractions contained a functional editing enzyme that contained both apobec-1 and the complementing activity. However, only the complementing activity was eluted with KCl, as these fractions did not edit apoB mRNA unless recombinant apobec-1 was added to the reaction (Fig. 4C). Western blot analysis also confirmed the absence of apobec-1 in the salt-eluted fractions, which demonstrates that the lack of editing activity was not due to stoichiometric inhibition by excess apobec-1 (data not shown). The apobec-1 affinity chromatography step resulted in a 300-fold increase in the specific activity of the complementing activity. No complementing activity was detected when similar elution experiments were performed with Ni-NTA resin.

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**DISCUSSION**

This study is the first characterization of the complementing activity in a mammalian system. We show that this activity in baboon kidney is a 65-kDa protein or protein complex that interacts with apobec-1 in vitro in the absence of apoB mRNA to generate a catalytically active enzyme. Although it has been difficult to purify the native editing enzyme to homogeneity in the past, this was primarily due to the lack of a high affinity purification step. Our results demonstrate that an apobec-1 affinity column can be used to isolate either a holoenzyme that contains both apobec-1 and the complementing activity, or the complementing activity alone. Apobec-1 is a 27-kDa protein that is known to dimerize, although the functional significance of this dimerization is not known (29). It is intriguing that the sum of the sizes of an apobec-1 dimer and the 65-kDa complementing activity is consistent with the minimal molecular size of 125 kDa that was previously reported for the editing complex in baboon (11) and rat (14) enterocytes. However, our studies have not definitively established that the complementing activity is a subunit of the native editing enzyme.

Our in vitro results suggest that the proteins involved in editing have a high affinity for each other, and that a significant lag period is not required for the interaction of apobec-1 and the complementing activity with each other, or with apoB mRNA. This is in contrast to studies by Smith and colleagues who suggested that a long lag period was required for the assembly of higher order complexes, or editosomes, on apoB mRNA (15, 30). The hypothesis that the editing enzyme may be associated with other proteins involved in RNA processing is attractive, since several lines of indirect evidence suggest that editing may occur during the splicing and polyadenylation of apoB mRNA in vivo (2, 19, 31). However, several laboratories were unable to demonstrate a lag period for the in vitro editing reaction with the native enzyme (11–13). In addition, it has been reported that the enzyme has a buoyant density of pure protein, without a nucleic acid component (12). This discrepancy may reflect differences in extract preparation, since nuclear, cytosolic S100, and whole cell extracts have all been used as sources of the editing enzyme by various laboratories.

Our results also confirm previous studies which demonstrated that apobec-1 and complementing activity need to interact in a defined ratio (18, 19). The decrease in editing efficiency that occurs when apobec-1 or the complementing activity are in excess may be due to the formation of nonfunctional complexes between homologous species, in contrast to a functional heterologous interaction between apobec-1, the complementing activity, and apoB mRNA. The domain in apobec-1 that interacts with the complementing protein appears to be highly conserved, since rat apobec-1 can interact with the complementing activity from baboon (this study), chick (16), and human (16, 32), and rabbit apobec-1 can interact with rat complementing activity (21). Although apobec-1 is not a conservative protein (33), the leucine-rich region in the carboxyl terminus of apobec-1 is highly conserved across species, and it has been proposed that this sequence may interact with the complementing activity (16, 18, 21, 29). In this study, we showed that deletion of the leucine-rich region reduced editing efficiency to 0.2% of wild type levels, but that the truncated protein was still able to interact with the complementing activity in in vitro binding assay. These results suggest that the domain for interacting with the complementing activity lies within amino acids 1–170 of apobec-1, and experiments are currently in progress to test this hypothesis. Although the function of the leucine-rich region is not known, it may be involved in RNA-binding (18) or homodimerization (29).

In addition to the 65-kDa protein reported here, several other candidate proteins for the complementing activity have been proposed. An “enhancement factor” of 49 kDa has been partially purified from chick enterocytes, which stimulated editing when added to the native editing enzyme in rat enterocyte extracts in vitro (34). UV cross-linking experiments have identified proteins of 60–66 kDa (p66) and 40–44 kDa (p40) in rat liver and enterocyte extracts, which bind to apoB mRNA downstream from the editing site at nucleotides 6671–6674 (15, 17, 35). However, no direct functional evidence has been presented demonstrating that these binding proteins have complementing activity or that they are involved in editing. Schock et al. (36) identified a 240-kDa protein or protein com-

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**Fig. 5. Complementing activity interacts with the apobec-1/Kpn deletion mutant.** A, proteins purified by nickel affinity chromatography from bacteria transformed with vector (lane 1), wild type apobec-1 (lane 2), or the apobec-1/Kpn deletion mutant (lane 3) were analyzed by Western blotting using a rabbit anti-apobec-1 peptide antibody. Molecular mass markers are indicated on the right. **B,** in vitro binding experiments were performed with the partially purified complementing activity and apobec-1-Ni-NTA or apobec-1/Kpn-Ni-NTA beads, as described in Fig. 4A. The starting material and unbound fractions (15 μg) were assayed for complementing activity in the presence of purified apobec-1. **Lane 1,** buffer control; **lane 2,** starting material; **lane 3,** unbound to apobec-1-Ni-NTA; **lane 4,** unbound to apobec-1/Kpn-Ni-NTA. **C,** partially purified complementing activity was bound to apobec-1/Kpn-Ni-NTA (0.35 ml) and bound proteins were eluted with 0.4 M KCl as described in Fig. 4C. Aliquots of each fraction (10 μl) were assayed for complementing activity in the presence of recombinant wild type apobec-1. Affinity column was comparable (within 2-fold) to the recovery from the wild type apobec-1 column in three separate experiments.
plex in rat liver extracts that may be required for the efficient editing of apoB mRNA in vitro, but the ability of p240 to complement recombinant apobec-1 was not tested. Several candidate auxiliary proteins in extracts of McArdle cells have also been reported to bind to an apobec-1-affinity resin in two recent preliminary studies (37, 38).

The simplest model of the editing enzyme is that it is composed of the catalytic subunit, apobec-1, and an RNA-binding subunit that docks apobec-1 to its target cytidine in apoB mRNA. Although this model is consistent with our data that suggests the apobec-1 subunit that docks apobec-1 to its target cytidine in apoB mRNA. Although this model is consistent with our data that suggests that the complementing activity represents the RNA-binding subunit of the enzyme. Recombinant apobec-1 expressed in bacteria has an RNA-binding activity, with a preference for AU-rich sequences (28, 39). Binding was of low specificity, which suggests that the sequence-specific recognition of apoB mRNA may require the complementing activity or the holoenzyme. The widespread expression of the complementing activity in tissues which do not synthesize apoB mRNA or apobec-1 indicates that this activity may have other cellular functions (16, 19–21). Although the complementing activity may function as a sequence-specific RNA-binding protein which has other roles in RNA metabolism. The complementing activity may serve as an RNA chaperone which facilitates the folding of apoB mRNA into a structure that is a more favorable substrate for the enzyme. The activity may also stimulate the binding of apobec-1 to apoB mRNA, or the dissociation of products after editing has occurred. Similar functions have been reported for the nonspecific RNA-binding proteins, HnRNP A1 and p7 nucleocapsid proteins, which facilitate hammerhead ribozyme catalysis (40, 41).

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