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Identification of domains in apobec-1 complementation factor required for RNA binding and apolipoprotein-B mRNA editing

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ABSTRACT

The C-to-U editing of apolipoprotein-B (apo-B) mRNA is catalyzed by an enzyme complex that recognizes an 11-nt mooring sequence downstream of the editing site. A minimal holoenzyme that edits apo-B mRNA in vitro has been defined. This complex contains apobec-1, the catalytic subunit, and apobec-1 complementation factor (ACF), the RNA-binding subunit that binds to the mooring sequence. Here, we show that ACF binds with high affinity to single-stranded but not double-stranded apo-B mRNA. ACF contains three nonidentical RNA recognition motifs (RRM) and a unique C-terminal auxiliary domain. In many multi-RRM proteins, the RRMs mediate RNA binding and an auxiliary domain functions in protein–protein interactions. Here we show that ACF does not fit this simple model. Based on deletion mutagenesis, the RRMs in ACF are necessary but not sufficient for binding to apo-B mRNA. Amino acids in the pre-RRM region are required for complementing activity and RNA binding, but not for interaction with apobec-1. The C-terminal 196 amino acids are not absolutely essential for function. However, further deletion of an RG-rich region from the auxiliary domain abolished complementing activity, RNA binding, and apobec-1 interaction. The auxiliary domain alone did not bind apobec-1. Although all three RRMs are required for complementing activity and apobec-1 interaction, the individual motifs contribute differently to RNA binding. Point mutations in RRM1 or RRM2 decreased the K_d for apo-B mRNA by two orders of magnitude whereas mutations in RRM3 reduced binding affinity 13-fold. The pairwise expression of RRM1 with RRM2 or RRM3 resulted in moderate affinity binding.

Keywords: ACF; apo-B; apobec-1; RNA editing

INTRODUCTION

RNA–protein interactions play a critical role in regulating eukaryotic gene expression at the posttranscriptional level. RNA-binding proteins mediate the processes of mRNA splicing, polyadenylation, transport to the cytoplasm, translation, and turnover. In mRNA editing, the sequence of the mRNA is changed after transcription by the insertion, deletion, or modification of nucleotides (Smith et al., 1997; Gott & Emeson, 2000). By altering the coding capacity of the transcript, editing generates alternative forms of the protein that have different biological functions. In mammals, the characterized examples of mRNA editing involve single nucleotide changes that are generated by site-specific deamination reactions that convert A → I or C → U (Smith & Sowden, 1996). Several mammalian mRNAs, including the glutamate and serotonin 5-HT_{2c} receptors, and hepatitis delta virus, undergo A → I editing events (Bass, 1997; Gott & Emeson, 2000). These conversions are catalyzed by a family of adenosine deaminases that act on double-stranded RNA (ADAR). The ADAR enzymes function as a single polypeptide that can bind to the substrate RNA and deaminate the target adenosines in the absence of other factors (Smith & Sowden, 1996; Bass, 1997; Gott & Emeson, 2000).

To date, there is only one example of C → U editing for which the editing machinery has been defined. The enzyme complex that edits mammalian apolipoprotein-B (apo-B) mRNA deaminates a genomically encoded cytidine at nt 6666 (Chan et al., 1997). This modification converts the codon CAA encoding glutamine in the full-length protein (apo-B100) to a premature stop codon, UAA (Chen et al., 1987; Powell et al., 1987). The edited mRNA codes for a truncated apo-B protein (apo-B48). In several species, including humans, editing of apo-B mRNA is restricted to the intestine whereas the liver synthesizes only the full-length protein (Greeve et al.,

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1993). The two forms of apo-B perform distinct roles in the synthesis, metabolism, and transport of plasma lipoproteins and in susceptibility to atherosclerosis (Chan, 1992; Innerarity et al., 1996).

The editing of apo-B mRNA requires sequences on apo-B mRNA that are recognized by the editing enzyme complex. An 11-nt mooring sequence (nt 6671–6681) located downstream of C^{6666} is critical for editing, and mutations in this sequence abolish or down regulate editing in vitro (Shah et al., 1991; Backus & Smith, 1991, 1992; Driscoll et al., 1993). The native editing holoenzyme has not been purified, but a minimal complex that edits apo-B mRNA in vitro has been defined. The catalytic subunit of the enzyme, apobec-1, is a cytidine deaminase that alone is not competent to edit (Teng et al., 1993; Navaratnam et al., 1993). We recently reported the purification and cloning of a novel RNA-binding protein, Apobec-1 complementation factor (ACF), that functionally complements apobec-1 to edit apo-B mRNA in vitro (Mehta & Driscoll, 1998; Mehta et al., 2000). Similar results were obtained by Greeve and colleagues, who isolated Apobec-1 stimulating protein (ASP), which is identical to ACF except for the presence of an eight amino acid insertion (Lellek et al., 2000). Both isoforms of ACF edit apo-B mRNA in vitro (Lellek et al., 2000; Mehta et al., 2000) and the two proteins appear to be generated by alternative splicing (A. Mehta & D.M. Driscoll, unpubl. observations). Our in vitro and in vivo experiments demonstrated that ACF specifically binds to the mooring sequence in apo-B mRNA and interacts with apobec-1 (Mehta et al., 2000). Based on these data, we proposed a model of the holoenzyme in which ACF functions as the RNA-binding subunit that docks apobec-1 to deaminate the upstream cytidine. Thus, in contrast to the ADARs, the catalytic and RNA-binding functions of the apo-B mRNA editing enzyme are encoded by two polypeptides.

ACF contains three nonidentical copies of the RNA recognition motif (RRM), a well-characterized RNA-binding motif present in a large number of proteins that are involved in RNA processing and metabolism (Burd & Dreyfuss, 1994). Each RRM domain spans 80–90 amino acids and is characterized by the presence of a less conserved RNP2 hexamer motif and a highly conserved RNP1 octamer motif, with several conserved amino acids interspersed across the domain (Burd & Dreyfuss, 1994; Nagai et al., 1995). RRM proteins have a modular structure, containing one to four RRM domains and additional regions referred to as auxiliary domains. Single RRM proteins often require flanking sequences for high affinity binding to their target RNAs. For proteins that contain multiple RRM domains, the RRM domains alone are generally sufficient for RNA-binding activity, with the auxiliary domain often functioning in protein–protein interactions (Burd & Dreyfuss, 1994). Furthermore, the function of the individual domain can vary such that each RRM may bind a different sequence or a subset of RRMs may be required for high affinity binding (Burd et al., 1991; Ginisty et al., 2001).

In this study, we show that ACF binds with high affinity to apo-B mRNA in vitro and that this interaction can be detected in extracts from cells that lack apobec-1 and editing activity. To identify functionally important domains in ACF, we performed deletion and site-directed mutagenesis experiments. We found that the RRMs alone did not bind apo-B mRNA and that the C-terminal auxiliary domain did not interact with apobec-1. Additional amino acids in the pre-RRM region and in the C-terminal auxiliary domain of ACF are required for complementing activity and high affinity binding to apo-B mRNA. Although all three RRMs are required for complementing activity and apobec-1 interaction, the individual motifs contribute differently to the RNA-binding activity of ACF.

RESULTS

Editing activity of ACF and apobec-1

We previously showed that ACF and apobec-1 are sufficient to edit apo-B mRNA in vitro, but a kinetic analysis was not performed in this study (Mehta et al., 2000). Figure 1 shows the editing activity of purified bacterially

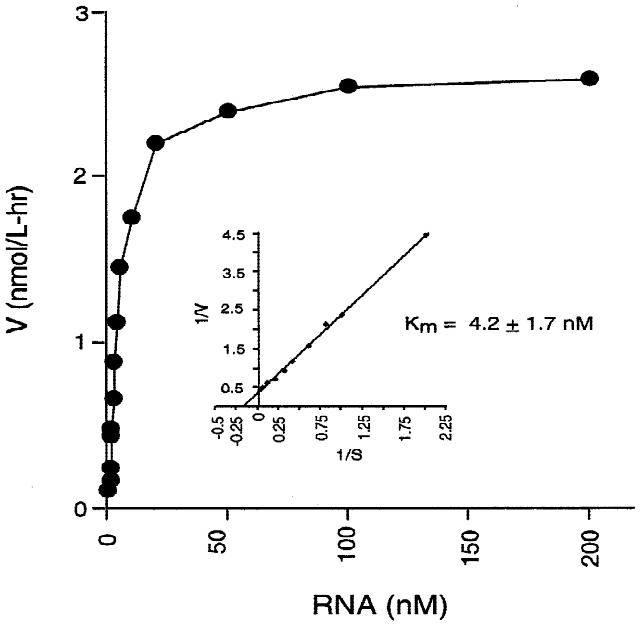


FIGURE 1. In vitro editing activity of ACF and apobec-1. Substrate dependence of the editing activity of purified recombinant Strep-tagged ACF and $(His)_6$-apobec-1. Editing assays were performed for 1 h at 37°C with 5 fmol ACF, 10 fmol apobec-1, and increasing concentrations of synthetic apo-B RNA. The reactions were stopped and analyzed by a poisoned primer extension assay (Mehta et al., 2000). The femtomoles of edited RNA were quantified by PhosphorImager analysis. Regression analysis of 1/s versus 1/v was performed according to Lineweaver–Burk.

expressed Strep-tagged ACF and $(His)_6$-apobec-1. The reaction followed Michaelis–Menten kinetics and reached substrate saturation at ~2.5 nmol/L-h. The preincubation of two of the three components of the reaction prior to the addition of the third did not change the kinetics of the reaction. The apparent K_m for Strep-tagged ACF and $(His)_6$-apobec-1 was 4.2 ± 1.7 nM.

