

# Molecular Cloning of Apobec-1 Complementation Factor, a Novel RNA-Binding Protein Involved in the Editing of Apolipoprotein B mRNA

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**The C-to-U editing of apolipoprotein B (apo-B) mRNA is catalyzed by a multiprotein complex that recognizes an 11-nucleotide mooring sequence downstream of the editing site. The catalytic subunit of the editing enzyme, apobec-1, has cytidine deaminase activity but requires additional unidentified proteins to edit apo-B mRNA. We purified a 65-kDa protein that functionally complements apobec-1 and obtained peptide sequence information which was used in molecular cloning experiments. The apobec-1 complementation factor (ACF) cDNA encodes a novel 64.3-kDa protein that contains three nonidentical RNA recognition motifs. ACF and apobec-1 comprise the minimal protein requirements for apo-B mRNA editing in vitro. By UV cross-linking and immunoprecipitation, we show that ACF binds to apo-B mRNA in vitro and in vivo. Cross-linking of ACF is not competed by RNAs with mutations in the mooring sequence. Coimmunoprecipitation experiments identified an ACF-apobec-1 complex in transfected cells. Immunodepletion of ACF from rat liver extracts abolished editing activity. The immunoprecipitated complexes contained a functional holoenzyme. Our results support a model of the editing enzyme in which ACF binds to the mooring sequence in apo-B mRNA and docks apobec-1 to deaminate its target cytidine. The fact that ACF is widely expressed in human tissues that lack apobec-1 and apo-B mRNA suggests that ACF may be involved in other RNA editing or RNA processing events.**

Base modification editing of mRNAs involves the conversion of single nucleotides within the coding region of a transcript. A number of mRNAs undergo site-specific deamination reactions that convert A→I or C→U. These modifications result in the synthesis of alternative forms of the protein which have different biological functions (41). The A→I editing of the glutamate receptor, serotonin 5-HT<sub>2C</sub> receptor, and hepatitis delta virus mRNAs is catalyzed by a family of adenosine deaminases known as ADAR. These enzymes act on double-stranded RNA and function as a single polypeptide which has both RNA-binding and catalytic activities (3, 38, 39). C-to-U conversions occur in mRNAs of *Physarum polycephalum*, plants, and mammals, but in most cases, the *cis*-acting sequences and *trans*-acting factors have not been identified (39).

The best characterized example of C→U editing is the editing of mammalian apolipoprotein-B (apo-B) mRNA. Apo-B is a structural component of plasma lipoproteins and a significant risk factor for the development of atherosclerosis (7). The editing of apo-B mRNA involves the site-specific deamination of C<sup>6666</sup> to U, which converts codon 2153 from a glutamine codon, CAA, to a premature stop codon, UAA (9, 34). The full-length and truncated forms of apo-B have distinct functions in lipoprotein metabolism and atherosclerosis susceptibility (21). Although apo-B mRNA editing is restricted to the intestine in most mammals, it is detected in the livers of some species, including rodents (16).

The editing of apo-B mRNA is catalyzed by a multiprotein complex that recognizes an 11-nucleotide mooring sequence at positions 6671 to 6681 downstream from the editing site. The molecular composition of the editing activity has not been defined. It has been proposed that editing in vitro occurs on a large 27S macromolecular complex, or editosome, which slowly assembles on apo-B mRNA (40). However, we and others have reported a smaller molecular size of 120 to 125 kDa for the holoenzyme (10, 32). To date, only the catalytic subunit of this complex, apobec-1, has been identified. Apobec-1 is a zinc-dependent cytidine deaminase that requires additional proteins, or auxiliary factors, to edit apo-B mRNA (31, 42).

The number of auxiliary factors required for apo-B mRNA editing in vitro, as well as their identity and function, is a subject of controversy. An activity that functionally complements apobec-1 has been detected in a wide variety of tissues, including many that do not synthesize apo-B or apobec-1 (13, 15, 43, 44). Partial purification of auxiliary factors by apobec-1 affinity chromatography revealed a complex pattern of polypeptides and activities, but only a 1,200-fold purification was achieved in this study (45). Several candidates for the auxiliary factors have been proposed based on their ability to bind to apo-B mRNA or apobec-1. These include p60 and p40, which UV cross-link to apo-B mRNA (20, 22, 32); ABBP-1 (an alternatively spliced form of hnRNP A/B) and hnRNP C1, which interact with apobec-1 (17, 25); and AUX240, a 240-kDa protein-containing complex associated with the editosome (35). However, none of these proteins have been shown to possess complementing activity and their requisite involvement in editing has not been established.

Using a functional approach, we previously characterized and purified a 65-kDa protein that complements apobec-1 to edit apo-B mRNA in vitro (27, 28). Here, we report the mo-

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lecular cloning and identification of this novel 64.3-kDa protein, which we named apobec-1 complementation factor (ACF). We show that ACF and apobec-1 comprise the minimal protein requirements for specific and efficient editing of apo-B mRNA *in vitro*. We also present evidence that ACF is involved in editing *in vivo*. Our data support a model of the enzyme in which ACF functions as the RNA-binding subunit that binds to the mooring sequence and docks apobec-1 to deaminate C<sup>666</sup>. This is the first example of an RNA editing activity composed of two polypeptides.

#### MATERIALS AND METHODS

**Peptide sequencing.** The 65-kDa complementing protein was purified from baboon kidney whole-cell extracts by RNA affinity chromatography with a 280-nucleotide synthetic apo-B RNA as the ligand as previously described (28). The purified protein (2 pmol) was excised from a Coomassie blue-stained gel and digested *in situ* with trypsin (37). The digest was analyzed by capillary liquid chromatography (LC)-electrospray mass spectrometry (MS), and peptide amino acid sequences were characterized by collisionally activated dissociation (CAD) using LC-electrospray-tandem MS. The LC-MS systems consisted of a Finnigan TSQ7000 system with an electrospray ion source interfaced to a POROS 10RC reversed-phase capillary column and a Finnigan LCQ system with a Protana nanospray ion source interfaced to a Phenomenex Jupiter C<sub>18</sub> reversed-phase capillary column. The peptide amino acid sequences were determined by manual interpretation of the CAD spectra.

