C to U editing of apolipoprotein B (apoB) mRNA involves the interaction of a multicomponent editing enzyme complex with a requisite RNA sequence embedded within an AU-rich context. This enzyme complex includes apobec-1, an RNA-specific cytidine deaminase, and apobec-1 complementation factor (ACF), a novel 65-kDa RNA-binding protein, that together represent the minimal core of the editing enzyme complex. The precise composition of the holo-enzyme, however, remains unknown. We have previously isolated an enriched fraction of S100 extracts, prepared from chicken intestinal cells, that displays apoB RNA binding and which, following supplementation with apobec-1, permits efficient C to U editing. Peptide sequencing of this most active fraction reveals the presence of ACF as well as GRY-RBP, an RNA-binding protein with ~50% homology to ACF. GRY-RBP was independently isolated from a two-hybrid screen of chicken intestinal cDNA. GRY-RBP binds to ACF, to apobec-1, and also binds apoB RNA. Experiments using recombinant proteins demonstrate that GRY-RBP binds to ACF and inhibits both the binding of ACF to apoB RNA and C to U RNA editing. This competitive inhibition is rescued by addition of ACF, suggesting that GRY-RBP binds to and sequesters ACF. As further evidence of the role of GRY-RBP, rat hepatoma cells treated with an antisense oligonucleotide to GRY-RBP demonstrated an increase in C to U editing of endogenous apoB RNA. ACF and GRY-RBP colocalize in the nucleus of transfected cells and, in cotransfection experiments with apobec-1, each appears to colocalize in a predominantly nuclear distribution. Taken together, the results indicate that GRY-RBP is a member of the ACF gene family that may function to modulate C to U RNA editing through binding either to ACF or to apobec-1 or, alternatively, to the target RNA itself. Apolipoprotein B (apoB) is an abundant gene product expressed in the mammalian small intestine and liver and plays a central role in the transport of cholesterol and triglyceride in plasma (1). Two forms of apoB exist, designated on a centile scale as apoB100 and apoB48 (2). ApoB48 is generated as a result of a site-specific, posttranscriptional C to U deamination of the nuclear transcript that in turn results in translational termination of the (edited) apoB RNA (3, 4). ApoB RNA editing is particularly active in the mammalian small intestine but also in the liver of certain species, including the mouse and rat (5). Since apoB RNA editing eliminates an important functional domain from the C terminus of the protein (reviewed in Ref. 2), the molecular mechanisms underlying this organ-specific partitioning have been extensively investigated to understand the presumed advantage of this specialized genetic adaptation.

Enzymatic deamination of apoB RNA exhibits important requirements in both the cis-acting RNA elements and in the trans-acting protein factors that restrict this reaction largely to a single, canonical site (6–15). The cis-acting elements have been well characterized and include an 11-nucleotide (nt) region referred to as a “mooring sequence,” located 4 nt downstream of the edited C, an AU-rich bulk RNA context, and other “efficiency elements” flanking a ~30-nt region representing the minimal editing cassette (6–15). This cassette is located within a region that exhibits important secondary structure, including a stem-loop conformation that is predicted to position the edited C in a favorable configuration relative to the active site of the deaminase (10, 12, 16, 17). C to U editing of apoB RNA is mediated by an enzyme complex that includes a single catalytic subunit, apobec-1, as well as additional proteins that together represent the holoenzyme (18). Apobec-1 is a zinc-dependent cytidine deaminase that exhibits RNA-binding affinity for AU-rich sequence elements (16). Targeted deletion of apobec-1 eliminated C to U editing of apoB mRNA, demonstrating that no functional redundancy exists in the catalytic deamination of this RNA (21–23). Nevertheless, although absolutely required for C to U editing of
apob RNA, apobec-1 alone is not sufficient (11, 18). Specifically, recombinant apobec-1 will deaminate a monomeric cytidine substrate but alone exhibits no deaminase activity on an RNA template (24, 25). This observation coupled with earlier studies in which C to U editing activity was found to exist in a higher order complex provide support for the proposal that apoB RNA editing is mediated by a heteromeric enzyme complex whose composition and functional organization facilitates catalytic deamination of the targeted base (11, 26, 27).