RNA-binding activity of ACF

ACF binds apo-B mRNA in a mooring sequence-specific manner in both UV crosslinking and EMSA assays (Mehta et al., 2000; Blanc et al., 2000). To determine the affinity of ACF for apo-B mRNA, we performed filter binding experiments using 1 ng (11 fmol) of a 280-nt ^{32}P-labeled apo-B100 RNA and increasing amounts of purified Strep-tagged ACF. The dissociation constant, K_d, was calculated as the protein concentration at which 50% of the RNA bound at saturation was retained on the filter. As shown in Figure 2A, ACF binds single-stranded apo-B RNA with relatively high affinity, with an apparent K_d of 8.0 ± 3.2 nM. No gel shift was detected when antisense apo-B RNA was used as a probe. Strep-tagged ACF did not bind to double-stranded apo-B RNA in a filter binding assay ($K_d > 1$ μM; Fig. 2A), or in an EMSA assay (data not shown).

Association of ACF with apo-B mRNA in HepG2 cells

Based on our previous coimmunoprecipitation (co-IP) experiments, ACF is associated with apo-B mRNA in McArdle 7777 cells, a rat hepatoma cell line that edits endogenous apo-B mRNA (Mehta et al., 2000). ACF and apo-B mRNA are also expressed in HepG2 cells, a human hepatoma cell line that lacks apobec-1 and editing activity (Giannoni et al., 1994). We prepared extracts from HepG2 cells and performed co-IP experiments using an anti-ACF antibody directed against amino acids 4–18 of human ACF (Mehta et al., 2000). RNAs were extracted from the immune complexes and analyzed by reverse transcriptase-PCR using gene-specific primers. As shown in Figure 2B, the anti-ACF antibody, but not the preimmune serum, coimmunoprecipitated apo-B mRNA. No PCR products were obtained when reverse transcriptase was omitted from the reaction. In contrast, there was no detectable co-IP of the abundant GAPDH mRNA by the anti-ACF antibody. These results suggest that ACF is associated with apo-B mRNA in vivo even in cells that are not competent to edit.

Deletion mutagenesis of ACF

Unlike apobec-1, which is only ~70% conserved across species, ACF is highly conserved, with >95% amino acid identity between the human (Lellek et al., 2000; Mehta et al., 2000; Blanc et al., 2001b), baboon (Mehta et al., 2000), chicken (Blanc et al., 2001b), and mouse (data not shown) proteins. Figure 3A shows a schematic of ACF, which is predicted to contain three non-identical RRM domains in the N-terminal half of the protein and a unique C-terminal auxiliary domain. As shown in Figure 3B, the RRM domains of ACF share significant homology with the three RRM domains of GRY-RBP, an RNA-binding protein that has been implicated in the regulation of apo-B mRNA editing in vivo (Blanc et al., 2001b). The auxiliary domain of ACF is characterized by the presence of six RG dipeptides, four of which are clustered in an RG-rich region between amino acids 380 and 402, and a weak double-stranded RNA-binding motif between amino acids 446 and 523 (Fig. 3A). To identify functionally important domains in ACF, we performed deletion mutagenesis of the human ACF cDNA. The N- and C-terminal deletion mutants are termed DM*x-y*, where *x-y* refers to the positions of the amino acids being expressed. All mutants were tested in at least three independent experiments. Results from representative experiments are shown in the figures, and the quantitative data for key mutants are summarized in Figure 6.

N-terminal sequences in ACF are required for complementing activity and RNA binding

In addition to the homology in the RRM domains, amino acids 17–46 in the pre-RRM region of ACF share 63% identity (80% similarity) with the pre-RRM region of GRY-RBP (Fig. 3B), which suggests that these residues may be functionally important. We generated a series of N-terminal deletion mutants of ACF that were expressed by in vitro translation in the presence of ^{35}S-methionine (Fig. 4A). Equal amounts of translation products were assayed for their ability to functionally complement apobec-1 to edit a 280-nt synthetic apo-B RNA in vitro. Under the conditions that were used, ~50% of the RNA was edited by apobec-1 and wild-type ACF, which is referred to as 100% complementing activity (Fig. 4B). The removal of the N-terminal 13 amino acids in ACF (DM14-586) reduced complementing activity to ~33% of wild-type levels, whereas activity was abolished by the deletion of two additional amino acids (DM16-586) or further truncations (Figs. 4B and 6).

The mutants were also analyzed for their ability to interact with apobec-1 in vitro using a co-IP assay (Mehta et al. 2000). Wild-type and mutant ACF translation products were incubated in the presence or absence of in vitro translated apobec-1 that was tagged with an HA peptide. As shown in Figure 4C, an anti-HA monoclonal antibody could coimmunoprecipitate HA-tagged apobec-1 and wild-type ACF when the two proteins were posttranslationally mixed. Although DM16-586, DM36-586, and DM52-586 lacked complementing activity, these proteins were still able to bind HA-tagged

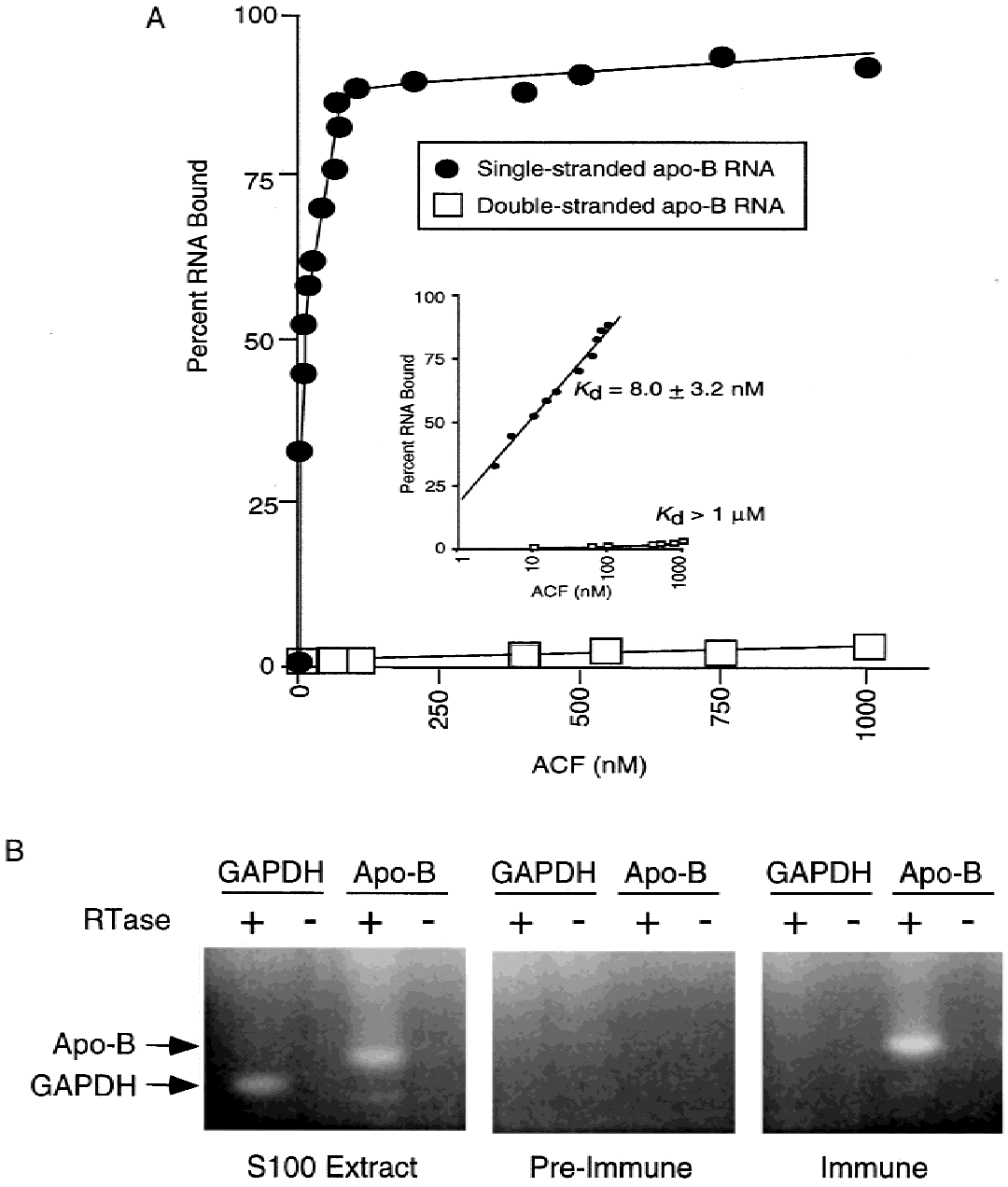
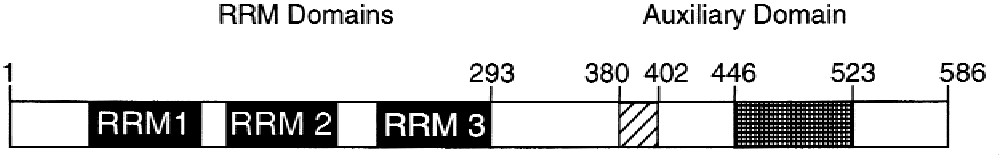