**cDNA cloning.** Peptide sequences were used to query the expressed sequence tag (EST) and nonredundant GenBank databases with the tBLASTn program. Two EST clones (accession no. N77737 and AA678055) which are predicted to contain seven of the peptide sequences were identified. The clones were obtained from the American Type Culture Collection (ATCC) and found to contain ~1 kb of nonoverlapping sequence. To isolate a full-length cDNA, both EST clones were used to screen primary and secondary membranes of a human universal cDNA library array (Stratagene). One positive cDNA clone which hybridized to both probes was identified. The cDNA was sequenced and found to contain an insert of 1.95 kb which encodes an open reading frame of 586 amino acids. To confirm the sequence of the coding region, baboon kidney cDNA (prepared with the Stratagene ZAP Express cDNA Synthesis Kit) and human intestine cDNA (Clontech) were used to PCR amplify the ACF cDNA with start (5'CCATGG AATCAAATCACAATCCG3') and stop (5'TCTAGAGTACCTCAGAAGG TGCCATATCCATC3') primers. Both strands of six human and four baboon clones were sequenced by automated DNA sequencing (Applied Biosystems). Homology searches of the National Center for Biotechnology Information databases were performed with the BLAST program. Protein sequence motifs were identified by the PROSITE and PSORT programs and by comparing the ACF sequence with the consensus RNP2 and RNP1 sequences (4).

**Expression of ACF.** Multiple tissue Northern blots (Clontech) were probed with Express-Hyb solution according to the manufacturer (Clontech). Multiple tissue cDNA panels (Clontech) were screened for the ACF cDNA by PCR using the start and stop primers described above. Control primers for the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA were supplied by Clontech.

**Recombinant proteins.** The apobec-1 and ACF cDNAs were subcloned as His<sub>6</sub>-tagged proteins in the bacterial expression vector pQE30 (Qiagen). The proteins were purified by Ni-NTA chromatography under native conditions as previously described (27). His<sub>6</sub>-ACF was further purified by apo-B RNA affinity chromatography (28).

**In vitro editing assays.** Editing assays were performed as previously described (28). Reactions contained 1 ng of synthetic apo-B RNA and 0.5 to 1.5 ng of recombinant His<sub>6</sub>-apobec-1 and His<sub>6</sub>-ACF. To calculate percent editing, the apo-B100 and apo-B48 extension products were quantified with a Phosphor-Imager (Molecular Dynamics).

**UV cross-linking.** UV cross-linking assays were performed with purified recombinant His<sub>6</sub>-ACF (0.5 ng) and a 280-nucleotide <sup>32</sup>P-labeled apo-B RNA (1 ng) as previously described (28). All reactions contained heparin (0.2 mg/ml), tRNA (0.2 mg/ml), and salmon sperm DNA (0.2 mg/ml). After UV cross-linking and treatment with RNase A, the samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

**Transient transfections.** For expression in Cos-7 cells, the coding region of the human ACF cDNA was cloned into pCR3.1-Uni (Invitrogen) under the control of the cytomegalovirus (CMV) promoter. The plasmid EE8, which contains the rat apobec-1 cDNA in pCDNA1/Amp, has been described (13). DNA from the vector pCR3.1-Uni was used as a negative control. Cos-7 cells were transfected with lipofectamine (Life Technologies) according to the manufacturer's instructions. At 48 h posttransfection, the cells were lysed in buffer D (28) containing 0.5% NP-40. Protein concentrations were determined with the Protein Assay Reagent (Bio-Rad).

**Antibodies and Western blotting.** Rabbit antibodies to synthetic peptides corresponding to amino acids 4 to 18 and 408 to 422 of human ACF were generated, ACF(4-18) and ACF(408-422), respectively. Conjugated synthetic peptides were injected into rabbits by standard methods (BioSynthesis, Inc.). For Western blot

analysis, extracts from transfected cells (10 μg) were resolved by SDS-PAGE (15% acrylamide) and the proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% nonfat dry milk in PBST (phosphate-buffered saline containing 0.2% Tween 20) for 1 h at room temperature. Western analysis was performed with anti-ACF(4-18) (1:7,500 dilution) or anti-apobec-1 (1:1,000 dilution) antibodies and developed with the Proto-blot alkaline phosphatase detection system (Promega).

**Coimmunoprecipitation experiments.** The anti-ACF(4-18) antibody and the preimmune sera were coupled to protein A-agarose with dimethylpimelimidate (Sigma). Cell extracts (100 μg) were incubated with 25 μl of the coupled antibody resins for 2 h at 4°C. The immunoprecipitates were washed five times in NET buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40). The proteins were eluted and analyzed by SDS-PAGE and Western blotting.

**Immunodepletion experiments.** Rat liver extracts (200 μg) were incubated with the anti-ACF(4-18) or anti-ACF(408-422) antibodies, or the respective preimmune sera (5 μl) in NET buffer (0.1 ml) for 2 h at 4°C. The immune complexes were removed by incubation with 25 μl of protein A-agarose (Boehringer Mannheim), and the supernatants were analyzed in an *in vitro* editing assay. The beads containing the immunoprecipitated complexes were washed three times in NET buffer, resuspended, and added directly to *in vitro* editing reactions containing 1 ng of synthetic apo-B RNA.