Over the last several years, many different proteins have been proposed to function as auxiliary or complementation factors, including candidates ranging in molecular mass from 40 to 240 kDa (11, 13, 26–30). Despite intensive effort, until very recently none of these has met the criteria of an authentic enzymatic cofactor in C to U editing. For the purposes of this report, we refer to ACF to describe this protein and its functions.

Identification of GRY-RBP as an ApoB RNA-binding Protein

Materials and Methods

Identification of GRY-RBP by Protein Sequence Analysis—Chicken enterocyte S100 extracts were prepared as described previously and fractionated through a 25-ml Blue-Sepharose column (Amersham Pharmacia Biotech) as detailed in Ref. 17. The material eluting in fractions 16–19 was pooled and used for UV cross-linking to a 55-nucleotide oligonucleotide substrate but alone exhibits no deaminase activity on an RNA template (24, 25). This observation coupled with earlier studies in which C to U editing activity was found to exist in a higher order complex provide support for the proposal that apoB RNA editing is mediated by a heteromeric enzyme complex whose composition and functional organization facilitates catalytic deamination of the targeted base (11, 26, 27).

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mm MgCl₂, 0.5% skim milk; 2% bovine serum albumin; 0.1% Tween 20) for 16 h at 30 °C. Filters were washed three times in buffer C (buffer A plus 2.5 mm MgCl₂, 0.1% Tween 20), dried at room temperature, and subjected to autoradiography.

Antisense Oligonucleotide Experiments—An antisense morpholino-oligonucleotide to GRY-RBP (5'-GCTCGGTTTCACTCGTGGCGTGC-3', 5' located at nt +479) and a scrambled control morpholino-oligonucleotide were prepared by Gene Tools, LLC, Corvallis, OR. McArdle rat hepatoma cells were plated on a 35-mm culture dish and grown to 70% confluence. Oligonucleotides (final concentration, 5 μM) were mixed with delivery reagent (EPEI, Gene Tools) in serum-free Dulbecco's modified Eagle's medium and layered over the cells. After incubation for 3 h, the delivery solution was removed, replaced with complete Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and the cells incubated for 48 h. RNA was extracted, and apoB RNA editing was determined by primer extension assay.

Immunofluorescence Microscopy—Rat apoBec-1 cDNA was cloned into pCMV-Tag2B vector or 3B and expressed as an N-terminal FLAG-tagged or an N-terminal c-Myc-tagged fusion protein, respectively. Human GRY-RBP cDNA was cloned into pCMV-Tag 3B and expressed as an N-terminal c-Myc-tagged fusion protein. Human ACF was expressed from pCMV-Tag2B vector as an N-terminal FLAG-tagged fusion protein. COS-7, HepG2, and McArdle cells (ATCC) were grown on cover slips and transiently transfected with 1–2 μg of plasmid DNA using FUGENE 6 (Roche Molecular Biochemicals). The cells were fixed 48 h after transfection with 3.7% formaldehyde, permeabilized with 0.5% Triton X-100, and probed with mouse monoclonal anti-Myc IgG (SC-40, Santa Cruz Biotechnology) and rabbit anti-FLAG IgG (1:5000, Zymed Laboratories Inc.), followed by Cy3- or FITC-conjugated secondary IgG, respectively (Jackson ImmunoResearch). For confocal microscopy, nuclei were visualized using TO-PRO-3 iodide (Molecular Probes). Preparations were imaged with a 63× plan neofluar objective and a 3CCR camera (DAGE-MTI, Inc.). A Zeiss Attoarc variable intensity lamp was used to illuminate the entire image giving a range of intensities covering every plane of the cells in that image. Pictures were processed using Adobe Photoshop 4.0 software. For standard immunofluorescence microscopy, transfection and antibody staining was carried out as described above using cells grown on coverslips. Stained cells were mounted with Vectashield and nuclei imaged with DAPI (Vector). Images were obtained with a Zeiss Axioskop 2 MOT microscope equipped with a 40× plan neofluar objective and a 3CCD camera (DAGE-MTI, Inc.). A Zeiss Attocore variable intensity lamp was used with filter sets designed for Cy3, FITC, and DAPI. Images were processed using Adobe Photoshop 4.0 software.