FIGURE 2. RNA-binding activity of ACF in vitro and in vivo. **A**: Filter-binding assays were performed with increasing concentrations of purified Strep-tagged wild-type ACF and 1 ng ^{32}P-labeled synthetic apo-B RNA. Double-stranded RNA was prepared by annealing ^{32}P-labeled sense and antisense strands of the 280-nt apo-B RNA. The amount of bound RNA was determined by binding to nitrocellulose filters. The apparent dissociation constant, K_d, was calculated as the concentration of protein at which 50% of the RNA bound at saturation is retained on the filter. **B**: Extracts from HepG2 cells were incubated with either preimmune serum or anti-ACF antibody, and the immune complexes were isolated using Protein A-agarose. The coimmunoprecipitated RNAs were extracted from the beads and used as templates for reverse transcriptase-PCR using primers for apo-B or GAPDH as indicated. The PCR products were resolved by 2% agarose gel electrophoresis. The first panel shows the products obtained from the total starting extract. The positions of the GAPDH and apo-B products are indicated on the left.

apobec-1 in vitro. Further deletion of 129 amino acids from the N-terminus (DM130-586) abolished interaction with apobec-1 (Figs. 4C and 6A). Interestingly, a mutant expressing the C-terminal half of ACF (DM296-586) did not interact with HA-tagged apobec-1 (Fig. 4C). Thus, sequences in the auxiliary domain of ACF are necessary but not sufficient for binding to apobec-1.

Because of high background from the RRL, we could not detect RNA–protein complexes with in vitro translated ACF. For RNA-binding studies, the mutants were expressed in bacteria as Strep-tagged proteins and purified on Streptactin-affinity resin under native conditions (Fig. 4D). All of the bacterially expressed mutants gave the same results as the in vitro translated mutant

A



B

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ACF   6   KSGDGLSGTQK---EAALRALVQRTGYSLVQENGQRKYGGPPPG--WDAAPPERGCEIFI   60
          K G  ++ + K   EA ++AL++RTGY+L    GQRKYGGPPP   +    P  G EIF+
GRY 112   KQGTKVADSSKGPDEAKIKALLERTGYTLDVTTGQRKYGGPPPDSVYSGQQPSVGTEIFV  166

                              RRM1
ACF  61   GKLPRDLFEDELIPLCEKIGKIYEMRMMMD-FNGNNRGYAFVTFSNKVEAKNAIKQLNNY  119
          GK+PRDLFEDEL+PL EK G I+++R+MMD   G NRGYAFVTF  K  A+ A+K  NN+
GRY 167   GKIPRDLFEDELVPLFEKAGPIWDLRLMMDPLTGLNRGYAFVTFCTKEAAQEAVKLYNNH  226

                                                          RRM2
ACF 120   EIRNGRLLGVCASVDNCRLFVGGIPKTKKREEILSEMKKVTEGVVDVIVYPSAADKTKNR  179
          EIR+G+ +GVC SV N RLFVG IPK+K +E+IL E  KVTEG+ DVI+Y    DK KNR
GRY 227   EIRSGKHIGVCISVANNRLFVGSIPKSKTKEQILEEFSKVTEGLTDVILYHQPDDKKKNR  286

ACF 180   GFAFVEYESHRTAAMARRKLLPGRIQLWGHGIAVDWAEPEVEVDEDTMSSVKILYVRNLM  239
           F F+EYE H+TAA ARR+L+ G++++WG+   V+WA+P  + D + M+ VK+L+VRNL
GRY 287   SFCFLEYEDHKTAAQARRRLMSGKVKVWGNVGTVEWADPIEDPDPEVMAKVKVLFVRNLA  346

                              RRM3
ACF 240   LSTSEEMIEKEFNNIKPGAVERVKKIRDYAFVHFSNRKDAVEAMKALNGKVLDGSPIEVT  299
           + +EE++EK F+    G +ERVKK++DYAF+HF  R  AV+AM+ +NGK L+G  IE+
GRY 347   NTVTEEILEKAFSQF--GKLERVKKLKDYAFIHFDERDGAVKAMEEMNGKDLEGENIEIV  404

                      *   *
ACF 300   LAKPVDKDSYVRYTRGTGGRGTMLQGEYTYSLGQVYDPT--TTYLGASVFYAPQTYAAI  356
           AKP D+    R  +    +  M    Y Y    +  PT      G       P Y
GRY 405   FAKPPDQKRKERKAQRQAAKNQMYDDYYYYGPPHMPPPTRGRGRGGRGGYGYPPDYYG  462

                                  *    *    *         *
ACF 357   --PSLHFPATKGHLSNRAIIRAPSV---RGAAGVRGLGGRG-YLAYTGLGRG  402
                      ++R     P          G RG GGRG   A    GRG
GRY 463   GYEDYYDYYGYDYHNYRGGYEDPYYGYEDFQVGARGRGGRGARGAAPSRGRG  513
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FIGURE 3. Domain structure of ACF. **A**: Schematic of human ACF showing the three RRM domains and the C-terminal auxiliary domain. The hatched box indicates the >60% RG-rich region (amino acids 381–402), and the stippled box indicates the position of a putative double-stranded RNA-binding motif (amino acids 446–523). **B**: Sequence alignment of human ACF and GRY-RBP with the conserved residues shown in the middle. The positions of the three RRM domains are shown and the six RG dipeptides are indicated by the asterisks.

proteins when assayed for complementing activity (data not shown). When tested in an EMSA assay, DM14-586 formed a complex with ^{32}P-labeled apo-B RNA, although the signal was reduced compared to the wild-type protein. However, no RNA–protein complexes were detected with DM16-586 or with further N-terminal deletions (Fig. 6). Based on filter-binding assays, the mutant DM14-586 exhibited a K_d for apo-B mRNA of ~23 nM, which is a threefold lower affinity than wild-type ACF (Figs. 4E and 6). The removal of the N-terminal 15 (DM16-586) or 35 amino acids (DM36-586) drastically reduced the binding affinity to ~230 nM and >1 μM, respectively (Figs. 4E and 6), which is consistent with the EMSA data. These results suggest that the amino acids in the pre-RRM region of ACF contribute to high affinity binding to apo-B mRNA. DM296-586, which contains the C-terminal auxiliary region alone, did not bind to apo-B RNA in a filter-binding assay ($K_d > 1$ μM; Fig. 4E.).