**In vivo association of ACF and apo-B mRNA.** Nuclear extracts (1 mg) prepared from McArdle 7777 cells were incubated with the anti-ACF(4-18) antibody or preimmune serum, and the immune complexes were trapped on protein A-agarose. After washing in NET buffer, the RNA was extracted from the beads in 250 μl of Trizol (Life Sciences) according to the manufacturer's protocol. The RNAs were resuspended in 10 μl of water, and 2 μl was used as the template in oligo(dT)-primed first strand synthesis with avian myeloblastosis virus reverse transcriptase (Life Sciences). The cDNAs were amplified by PCR for 35 cycles with primers specific for rat apo-B (11) or GAPDH (Clontech). The apo-B (0.38 kb) and GAPDH (~1 kb) products were resolved on 1.2% agarose gels.

**Nucleotide sequence accession number.** The ACF cDNA sequence has been deposited in GenBank (accession no. AF209192).

#### RESULTS

**Cloning of the ACF cDNA.** To obtain peptide sequences for molecular cloning experiments, the 65-kDa complementing protein was purified from baboon kidney extracts by RNA affinity chromatography as previously described (28). The purified protein (2 pmol) was subjected to tryptic digestion and microsequence analysis. Twenty-three peptides were obtained from two independent experiments, and the sequences were used as queries in database searches. We identified two sequences in the human EST database which were predicted to encode seven of the peptides. The EST clones were obtained from ATCC, analyzed by DNA sequencing, and found to contain nonoverlapping sequences. To obtain a full-length cDNA, the clones were used to screen a human cDNA library array. Both EST clones hybridized to a single cDNA that contained an insert of 1.95 kb. The cDNA contains a 5' untranslated sequence of 140 nucleotides, a coding region of 1,761 nucleotides, and a 3' untranslated sequence of 44 nucleotides. The initiating methionine shown in Fig. 1A is the most likely start site for translation since it is the first ATG in the cDNA that gives rise to a continuous open reading frame. To confirm the sequence of the coding region, oligonucleotide primers corresponding to the translation start and stop sites were used in PCR to amplify multiple baboon kidney and human intestine cDNAs. There were only three nucleotide differences and no amino acid differences between the baboon and human sequences.

**Analysis of the ACF cDNA sequence.** Figure 1A depicts the deduced amino acid sequence derived from the cDNA, which we named ACF. The open reading frame contains 20 of the 23 peptides that were obtained by mass spectrometry (Fig. 1A). The ACF cDNA encodes a 586-amino-acid protein with a predicted molecular mass of 64,274 Da. This is in good agreement with our previous studies, which demonstrated that the complementing activity has a native molecular mass of 65 ± 10 kDa by gel filtration chromatography (27) and that the purified protein migrates as a 65-kDa protein on SDS-PAGE (28).

The N-terminal region of ACF contains three nonidentical

**A.**

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10      20      30      40      50
MESNHKSGDGLSGTQKEAALRALVQRTGYSLVOENGORKYGGPPPGWDA
60      70      80      90      100
PPERGCEIFIGKLPRDLFEDELIPLCEKIGKIYEMRMMDFNGNNRGYAF
110     120     130     140     150
VTFSSNKVEAKNAIKQLNNYEIRNGRLLGVCASVDNCRLFVGGIPKTKKRE
160     170     180     190     200
EILSEMKKVTEGVVDVIVYPSAADKTKNRGFAFVEYESHRTAAMARKKLL
210     220     230     240     250
PGRIQLWGHGIAVDWAEPEVEVDEDTMSSVKILYVRNLMLSTSEEMIEKE
260     270     280     290     300
FNNIKPGAVERVKKIRDYAFVHFSNRKDAVEAMKALNGKYLDGSPIEVTL
310     320     330     340     350
AKPVDKDSYVRYTRGTGGRTMLQGEYTYSLGQVYDPTTTYLGAPVFYAP
360     370     380     390     400
QTYAAIPSLHFPATKGHLSNRAIIRAPSVRGAAGVRGLGGRGYLAYTGLG
410     420     430     440     450
RGYQVKGDKREDKLYDILPGMELTPMNPVTLKPOGIKLAPQILEEICQKN
460     470     480     490     500
NWGQPVYQLHSAIGQDQRQLFLYKITIPALASONPAIHPFTPPKLSAFVD
510     520     530     540     550
EAKTYAAEYTLQTLGIPTDGGMATAAAAAATAFPGYAVPNATAPVSAA
560     570     580
QLKQAVTLGQDLAAAYTTYEVYPTFAVTARGDGYGTF
    
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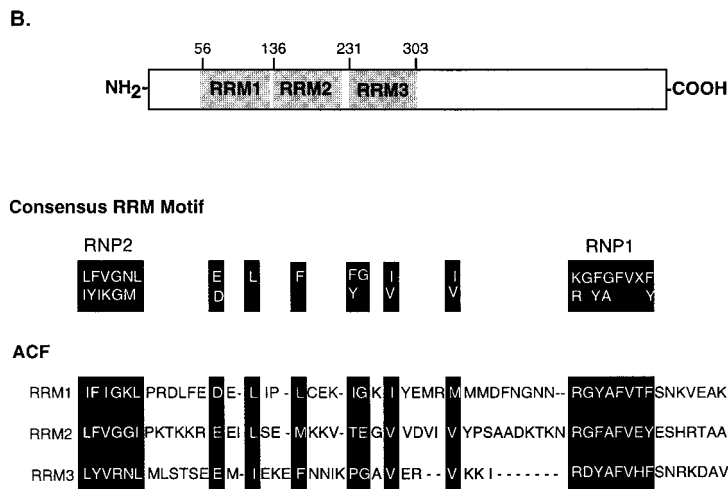


FIG. 1. Protein sequence of ACF. (A) The deduced amino acid sequence of the ACF cDNA is shown. The peptide sequences obtained from mass spectrometric analysis are underlined. (B) A diagram of the three RRM motifs in ACF and the alignment of residues 58 to 123, 138 to 208, and 233 to 293 with the consensus RRM motif (4) containing the conserved RNP2 hexamer and RNP1 octamer sequences.