Miscellaneous Methods—Primer extension analysis of apoB RNA editing was conducted as detailed previously (24). Coimmunoprecipitation assays were conducted using Myc-GRY-RBP, FLAG-ACF, apoBec-1, and GST-apoBec-1, expressed either as recombinant proteins or, where indicated, from in vitro translation using a Toto-Coupled Reticulo-locyte Lysate (Promega). Interaction studies were performed at 30 °C for 30 min in 200 μl of binding buffer (20 mM Pipes (pH 7.9), 100 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 0.4% Nonidet P-40). ACF and GRY-RBP were coimmunoprecipitated from cell lysates following transient transfection as described above. Following nondenaturing cell lysis, the target proteins were immunoprecipitated with 5 μl of the appropriate antiiserum, including mouse monoclonal anti-FLAG (Sigma) or rabbit polyclonal anti-Myc (Sigma) antibody at 4 °C for 60 min with agitation. The complexes were collected on protein A-agarose beads that were washed extensively and analyzed by 12% SDS-PAGE and autoradiography. Where indicated, the material coprecipitating on the protein A-agarose beads was eluted in denaturing buffer, subjected to SDS-PAGE, and visualized by Western blotting with the appropriate antisera. Yeast two-hybrid interaction assays were conducted with ACF and GRY-RBP cDNAs, which were cloned into either pSB202 or pDG vectors (35) or both, and their interaction with wild-type apoBec-1 and various C- or N-terminal deletion mutants was determined as described (35). Construction of a yeast two-hybrid library used 5 μg of poly(A)⁺ RNA, isolated from chicken enterocytes, packaged in pAD-GAL4 using the HybriZAP two-hybrid gigapack cloning kit (Stratagene). Rat apoBec-1 cDNA was cloned into pBD-Gal4 as the bait using standard methods, and 10 μg of each plasmid was used to cotransform yeast (YEP-2) as detailed by the manufacturer (Stratagene).

RESULTS AND DISCUSSION

Biochemical and Genetic Isolation of Proteins That Bind to ApoB RNA and to ApoBec-1—We reasoned that proteins that function as integral components in the apoB RNA editing complex should exhibit binding activity toward both the substrate (apoB RNA) and also the catalytic subunit of the holoenzyme (apobec-1). Partially purified chick intestinal S100 extracts were UV cross-linked to radiolabeled apoB mRNA (13), and the cross-linked protein(s) of molecular weight ~p65 were identified by autoradiography (Fig. 1). The material contained in these pooled fractions was previously demonstrated to be enriched in apoB RNA editing complementation activity, although its composition was unknown (17). 70 peptides were obtained in this screen of which the sequence of 6, shown in Fig. 1, matched unambiguously to GRY-RBP, whereas 5 others matched to a sequence recently identified as ACF by Driscoll and co-workers (31). These results suggest that fractionated chicken intestinal extracts, a source of enriched complementation activity, contain at least two proteins that can be identified through interaction with apoB RNA. The demonstration that ACF copurifies with other protein(s) is reminiscent of the recent report of Greene and colleagues (32) in which fractionated rat liver nuclear extracts revealed the presence of other pro-
teins in addition to ACF/ASP in the most highly enriched editing fraction.