Amino acids 378–391 in ACF are essential for complementing activity, RNA binding and apobec-1 interaction

Figure 5A shows the SDS-PAGE analysis of in vitro-translated proteins that were obtained from a series of C-terminal deletions of ACF. As shown in Figures 5B and 6, the removal of the C-terminal 66 amino acids of ACF (DM1-520) reduced complementing activity to ~60% compared to wild-type ACF. Further deletion from the C-terminus reduced complementing activity to ~20% in DM1-451 and 13% in DM1-391. These last two deletions lack the putative double-stranded RNA-binding domain, which suggests that this motif may be impor-

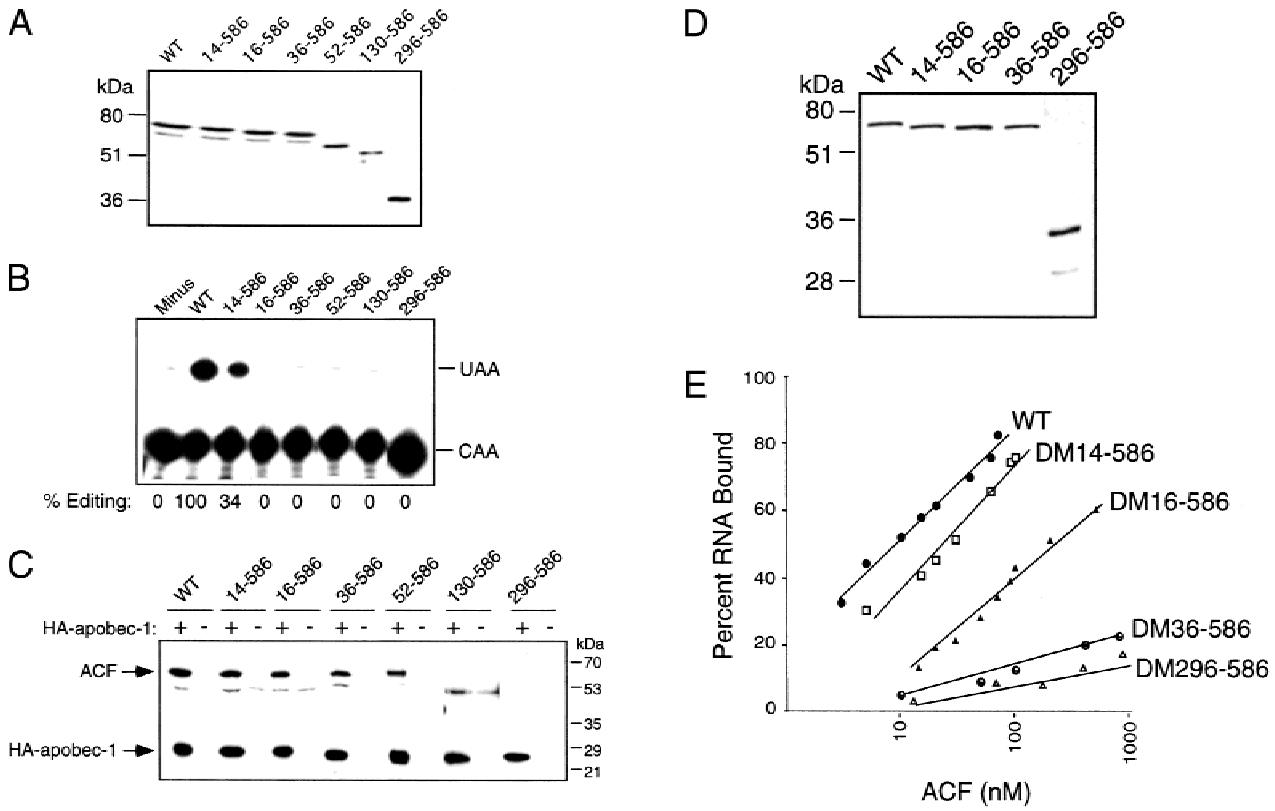

FIGURE 4. Expression and functional analysis of N-terminal deletion mutants. **A**: Wild-type ACF and the N-terminal deletion mutants were in vitro translated and the translation products were resolved by SDS-PAGE. The mutants are identified by the amino acids that are expressed. The faint bands below the major translation products may represent internal initiation, premature termination or degradation. **B**: Wild-type and mutant ACF translation products (5 fmol) were assayed for complementing activity in an editing assay containing in vitro-translated apobec-1 (10 fmol) and a 280-nt synthetic apo-B RNA. Minus refers to reactions performed in the absence of ACF to determine the background of the assay (typically 0.1–0.3%), which was subtracted out. The reactions were analyzed by a poisoned primer extension assay (Mehta et al., 2000). The positions of the products from the unedited (CAA) and edited (UAA) RNAs are indicated. **C**: Wild-type and mutant ACF translation products were incubated in the presence or absence of in vitro-translated HA-tagged apobec-1 as indicated. The reactions also contained an anti-HA monoclonal antibody and Protein A-Sepharose. After extensive washing, the immunoprecipitated complexes were analyzed by SDS-PAGE and autoradiography. **D**: Purified recombinant Strep-tagged proteins were resolved by SDS-PAGE and analyzed by western blotting stained with an anti-Strep tag antibody. **E**: Filter-binding assays were performed with increasing concentrations of purified Strep-tagged wild-type or mutant ACF as described in the legend to Figure 2A. The results are normalized to the percent RNA bound at saturation. The calculated K_d values are shown in Figure 6.

tant for full complementing activity but that it is not absolutely essential. This domain is not required for specificity, as a mutant apo-B RNA with three mutations in the mooring sequence was not edited in an in vitro assay containing DM1-451 or DM1-391 and apobec-1 (data not shown). DM1-377 and further C-terminal deletions were inactive in the in vitro editing assay (Fig. 5B). As shown in Figure 5C, amino acids 378–391 are also required for binding to HA-tagged apobec-1 in vitro, as the anti-HA antibody did not immunoprecipitate DM1-377 and DM1-331 above background levels.

The C-terminal deletion mutants were also expressed in bacteria as Strep-tagged proteins (Fig. 5D). We found that DM1-451 and DM1-391 bound to ^{32}P-labeled apo-B RNA in an EMSA assay, but that no RNA–protein complexes were detected with DM1-377, DM1-331, or DM1-293 (see schematic in Fig. 6). Similar results were obtained when the mutant proteins were assayed for RNA-binding activity by UV crosslinking or when EMSA assays were performed using crude bacterial extracts instead of purified recombinant proteins (data not shown). Therefore, the loss of RNA-binding activity in the mutant proteins is not due to inactivation during purification. Filter-binding studies showed that the C-terminal amino acids 521–586 are dispensable for high affinity binding, as DM1-520 binds to apo-B RNA with an apparent K_d of ~15 nM, which is only twofold higher than the wild-type protein (Figs. 5E and 6). The mutants DM1-451 and DM1-391 bind with K_ds of ~30 nM and 70 nM, respectively. In contrast, DM1-377, which lacks an additional 14 amino acids, and DM1-293, which expresses only the RRM domains, exhibited K_ds $\geq$ 1 μM (Figs. 5E and 6). Thus, sequences