copies of an RNA recognition motif (RRM), a conserved 80-amino-acid sequence that functions in binding to RNA (4). RRM domains are composed of 80 to 90 amino acids and contain two short conserved sequences, a ribonucleoprotein 2 (RNP2) hexamer and an RNP1 octamer, with a number of conserved residues in between. An alignment of the RRM motifs in ACF with the consensus RRM sequence is shown in Fig. 1B. This region in ACF shows 51 and 53% amino acid identity, respectively, to human heterogeneous nuclear RNP R (accession no. AF00364) and human Gry-rbp (accession no. AF037448), an RNA-binding protein of unknown function. ACF contains a potential nuclear localization signal of the simian virus 40 T antigen type (<sup>144</sup>PKTKKRE<sup>150</sup>), which is

consistent with the fact that apo-B mRNA editing is a nuclear event (23). The C-terminal region of ACF (residues 304 to 586) does not share significant homology with any proteins in the nonredundant databases. Interestingly, there are six RG dipeptides between amino acids 314 and 402. Similar RG clusters are present in a human adenosine deaminase editing enzyme, but their functional significance is not known (33).

**Expression of human ACF.** In humans, the expression of apobec-1 is restricted to the small intestine (19, 24). To examine the distribution of ACF mRNA in primate tissues, we performed a Northern blot analysis. Two ACF transcripts of 9.5 and 2.2 kb were detected in poly(A)<sup>+</sup> RNA from human liver, kidney, and pancreas (Fig. 2A). Similar-size transcripts

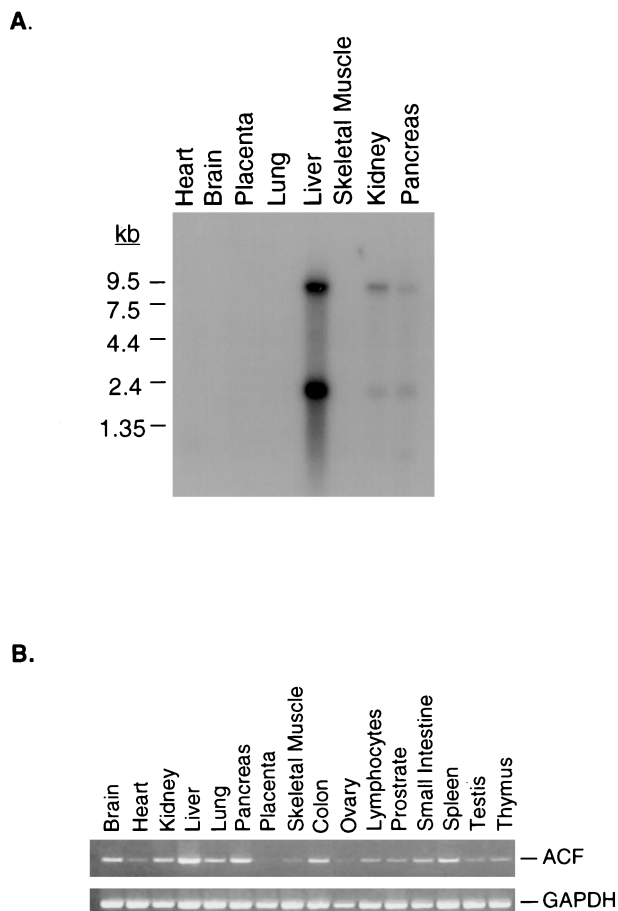


FIG. 2. Expression of ACF mRNA. (A) A human tissue Northern blot (Clontech) was probed with  $^{32}\text{P}$ -labeled ACF cDNA insert. The positions of RNA molecular weight markers are shown on the left. (B) Multiple tissue cDNA panels (Clontech) were analyzed for the ACF and GAPDH cDNAs by PCR.

were also detected in baboon kidney and small intestine (data not shown). Assuming a poly(A) tail of  $\sim 200$  nucleotides, the 1.94-kb ACF cDNA most likely corresponds to the 2.2-kb transcript. The origin of the 9-kb transcript is not known, but it may represent an alternatively spliced form of ACF. Because the ACF mRNA is of low abundance, we also screened multiple tissue cDNA panels by PCR using gene-specific primers for ACF or GAPDH. As shown in Fig. 2B, the ACF cDNA was detected in many, but not all, human tissues in addition to small intestine. This distribution is consistent with previous studies which showed that the complementing activity is widely but not ubiquitously expressed in baboon and rabbit tissues (10, 44). Whether the PCR products are derived from the 2.2- or 9-kb ACF transcript is currently under investigation. The housekeeping gene, which encodes GAPDH, was expressed in all tissues examined (Fig. 2B).

**ACF complements apobec-1 in vitro.** For functional studies, ACF and apobec-1 were expressed in bacteria as His<sub>6</sub>-tagged proteins and purified under native conditions by Ni-NTA chromatography (27). His<sub>6</sub>-ACF was further purified by apo-B RNA affinity chromatography as previously described (28). Based on SDS-PAGE analysis, the final purified fractions contained a single prominent polypeptide with a mass of 65 kDa for His<sub>6</sub>-ACF and 27 kDa for His<sub>6</sub>-apobec-1 (Fig. 3A). The

purified proteins were analyzed for their ability to edit a 280-nucleotide synthetic apo-B RNA in an in vitro editing assay. Although no editing was detected in reactions that contained His<sub>6</sub>-ACF or His<sub>6</sub>-apobec-1 alone, apo-B RNA was edited when both proteins were added to the reaction (Fig. 3B). Shah et al. found that mutations in the mooring sequence reduced or abolished editing by the native enzyme in rat intestinal extracts (36). As shown in Fig. 3B, editing by His<sub>6</sub>-apobec-1 and His<sub>6</sub>-ACF was also dependent on the mooring sequence since the recombinant proteins did not significantly edit a triple mutant RNA (GGAUGAGAAUA) or a U<sup>6678</sup>→G point mutant RNA (UGAUCAGGAUA) (bold indicates mutant nucleotide).