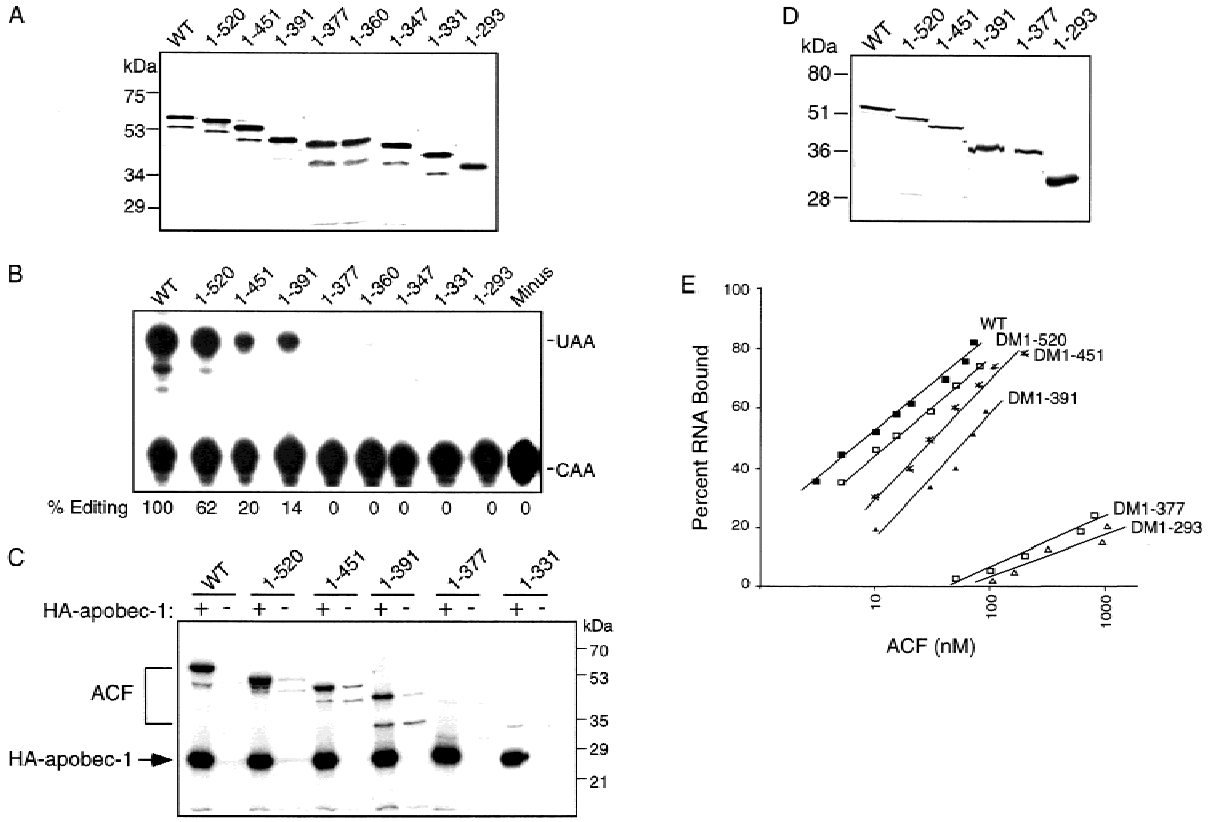

FIGURE 5. Expression and functional analysis of C-terminal deletion mutants. **A**: The in vitro translation products from the wild-type ACF and the C-terminal deletion mutants were resolved by SDS-PAGE. The mutants are identified by the amino acids that are expressed. **B**: Translation products were assayed for complementing activity as described in the legend to Figure 4B. The positions of the products from the unedited (CAA) and edited (UAA) RNAs are indicated. **C**: In vitro-translated wild-type ACF and C-terminal deletion mutants were assayed for their ability to interact with HA-tagged apobec-1 as described in the legend to Figure 4C. **D**: Purified recombinant Strep-tagged proteins were analyzed by western blotting as described in the legend to Figure 4D. **E**: Filter-binding assays were performed with purified Strep-tagged wild-type ACF or the C-terminal deletion mutants as described in the legend to Figure 4E.

outside the RRM domains are required for high affinity binding to apo-B RNA. However, the C-terminal auxiliary region alone does not appear to contain an independent RNA-binding domain, as DM296-586 had a very low affinity for apo-B RNA (Figs. 4E and 6).

To define a minimal functional protein, we generated ACF mutants that lacked the first 13 amino acids but differed in the number of C-terminal amino acids. The mutants were assayed for their ability to functionally complement apobec-1 in an in vitro editing assay. The smallest functional protein that we identified was DM14-391, which expresses 378 amino acids. Both DM14-391 and DM14-451 had low levels of activity ($<$15% of wild type), suggesting that these deletions severely compromise ACF function. The only deletion mutant that had robust complementing activity (60% of wild type) was DM14-520, which expresses 506 amino acids or 86% of the full-length protein.

Role of the RRM domains

To assess the relative contributions of the individual RRM domains in ACF, we made point mutations in each of the three RRM domains by converting either the second amino acid (F or Y) in the RNP2 motif or the conserved F residue in the RNP1 motif to alanine (Fig. 7A). Mutation of these conserved residues in other RRM proteins has been shown to eliminate RNA-binding activity (Nagai et al., 1995). None of the ACF point mutants were able to complement apobec-1 in an in vitro editing assay or interact with HA-tagged apobec-1 in the co-IP assay. As shown in Figure 7A, point mutations in RRM1 (F59A and F100A) and RRM2 (F139A and F183A) drastically reduced the affinity for apo-B mRNA by two orders of magnitude (K_ds $>$ 800 nM). In contrast, both RRM3 mutants (Y234A and F270A) showed only a $\sim$13-fold reduction in affinity, binding with K_ds of $\sim 105 \pm 6.2$ and 110 ± 4.7 nM, respectively.

As shown in Figure 6, DM130-586, which contains RRMs 2 and 3, had a very low affinity for apo-B mRNA. We also generated two internal deletion mutants that lack either RRM2 (IDMΔRRM2) or RRM3 (IDMΔRRM3) as shown in Figure 7B. Both deletions abolished complementing activity, interaction with HA-tagged apobec-1, and RNA-binding activity in an EMSA assay (data not shown). We measured a K_d of $\sim$55 nM for IDMΔRRM3,

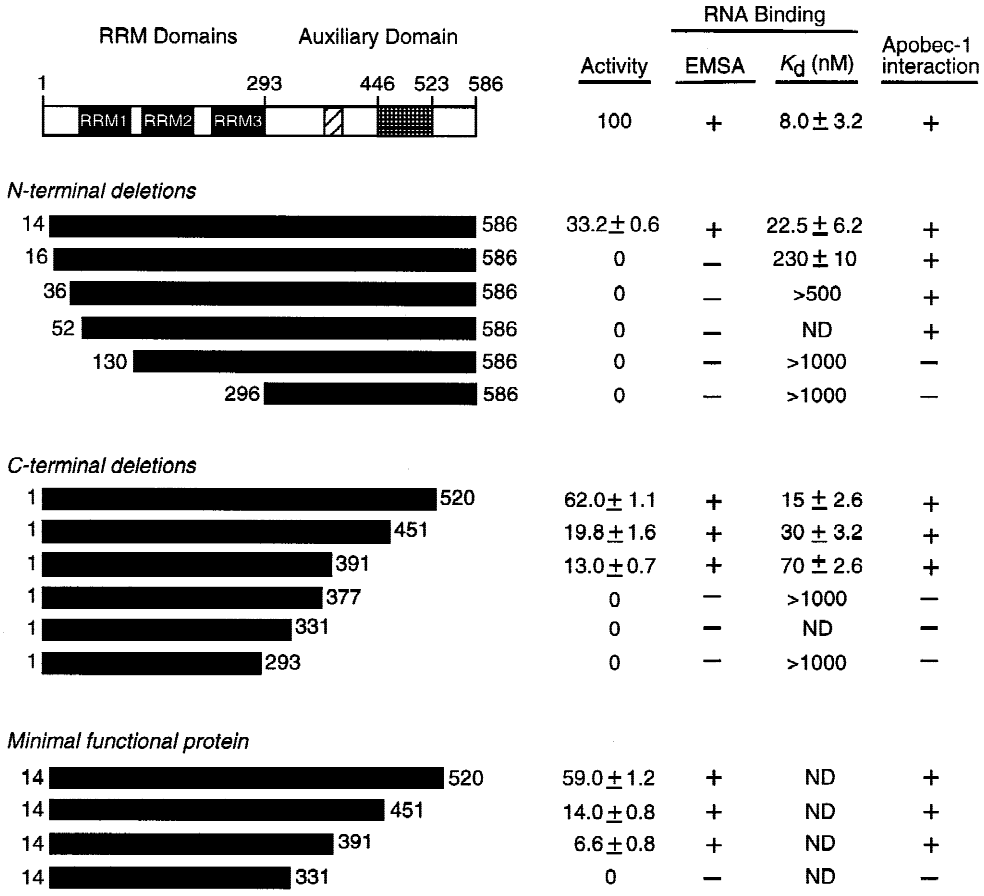

FIGURE 6. Summary of the data from N- and C-terminal deletion mutants of ACF. The structure of wild-type ACF is shown at the top. The three RRM domains are indicated as RRM1, RRM2, and RRM3. The hatched and stippled bars represent the RG-rich region and the putative double-stranded RNA-binding domain, respectively. A diagram of key N- and C-terminal deletion mutants is shown on the left. All mutants were tested in at least three independent experiments, and the results were quantified by PhosphorImager analysis. The results for complementing activity are expressed relative to the activity of the full-length ACF (average % ± *SD*). The K_ds for apo-B mRNA were obtained from filter-binding experiments and were calculated as the protein concentration at which 50% of the RNA was bound (average nM ± *SD*). The results from the EMSA assays and apobec-1 binding assays are expressed qualitatively (+ or −). ND: not determined.

which is 7-fold lower than wild-type ACF (Fig. 7B). Surprisingly, although point mutations in RRM2 were severely deleterious, deletion of RRM2 caused only a 14-fold reduction in binding affinity. Taken together, our results suggest that all three RRMs in ACF are required for high affinity binding to apo-B mRNA.