The primer extension assay that is used to analyze apo-B mRNA editing only detects nucleotide changes at C<sup>6666</sup> (12). To further confirm that editing by ACF and apobec-1 is specific, the products of the in vitro editing reaction mixture were cloned and twenty clones were analyzed by DNA sequencing as previously described (11). We found that six clones were edited at C<sup>6666</sup> and that no other nucleotides in the 280-nucleotide substrate were modified by the recombinant proteins. The other 14 clones were not edited at any position.

In addition to being specific, the editing activity of His<sub>6</sub>-ACF and His<sub>6</sub>-apobec-1 is also very efficient. The time course experiment in Fig. 3C compares the editing activity of the recombinant proteins with the native editing enzyme in rat liver extracts. After 3 h of incubation at 30°C, 69% of the RNA (7.6 fmol) was edited by 1.5 ng of His<sub>6</sub>-ACF and His<sub>6</sub>-apobec-1, whereas only 18% of the RNA (2 fmol) was edited by 60  $\mu\text{g}$  of liver extract. These experiments were done by using a 2:1 molar ratio of His<sub>6</sub>-apobec-1 to His<sub>6</sub>-ACF (12 fmol of holoenzyme) since it has been proposed that the catalytically active form of apobec-1 is a dimer. However, it is important to note that all of the recombinant protein may not be fully active or assemble into an enzyme complex. When the reactions were incubated at 37°C instead of 30°C, over 80% of the RNA was edited by the recombinant proteins after 2 h (data not shown).

**ACF interacts with apo-B mRNA in vitro.** The purified complementing protein UV cross-linked to the wild-type apo-B RNA but not to the triple mutant RNA with three mutations in the mooring sequence (28). To study RNA-protein interactions with the recombinant protein, UV cross-linking experiments were performed with purified His<sub>6</sub>-ACF and  $^{32}\text{P}$ -labeled wild-type apo-B RNA (Fig. 4). All reactions contained tRNA and salmon sperm DNA as nonspecific competitors. As shown in Fig. 4, cross-linking of His<sub>6</sub>-ACF to the probe was competed by a fivefold molar excess of the unlabeled wild-type apo-B RNA. In contrast, the U<sup>6678</sup>→G mutant RNA required a 50-fold molar excess for effective competition. The triple mutant only partially competed at this concentration.

**ACF interacts with apobec-1.** Our previous studies established that the native complementing activity physically interacted with apobec-1 in an in vitro binding assay (27) and far-Western analysis (28). To study interactions between ACF and apobec-1, we performed coimmunoprecipitation experiments. Rabbit antibodies to synthetic peptides corresponding to amino acids 4 to 18 [anti-ACF(4-18)] and 408 to 422 [anti-ACF(408-422)] of human ACF were generated. These sequences were chosen because they are outside of the RRM domains, they are in hydrophilic regions predicted to be on the surface of the protein based on computer analysis (Lasergen), and they are conserved in mouse and rat ACF (A. Mehta and D. Driscoll, unpublished results). Plasmids containing the ACF and apobec-1 cDNAs under the control of a CMV promoter were transiently transfected into Cos-7 cells. After 48 h, the cells were lysed and analyzed by Western blotting. As shown in the top panel of Fig. 5A, the anti-ACF(4-18) antibody recog-

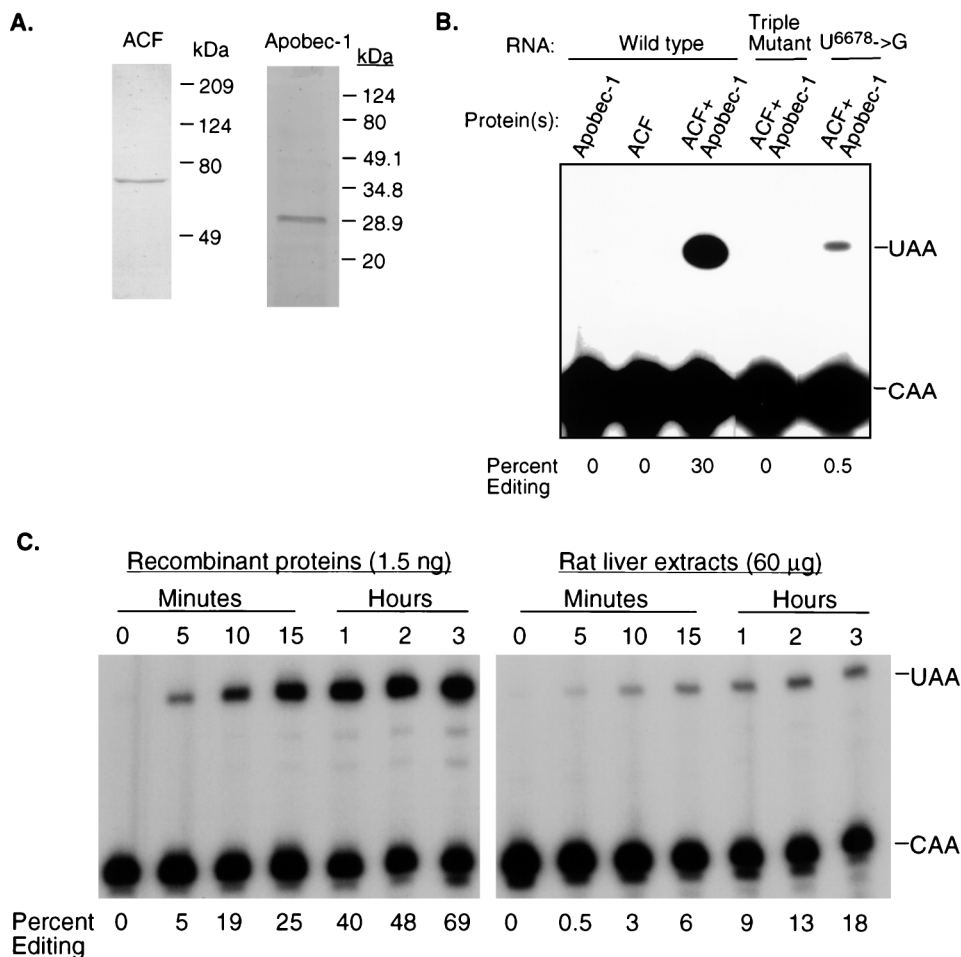


FIG. 3. ACF and apobec-1 edit apo-B RNA in vitro. (A) Purified recombinant His<sub>6</sub>-ACF and His<sub>6</sub>-apobec-1 were resolved by SDS-PAGE and stained with Coomassie blue (ACF) or silver (apobec-1). (B) Purified His<sub>6</sub>-apobec-1 (~0.5 ng) and His<sub>6</sub>-ACF (~0.6 ng) were added to in vitro editing assays containing wild-type apo-B RNA, the triple mutant RNA with three mutations in the mooring sequence, or the point mutant RNA (U<sup>6678</sup>→G). After incubation at 30°C for 2 h, the reactions were analyzed by a poisoned primer extension assay (28). The positions of the products from the edited (UAA) and unedited (CAA) RNAs are indicated. (C) In vitro editing assays were performed with His<sub>6</sub>-apobec-1 and His<sub>6</sub>-ACF (~1.5 ng) or with rat liver extracts (60 μg). After incubation at 30°C for the indicated times, the reactions were analyzed as described above.

nized a 65-kDa protein in extracts from cells transfected with the ACF cDNA but not with vector DNA. This antibody did not cross-react with apobec-1 since no reactivity was seen in extracts from apobec-1-transfected cells. The results of a Western analysis performed with an anti-apobec-1 antibody are

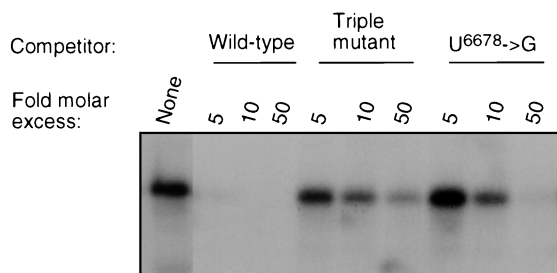


FIG. 4. ACF UV cross-links to apo-B RNA. Purified His<sub>6</sub>-ACF (0.6 ng) was used in UV cross-linking experiments with <sup>32</sup>P-labeled wild-type apo-B RNA. Competition experiments were performed with a 5- to 50-fold molar excess of the unlabeled wild-type apo-B RNA, the triple mutant RNA with three mutations in the mooring sequence, or the point mutant RNA (U<sup>6678</sup>→G) as indicated. Reactions were analyzed by SDS-8% PAGE and autoradiography.

shown in Fig. 5A (bottom panel). Cells cotransfected with the ACF and apobec-1 plasmids expressed both proteins based on Western blot analysis (Fig. 5A) and in vitro editing assays (data not shown).

To detect interactions between ACF and apobec-1, extracts from the transfected cells were immunoprecipitated with the anti-ACF(4-18) antibody under non-denaturing conditions. The immunoprecipitated complexes were analyzed for the presence of ACF and apobec-1 by Western blotting. As shown in Fig. 5B, the anti-ACF antibody coimmunoprecipitated ACF and apobec-1 when the two proteins were coexpressed. We also performed experiments with in vitro-translated ACF and apobec-1 that was tagged with a hemagglutinin (HA) peptide. An anti-HA monoclonal antibody coimmunoprecipitated ACF and HA-tagged apobec-1 when the two proteins were cotranslated or posttranslationally mixed (data not shown).

**Immunodepletion of editing activity with anti-ACF antibodies.** To test the hypothesis that ACF is involved in editing in vivo, we immunodepleted ACF from rat liver whole-cell extracts, which contain the native editing enzyme. We took this approach because neither of the anti-ACF antibodies inhibited editing in vitro. Extracts were incubated with anti-ACF(4-18)

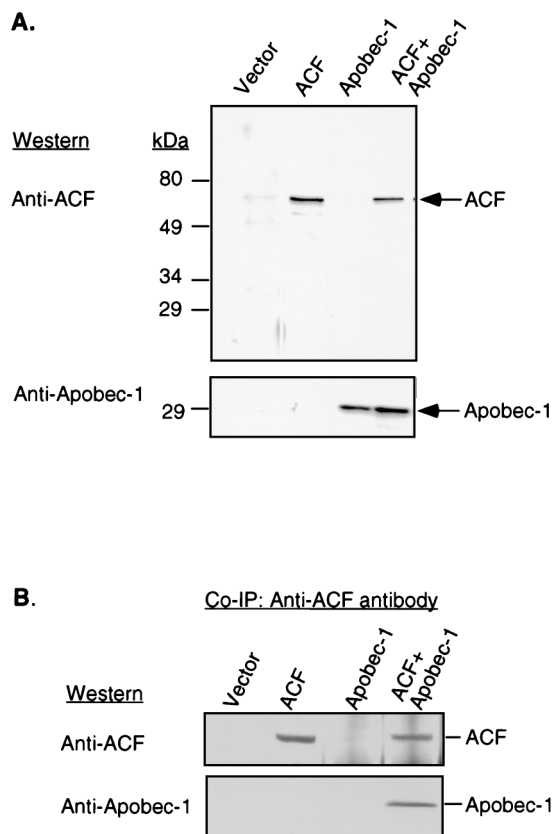


FIG. 5. ACF coimmunoprecipitates with apobec-1 in transfected cells. (A) Extracts from Cos-7 cells were transiently transfected with vector DNA or plasmids encoding the ACF and apobec-1 cDNAs as indicated. After 48 h, cell extracts were analyzed by Western blotting using anti-ACF(4-18) or anti-apobec-1 antibodies. (B) The extracts from transfected Cos-7 cells were immunoprecipitated with the anti-ACF(4-18) antibody coupled to protein A-Sepharose. After extensive washes, the complexes were resolved on SDS-12% PAGE, transferred to PVDF membranes, and analyzed by Western blotting using the anti-ACF(4-18) or anti-apobec-1 antibodies as indicated.