DISCUSSION

In this study, we undertook an investigation of the structure and function of ACF to begin to understand how this protein recognizes its target RNA. We found that the affinity of ACF for apo-B mRNA is relatively high. The recombinant protein binds its substrate in vitro with apparent K_d of ~8 nM, which is well within the range reported for other known sequence- or structure-specific RNA-binding proteins (Burd & Dreyfuss, 1994). Recently it was suggested that secondary structure in the region of the editing site may be important for apo-B mRNA editing (Richardson et al., 1998; Hersberger et al., 1999). Although different low-energy structures were proposed in two studies, in both cases the edited C was predicted to be in a single-stranded loop with the mooring sequence base paired to either a 5′ proximal element (Richardson et al., 1998) or a 3′ distal sequence (Hersberger et al., 1999). We showed that the affinity of ACF for double-stranded apo-B mRNA is over two orders of magnitude lower than for single-stranded RNA. We also found that deletion of a putative double-stranded RNA-binding domain from ACF did not completely abolish complementing activity or binding to apo-B mRNA, nor did it alter the specificity of the editing reaction. However, this deletion did reduce complementing activity and RNA-binding affinity by four- to fivefold, which suggests that amino acids 451–520 are important for function. However, it is important to note that our K_d values are only in vitro estimates and that the binding affinity may be different in vivo in the con-

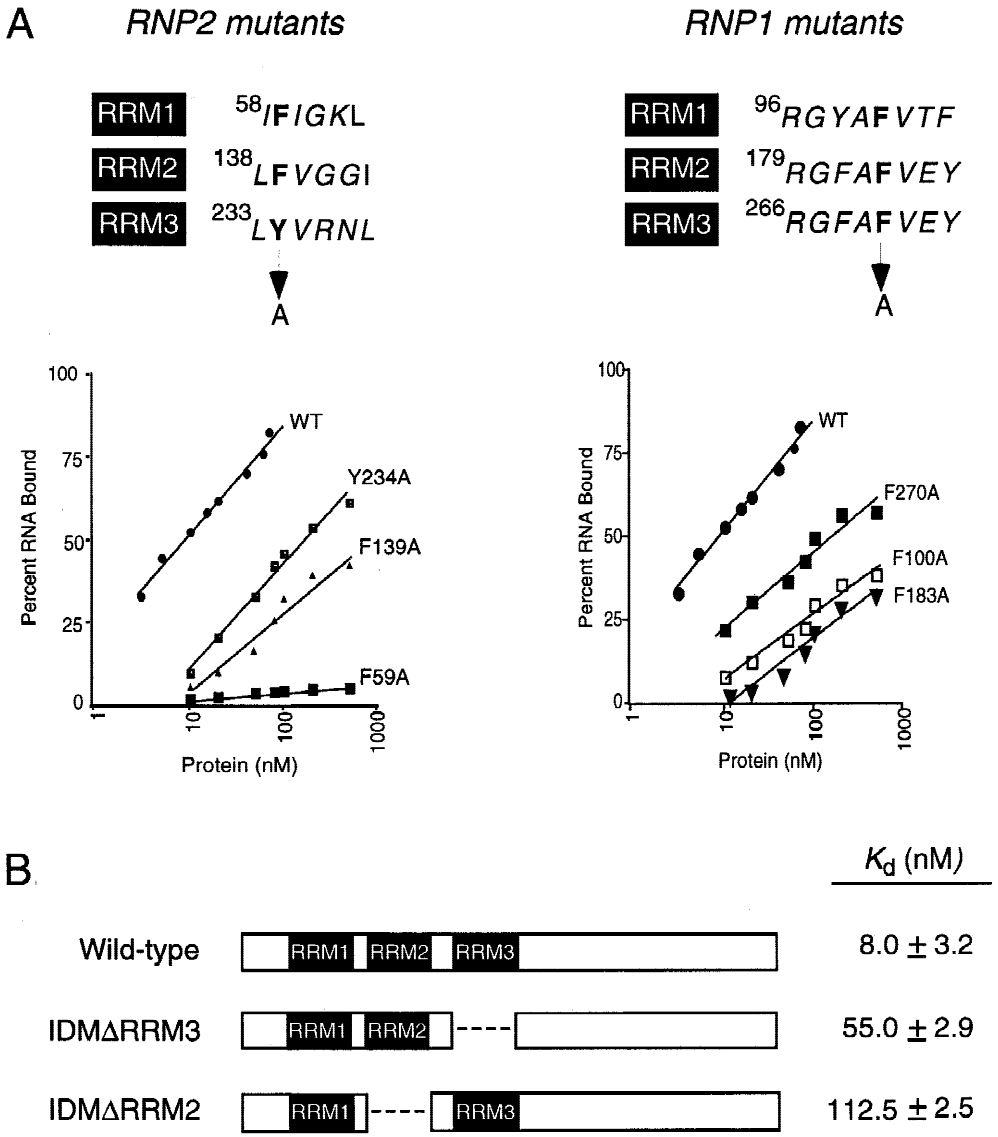

FIGURE 7. Mutagenesis of the RRM domains in ACF. **A**: Point mutations in the conserved RNP2 and RNP1 motifs were made in the three RRMs of human ACF as indicated. The mutants were expressed in bacteria as Strep-tagged proteins and analyzed for their ability to bind to ^{32}P-labeled apo-B RNA in a filter-binding assay as described in Figure 2A. The calculated K_d values are discussed in the text. **B**: RRM deletion mutants were analyzed in a filter-binding assay and the K_ds for apo-B mRNA were calculated as described in the legend to Figure 6.

text of the holoenzyme. In contrast to ACF, apobec-1 has a weak nonspecific RNA-binding activity (Anant et al., 1995; Navaratnam et al., 1995) with a K_d for apo-B mRNA of ~450 nM (Anant & Davidson, 2000). We found that the addition of recombinant apobec-1 did not change the affinity of ACF for apo-B mRNA (S. Murata & D.M. Driscoll, unpubl. observations), which suggests that the recognition of apo-B mRNA by the holoenzyme is mediated solely by ACF. This result should be interpreted cautiously, because all of the recombinant proteins may not assemble into an active enzyme complex.

In addition to our in vitro studies, we present evidence that suggests that ACF is associated with apo-B mRNA in vivo in HepG2 cells, a human hepatoma cell line that does not express apobec-1. In terms of physiological relevance, apo-B mRNA and ACF are both expressed in the livers of primates and rabbits, even though these tissues lack apobec-1 and are not competent to edit (Greeve et al., 1993; Giannoni et al., 1994; Hadjiagapiou et al., 1994). Our results raise the possibility that ACF has an additional function in the processing of apo-B mRNA that is independent of editing. In fact, ACF may play a more general role in mRNA metabolism, as ACF is widely expressed in other tissues that do not express apo-B mRNA (Mehta et al., 2000). This question is of particular interest because it was recently suggested that apobec-1 is involved in

the stabilization of mRNAs that undergo rapid degradation, including c-myc mRNA (Anant & Davidson, 2000). Experiments are currently in progress to identify other mRNA targets that are bound to ACF in vivo.

In many multi-RRM proteins, the RRM domains mediate RNA recognition whereas the auxiliary domain is involved in protein–protein interactions (Burd & Dreyfuss, 1994). This type of modular structure is reminiscent of transcription factors that contain distinct DNA-binding and transactivation domains. Our results suggest that ACF does not fit this simple model. The N-terminal half of ACF, which contains all three RRM domains, does not bind with high affinity to apo-B mRNA and the C-terminal auxiliary domain alone does not interact with apobec-1 in vitro. The two halves of ACF also did not function in *trans* to complement apobec-1 when they were added to the same in vitro editing reaction (data not shown). Both N- and C-terminal amino acids flanking the RRM domains are essential for optimal RNA-binding activity. These residues are also required for ACF to complement apobec-1 in an in vitro editing assay.