or anti-ACF(408-422) antibody or the respective preimmune sera under nondenaturing conditions. The immune complexes were removed by incubation with protein A-Sepharose, and the supernatants were analyzed in an *in vitro* editing assay. As shown in Fig. 6A, editing activity was immunodepleted by both anti-ACF antibodies but not by the preimmune sera. We were not able to detect endogenous ACF or apobec-1 in the immunoprecipitated complexes by Western blotting due to their low abundance. However, experiments in which recombinant ACF or apobec-1 were individually added back to the supernatants indicated that both proteins were greatly depleted by the anti-ACF antibodies. Editing activity in the depleted extracts was restored by the addition of both His<sub>6</sub>-ACF and His<sub>6</sub>-apobec-1 (data not shown). To test whether the immunoprecipitated complexes contained a functional holoenzyme, the resins (5 to 15  $\mu$ l) were analyzed in an *in vitro* editing assay. As shown in Fig. 6B, the complexes immunoprecipitated with the anti-ACF antibodies edited apo-B mRNA *in vitro*, which suggests that they contained both ACF and apobec-1. The edited RNA did not represent endogenous apo-B mRNA in the complex since no signal was detected when the complexes were assayed in the absence of exogenous substrate. Editing activity was not immunoprecipitated by the preimmune sera (Fig. 6B).

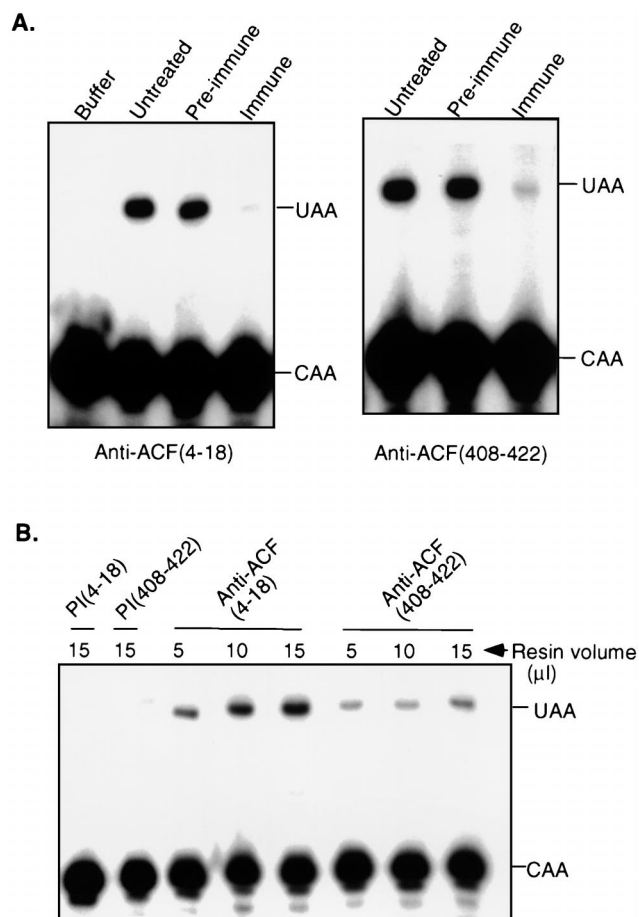


FIG. 6. Immunodepletion of editing activity from rat liver extracts. (A) Rat liver extracts were incubated with anti-ACF(4-18) or anti-ACF(408-422) antibodies or their respective preimmune sera (PI) as indicated. The immune complexes were removed by protein A-agarose and the supernatants were analyzed in an *in vitro* editing assay. (B) The protein A-Sepharose beads containing the immune complexes from above were extensively washed. Aliquots of the beads (5 to 15  $\mu$ l) were incubated with synthetic apo-B RNA in an *in vitro* editing assay.

**In vivo interactions between ACF and apo-B mRNA.** To detect interactions between ACF and apo-B mRNA *in vivo*, nuclear extracts were prepared from McArdle 7777 cells, a rat hepatoma cell line that synthesizes and edits apo-B mRNA. The extracts were immunoprecipitated with the anti-ACF(4-18) antibody or preimmune serum. RNA was extracted from the complexes and analyzed by reverse transcriptase PCR using gene-specific primers for apo-B, GAPDH, and actin. As shown in Fig. 7, apo-B mRNA was detected in the complexes immunoprecipitated with the anti-ACF(4-18) antibody but not with preimmune serum. No products were obtained in the absence of reverse transcriptase, which eliminates the possibility of contamination with genomic DNA. The anti-ACF antibody did not immunoprecipitate the abundant mRNAs encoding GAPDH (Fig. 7) or actin (data not shown).

## DISCUSSION

Since the discovery of apobec-1 in 1993, little progress has been made in identifying the other *trans*-acting factors that are required for the site-specific deamination of apo-B mRNA. Here we report the molecular cloning of ACF and demonstrate that ACF and apobec-1 comprise the minimal protein