ACF is not the only example of a multi-RRM protein that requires sequences flanking the RRM domains for function. Recently, Brewer and colleagues analyzed the structural determinants that are essential for the RNA-binding activity of AUF1, a protein that binds to AU-rich elements in the 3′ untranslated regions of unstable mRNAs (DeMaria et al., 1997). In addition to the two tandem RRM domains, the high affinity binding of AUF1 requires an alanine-rich region from the N-terminus and a short glutamine-rich region that is C-terminal to RRM2. Although the function of the glutamine-rich sequence is not known, deletion of the alanine-rich sequence abolished homodimerization, which suggests that protein–protein interactions are important for the RNA-binding activity of AUF1 (DeMaria et al., 1997). We wondered whether the N- or C-terminal regions of ACF are required for homodimerization, which might explain why these residues are important for function. However, we have not been able to detect self-association of recombinant wild-type ACF using in vitro pull-down assays that were performed in the absence or presence of synthetic apo-B RNA (data not shown). Furthermore, as judged by gel filtration, native ACF exists as a monomeric protein (Mehta & Driscoll, 1998).

Based on structural studies of a number of RRM proteins, the RRMs are globular domains that fold into a characteristic $\beta\alpha\beta\beta\alpha\beta$ secondary structure, and this structure is identical whether the RRM is bound to RNA or free in solution (Burd & Dreyfuss, 1994; Nagai et al., 1995). The conserved RNP2 and RNP1 motifs, which lie on the two central β-strands, are thought to provide a general surface for binding to RNA. The specificity of the RNA-binding activity may come from less conserved amino acids in the loops and termini of the RRM domains (Burd & Dreyfuss, 1994; Nagai et al., 1995). The RNP2 and RNP1 motifs in the three RRM domains of ACF share substantial homology with the corresponding motifs in GRY-RBP. This high degree of conservation suggests that the two proteins may be ancestrally related and this hypothesis is supported by phylogenetic analyses (Blanc et al., 2001b). The homology between ACF and GRY-RBP extends into the pre-RRM region, ~40 amino acids preceding the RNP2 motif of RRM1. Our mutagenesis data suggest that this sequence is functionally important. Deletion of the pre-RRM region from ACF abolished complementing activity and binding to apo-B mRNA. The loss of complementing activity in these mutants is not likely due to a folding problem, as the proteins were expressed in RRL at low concentrations. Furthermore, the mutant proteins are still capable of interacting with apobec-1 in an in vitro binding assay. The amino acids in the pre-RRM region of ACF may be involved in directly contacting the RNA, either as a separate structural domain or more likely, as part of an extended RRM1 domain.

We also found that amino acids 378–391 in the C-terminus of ACF are critical for complementing activity, RNA-binding, and apobec-1 interaction. This region is >60% RG-rich and contains three of the six RG dipeptides that are found in ACF. These residues may be involved in modulating both RNA–protein and protein–protein interactions, or they may be required for proper folding of the protein. Experiments are currently in progress to distinguish between these two possibilities. Regardless of the explanation, our results clearly show that the minimal functional protein is quite large, which is of interest for future structural studies.

In addition to functioning in RNA recognition, RRMs can overlap with domains required for protein–protein interaction or nuclear localization. Although we did not identify a discrete domain in ACF that is responsible for binding to apobec-1, our mutagenesis studies suggest that the auxiliary domain and all three RRM domains are required for apobec-1 interaction. Importantly, we were able to identify mutations in the pre-RRM region that abolished RNA binding but not interaction with apobec-1. These results suggest that the RNA-binding and apobec-1 interaction domains are overlapping but distinct. This finding is consistent with our previous studies that showed that ACF and apobec-1 can physically interact in vitro in the absence of apo-B mRNA (Mehta et al., 2000).

Binding of multi-RRM proteins to a specific RNA sequence or structure may require all of the RRMs or only a subset of the domains. A recent study shed light on how RRM domains may cooperate to determine the RNA-binding activity of a multi-RRM protein (Park et al., 2000). HuD, a human neuron-specific RNA-binding protein, contains three RRM domains, but only RRM1 and an additional RRM are essential for binding with nanomolar affinity. When the kinetics of complex formation were analyzed, it was found that the RRMs cooperate not only to increase the affinity of the RNA–protein

interaction but also to stabilize the formed complex (Park et al., 2000). In this study, we showed that all three RRMs in ACF are required for complementing activity and high affinity binding to apo-B RNA. However, the relative contributions of the individual RRM domains to the RNA-binding activity of ACF are different. Point mutations in RRM1 and RRM2 reduced binding affinity to ~1 μM, whereas mutations in RRM3 are much less deleterious to function. Interestingly, the pairwise expression of RRM1 with either RRM2 or RRM3 is sufficient to confer moderate RNA-binding affinity. Because there is no significant homology between RRM2 and RRM3 to suggest that the domains may be functionally equivalent, a more likely explanation for our results is that spatial positioning of the RRMs is important for function. In the case of the RRM2 deletion mutant, the RRM1 and RRM3 domains are adjacent, whereas they are separated by over 100 amino acids in the RRM2 point mutant. The cocrystal structures of poly(A)-binding protein with poly(A) RNA, or Sex-lethal protein with polypyrimidine tract of *transformer* mRNA have recently been determined (Deo et al., 1999; Handa et al., 1999). In both of these proteins that bind unstructured RNA, a single functional RNA-binding unit is created from two tandem RRMs that together form an RNA-binding cleft. ACF, which also binds single-stranded RNA, may have a similar structure. We also found that a deletion mutant that expresses only RRM2 and RRM3 has a very low affinity for apo-B RNA. Experiments are currently in progress to determine whether RRM1 is the major RNA-binding determinant in ACF and whether the pre-RRM region is part of an extended RRM1 domain or a distinct RNA-binding domain.

While this work was under review, Blanc et al. reported that the RNA-binding activity of ACF requires amino acids 150–380, which do not encompass RRM1 (Blanc et al., 2001a). However, it is important to note that these authors showed that RNA-binding and complementing activity were abolished by deletion of the 84 N-terminal amino acids that contain the pre-RRM region and part of RRM1, which is consistent with our conclusion that these residues are functionally important. Interestingly, an RRM1 deletion mutant exhibited significantly reduced levels of complementing activity and RNA binding as determined by UV cross linking (Blanc et al., 2001a), which is in contrast to our RRM1 point mutants that are completely inactive. These apparently discrepant results could be explained by spatial positioning. The RRM1 deletion mutant expresses the pre-RRM region positioned adjacent to RRMs 2 and 3, which may allow the protein to bind to apo-B mRNA with moderate affinity in the absence of RRM1.

In conclusion, our results suggest that both N- and C-terminal sequences are required for ACF function and that all three RRM domains may cooperate to mediate binding to apo-B mRNA. A deeper understanding of how ACF recognizes its target RNA will require structural analysis of RNA–protein complexes that are formed with ACF alone or with the holoenzyme. Finally the presence of three nonidentical RRMs that are not functionally equivalent may allow ACF to interact with several different mRNA sequences.

MATERIALS AND METHODS

Mutagenesis

All N- and C-terminal deletions were made by the polymerase chain reaction (PCR) using primers that flanked the required regions. All of the 5′ primers added an initiator methionine to the N-terminus (Table 1). The amplified fragments were cloned by TA-expression cloning in pCR 3.1 (Invitrogen). Internal deletion mutants were made by a combination of PCR amplification and restriction digestion cloning. The construct IDMΔRRM2, which expresses amino acids 1–125 and 218–586, was generated using the primer pairs IDM-Sense-5′ and IDM(1–125)-3′. After digestion with *Kas*I, the PCR products were cloned into the *Kas*I site of a PCR fragment amplified with primers IDM(218–586)-5′ and Antisense-C-3′, which amplify RRM3 and the C-terminal auxiliary domain of ACF. For generating the construct IDMΔRRM3, which expresses amino acids 1–224 and 283–586, the ACF cDNA was amplified by PCR using primer pairs IDM-Sense-5′ and IDM(1–224)-3′ and the products were digested with *Hin*dIII. The fragment was ligated to the *Hin*dIII-digested DNA that was amplified with the primers IDM(283–586)-5′ and Antisense-C-3′, which amplify the C-terminal auxiliary domain of ACF. Point mutations in the RRM domains were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. The sequences of all mutant constructs were confirmed by automated DNA sequencing.

In vitro translation

Plasmid DNAs were added directly to coupled T7 transcription/translation lysates (Quick-TNT; Promega) in the presence of ^{35}S-methionine (Amersham). Translation products were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by PhosphorImager analysis (Molecular Dynamics). The amount of protein was determined by quantitation of known amounts of ^{35}S-methionine spotted on a 3 MM filter paper and calculated based on an endogenous concentration of 5 μM cold methionine in the lysate as specified by the manufacturer. The values were adjusted for the different number of methionines in the wild-type ACF and mutant proteins.