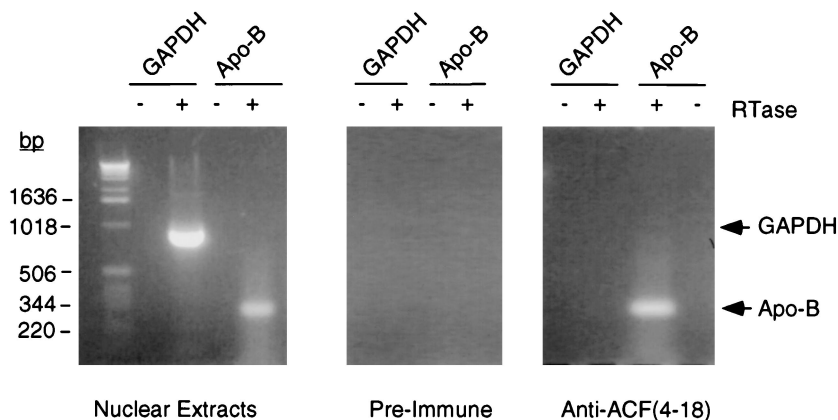


FIG. 7. In vivo association of ACF with apo-B mRNA. Nuclear extracts from McArdle 7777 cells were immunoprecipitated with the anti-ACF(4-18) antibody or preimmune serum as described in the legend to Fig. 6. RNAs extracted from nuclear extracts or the immune complexes were analyzed by reverse transcriptase PCR using gene-specific primers for GAPDH or apo-B. Reverse transcriptase (RTase) was included in the cDNA reaction mixture as indicated. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel. The positions of the GAPDH and apo-B products are indicated.

requirements for apo-B mRNA editing in vitro. The editing activity of ACF and apobec-1 is specific for C<sup>666</sup> and dependent on the mooring sequence. The activity of the recombinant enzyme is very robust, which suggests that additional auxiliary factors are not required for efficient editing in vitro. Our results support a model of the apo-B mRNA editing enzyme in which ACF functions as the RNA-binding subunit that binds to the mooring sequence in apo-B mRNA and docks apobec-1, the catalytic subunit, to deaminate the upstream cytidine. This is the first example of C→U editing in which the editing machinery has been defined.

Based on cDNA sequence analysis, the amino terminus of ACF contains three nonidentical RRM domains, a well-characterized RNA-binding motif found in many proteins involved in pre-mRNA processing. It should be noted that the ability to complement apobec-1 is not a general property of RRM-containing proteins since other RRM proteins have been shown to lack this activity (1, 17, 25). In proteins that contain multiple RRMs, the function of the individual motifs in RNA recognition can vary. Each RRM may bind a different sequence or contiguous RRMs may be required for specific binding (5, 6). It will be of interest to identify the smallest functional domain in ACF required for the recognition of apo-B mRNA. An important observation from our study is that ACF is widely expressed in human tissues that lack apobec-1 and apo-B mRNA. There are examples of other proteins that contain several RRM domains, bind different RNA sequences, and have multiple functions (6, 26). In addition to editing apo-B mRNA, ACF may be involved in editing other mRNAs by interacting with novel catalytic activities or in other RNA processing events.

The binding of ACF to apo-B mRNA is dependent on an intact mooring sequence, which supports the hypothesis that this protein functions as the RNA-binding subunit of the editing enzyme. However, apobec-1 has a weak nonspecific RNA-binding activity with a preference for AU-rich sequences (2, 29). The significance of this finding to apo-B mRNA editing has not been clear since the binding of apobec-1 to apo-B mRNA in vitro was competed by noneditable apo-B RNAs and irrelevant RNAs (2). Experiments are currently in progress to determine whether apobec-1 contributes to the sequence-specific recognition of apo-B mRNA in the context of the holoenzyme.

In addition to in vitro studies, we also provide evidence that

ACF is a component of the native editing enzyme. Immunodepletion experiments using two different anti-ACF antibodies demonstrate that ACF is required for rat liver extracts to edit apo-B mRNA. Although we could not detect apobec-1 and ACF in the immunoprecipitated complexes due to their low abundance, the complexes were capable of editing apo-B RNA in vitro. These results strongly suggest that a functional holoenzyme containing apobec-1 and ACF was generated on the resin. Furthermore, we found that McArdle 7777 cells contain coimmunoprecipitable complexes of ACF and endogenous apo-B mRNA. Definitive proof that ACF is involved in editing in vivo will require the elimination of ACF expression in animals through antisense or gene knockout strategies.

The number of auxiliary factors required for apobec-1 to edit apo-B mRNA in vitro has been a subject of debate. Smith et al. have proposed that apo-B mRNA editing in vitro is dependent on the assembly of a 27S editosome that contains multiple proteins (40). However, studies from other groups have challenged the editosome model (10, 14, 18). The data presented here demonstrate that specific and efficient editing of apo-B mRNA can be reconstituted in vitro with only ACF and apobec-1. The simplest model of the apo-B mRNA editing enzyme is that it is composed of an apobec-1 dimer (54 kDa) and an ACF monomer (65 kDa). This model is consistent with the minimal size of the native holoenzyme, which was observed to be 120 to 125 kDa (10, 32). Apobec-1 has been shown to dimerize in vitro (24), but whether apobec-1 exists as a dimer in the holoenzyme has not been established. Experiments to isolate a functional complex from the purified recombinant proteins in order to directly determine the stoichiometry of the subunits are presently in progress. It is important to note that our results do not exclude the possibility that the editing enzyme contains additional subunits. Although a minimal activity composed of ACF and apobec-1 can edit a small synthetic apo-B substrate in vitro, editing in vivo may occur on a large editosomal complex. It is likely that other factors are involved in the context of the nucleus, where the 43-kb pre-mRNA undergoes editing during splicing and polyadenylation (23). The editing mechanism is also regulated by developmental, hormonal, and dietary factors, which adds another layer of complexity (8).

In conclusion, the molecular cloning and identification of ACF reported here will greatly facilitate studies on the mechanism and regulation of apo-B mRNA editing. To date, the

only other system for which the editing activity has been defined is the A→I editing of mammalian mRNAs which is catalyzed by the ADAR gene family. Interestingly, the active sites of the ADARs have greater homology with the active site of apobec-1 than with the other adenosine deaminases (39). An important difference between the two systems is that the ADARs are capable of binding and deaminating their mRNA targets in the absence of other cofactors (3, 38). In contrast, the cytidine deaminase and RNA-binding activities of the apo-B mRNA editing enzyme are encoded by two different genes. Our model may have implications for the other examples of C-to-U editing that occur in mRNAs and tRNAs for which the editing machinery has not yet been defined (39).

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