Expression of Strep-tagged proteins

Wild-type and mutant ACF cDNAs were subcloned into the *Bam*HI and *Xho*I sites of pASK-IBA7 (Strep-tag II; Genosys), which allows proteins to be expressed in bacteria with a Strep-tag at the N-terminus. The DNAs were transformed into *Escherichia coli* strain BL21 (Promega). Bacteria were grown in LB medium to OD_{600} density of 0.5–0.7 and induced with anhydrotetracycline (0.2 μg/mL) for 3 h at 37 °C. The cells

TABLE 1. List of oligonucleotides.

Primer name	Sequence[a]
C-terminal deletions	
Sense N-5′	ATTCAGGATCCATGGAATCAAATCACAAATC
DM1-520-3′	GCATCTCGAGTCATCCATCAGTGGGGATGCCCAGGGT
DM1-451-3′	GCATCTCGAGTCAGTTATTTTTCTGACAAATCTCTTCTA
DM1-391-3′	GCATCTCGAGTCAACGGCCGCCCAGTCCTCTCACT
DM1-377-3′	GCATCTCGAGTCAAGGGGCTCGGATAATGGCTCTGTTGC
DM1-360-3′	GCATCTCGAGTCAATGAAGACTGGGAATTGCTGCATAGGTCTG
DM1-347-3′	GCATCTCGAGTCAGAAGACAGGAGCTCCAAGGTA
DM1-331-3′	GCATCTCGAGTCACAAAGAGTAGGTATACTCTCCTTG
DM1-293-3′	GCATCTCGAGTCAACCATCCAGCACCTTGCCATT
N-terminal deletions	
Antisense C-3′	GCATCTCGAGTCATCAGAAGGTGCCATATCCATCC
DM14-586-3′	ATTCAGGATCCATGACTCAGAAGGAAGCAGCCCTCCGCGCA
DM16-586-3′	ATTCAGGATCCATGACTCAGAAGGAAGCAGCCCTCCGCGCA
DM36-586-3′	ATTCAGGATCCATGGGACAAAGAAAATATGGTGGCCCTCCACCT
DM52-586-3′	ATTCAGGATCCATGCCTGAAAGGGGCTGGA
DM130-586-3′	ATTCAGGATCCATGTGTGCCAGTGTGGACAATGC
DM228-586-3′	ATTCAGGATCCATGTCTTCAGTGAAAATCCTA
DM296-586-3′	ATTCAGGATCCATGATTGAAGTCACCCTAGCA
Internal deletions	
IDM-Sense-5′	ATTCAGGATCCATGGAATCAAATCACAAAT
IDM(1–125)-3′	TAAGAGGCGCCCATTTCTAATTTCATAATTATT
IDM(218–586)-5′	ATTAGAAATGGGCGCCCAGAAGTAGAAGTTGATGAAGAT
IDM(1–224)-3′	ATATTTAAAGCTTTTTCATCAACTTCTACTTCTGGCTC
IDM(283–586)-5′	GCAGTTGAGGCTATGAAAGCTTTAAATGGCA
Point mutations	
F59A	GGCTGTGAAATT*GCA*ATTGGAAAACTTCCC
F100A	GCAACAATAGAGGATATGCA*GCT*GTAACATTTTCAAATAAAGTGG
F139A	GACAACTGCCGATTA*GCA*GTTGGGGGCATC
F183A	CCAAAAACCGAGGCTTTGCC*GCA*GTGGAGTATG
Y234A	TCAGTGAAAATCCTA*GCA*GTAAGAAATCTTATGCTGTC
F270A	CGAGACTATGCT*GCA*GTGCACTTCAGTAAC

[a]Nucleotides that vary from the wild-type sequence are shown in italics.

were pelleted and sonicated in Buffer W (100 mM Tris-HCl, pH 8.0, 1 mM EDTA) with protease inhibitors (Complete; Roche). The lysate was spun at 10,000 × *g* for 20 min at 4 °C. The supernatant was applied directly to a 0.5 × 10 cm column containing Streptactin-POROS equilibrated in Buffer W containing 25 mM KCl, and the bound protein was eluted with 2.5 mM desthiobiotin (Sigma) in Buffer W. Proteins were quantified using the Protein Assay Reagent (BioRad) and analyzed by western blotting using a streptavidin AP-conjugate (APBiotech) according to the manufacturer's instructions. To block endogenous biotin-containing proteins, membranes were incubated with 2 μg/mL avidin for 10 min. The blots were developed using western blue stabilized substrate for alkaline phosphatase (Promega).

Complementing activity assays

Complementing activity was assayed as previously described (Mehta et al., 2000). Briefly, equal amounts of wild-type or mutant ACF proteins (5 fmol) were added to in vitro editing assays containing 11 fmol of a 280-nt synthetic RNA corresponding to nt 6504–6784 of baboon apo-B100 mRNA, 10 fmol apobec-1, and 250 μg tRNA. The reactions were incubated at 37 °C for 1.5 h, which is within the linear range of the assay. The edited products were detected by poisoned primer extension analysis (Mehta et al., 2000). Reactions performed in the absence of protein were used to calculate the background of the assay, which is typically between 0.1 and 0.3%. The results for the mutant proteins were normalized to the complementing activity of wild-type ACF, which complements apobec-1 to edit ~40 to 50% of the RNA under these conditions.

Electrophoretic mobility shift assays (EMSA)

^{32}P-labeled apo-B RNA was synthesized as previously described (Mehta & Driscoll, 1998). Purified recombinant protein was incubated with 1 ng ^{32}P-labeled RNA in Dignam buffer D (20 mM HEPES, pH 7.9, 2.5 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 0.5 mM DTT) containing 250 μg tRNA. After incubation for 1 h at 30 °C, the reactions were treated with 0.5 U of RNase T1 at 30 °C for 10 min. The complexes were resolved on 4% native polyacrylamide gels in 1 × TGE (20 mM Tris, pH 7.4, 150 mM glycine, 20 mM EDTA). The gels were dried and analyzed using a PhosphorImager (Molecular Dynamics).

Determination of K_d values

Filter-binding assays were performed using purified bacterially expressed proteins. Increasing concentrations of protein were added to reactions containing 1 ng ^{32}P-labeled apo-B RNA and 250 μg tRNA in 100 μL Dignam buffer D. After incubation at 30 °C for 20 min, the reactions were filtered through nitrocellulose (Millipore) and washed with 5 mL of Dignam buffer D. The filters were dried and counted in a liquid scintillation counter. Reactions set up in the absence of recombinant protein served as negative controls to calculate the background, which was less than 5% of the input counts in any independent experiment. After the background was deducted, the results were normalized to the percent RNA bound. The linear portion of each curve was subjected to regression analysis, and the apparent K_d was calculated as the concentration of protein at which 50% of the RNA bound at saturation is retained on the filter.

Association of ACF and apo-B mRNA in HepG2 cells

S100 extracts (2 mg) were prepared from HepG2 cells as previously described (Mehta et al., 2000). Extracts were incubated with preimmune serum or with the anti-ACF(4–18) antibody, which is an antipeptide antibody directed against amino acids 4–18 in human ACF (Mehta et al., 2000). The immune complexes were trapped on protein A-agarose (Roche), and washed in NET buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 0.5% NP-40). RNA was extracted from the beads with Trizol (Invitrogen) according to the manufacturer's instructions. The RNAs were suspended in water and used for first-strand synthesis and PCR amplification using gene-specific primers as described earlier (Mehta et al., 2000). The PCR products were resolved by electrophoresis on 2% agarose gels. The primers for baboon apo-B and GAPDH generate products of 280 and 210 bp, respectively.

Apobec-1 interaction assay

The cDNA clone encoding rat apobec-1 tagged with a hemagglutinin (HA) peptide was provided by Dr. Shai Patel. Wild-type or mutant ACF translation products (5 fmol) were incubated with 5 ng anti-HA monoclonal antibody and 10 μL of protein A-agarose (Roche), in the presence or absence of 10 fmol of in vitro-translated HA-tagged apobec-1 (Mehta et al., 2000). The tubes were rocked at 4 °C for 1 h and washed five times with 0.5 mL of interaction buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.5% NP40, 5% glycerol). The protein A-agarose beads were boiled in 1× SDS sample buffer, and the supernatants were analyzed by SDS-PAGE and autoradiography.

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