Independent examination of interacting proteins was undertaken using a chicken intestinal library cloned into a yeast two-hybrid expression system, using rat apobec-1 as bait. This approach also revealed a strongly interacting clone, which was identified as GRY-RBP (data not shown). Searches with various regions of GRY-RBP identified several homologs including the EST image clone (N77737) recently identified as ACF by Driscoll et al. (31) and ASP by Greeve and colleagues (32). Clone N77737 was directly sequenced, and five separate nucleotide differences were identified from the reported sequence of Driscoll and coworkers (31). (C deletions at 84, 191, C insertions at 364, 437, and a G at 189). After correcting these differences, clone N77737 was found to align completely with the cDNA of the recently identified complementation factor, ACF (31). Analysis of the full-length chicken ACF cDNA demonstrated the predicted amino acid sequence (Fig. 2A) is identical to the human clone. This apparent conservation suggests an interesting paradox, since chicken apoB RNA is not edited (33). This functional limitation reflects at least two components. First, chicken intestine lacks apobec-1 (33, 36) and thus cannot mediate catalytic deamination of a target apoB RNA and, second, chicken apoB RNA is itself not an editable template (33). Thus, the evolutionary and functional significance of ACF expression in this setting must await further study.

Sequence Alignment and Phylogeny of GRY-RBP and ACF Isoforms—Full-length cDNAs encoding GRY-RBP and ACF were isolated from human liver and intestinal RNA as described, and their sequence alignments are shown in Fig. 2A. GRY-RBP contains a distinctive N-terminal extension (Fig. 2A) as well as three nonidentical RNA recognition motifs (RRMs) that appear conserved with those found in ACF (Fig. 2A). In addition, GRY-RBP contains a C-terminal, putative bipartite, nuclear localization sequence [Lys984-Arg-Lys-X2-Lys-Lys-Arg1578] that differs in sequence and location from that proposed in ACF (31). The C-terminal region of GRY-RBP, between residues 446 and 623, contains 20 RG clusters and 8 RGG repeats in an overall context of 36% arginine or glycine residues. This region, in particular the RGG repeats and RG clusters, may signify an RNA binding domain (37) that spans over 170 residues. By contrast, the C-terminal region of ACF contains 6 RG clusters within a domain spanning residues 311–402 that is composed of 28% arginine/glycine residues. Further analysis revealed three isoforms of ACF cDNA in human liver (data not shown). These include the cDNA clone reported by Driscoll et al. (31), a second isoform containing an 8-amino acid insertion (ASP, described by Greeve et al. (32), and a third form with a 55-amino acid deletion (data not shown). Sequence analysis of 10 independent clones isolated from adult human liver RNA revealed 6 encode the clone identified by Driscoll and co-workers (31) and 2 contain the 8-amino acid insertion and 2 contain the 55-amino acid deletion. Analysis of ACF cDNAs from human small intestine revealed that 3 of 9 clones contain the 8-amino acid insertion (data not shown), recently demonstrated by Greeve and colleagues (32) in their liver-derived clone. This insertion was present in the single clone analyzed from chicken small intestine. Phylogenetic analysis suggests that ACF and GRY-RBP represent two distinct members of a common ancestral gene family that is conserved from Dictostelium to mammals (Fig. 2B). Examination of the UNIGENE data base reveals that the EST corresponding to ACF is located on human chromosome 10, whereas GRY-RBP is located on chromosome 20.

Protein-Protein Interaction of GRY-RBP with ACF and with Apobec-1—Protein-protein interaction of GRY-RBP with ACF was examined by several complementary strategies. We first undertook immunoprecipitation of the products of radiolabeled in vitro translation mixed with epitope-tagged recombinant, unlabeled protein. In vitro translated Myc-GRY-RBP yielded a predominant species of ~80 kDa with smaller products that represent either internal methionine residues or partial degradation products, whereas in vitro translation of FLAG-ACF yielded a protein of ~67 kDa (Fig. 3A, lanes 1 and 2, respectively). Mixing radiolabeled ACF with cold, unlabeled Myc-GRY-RBP followed by immunoprecipitation with anti-Myc IgG revealed a physical interaction of these two proteins as demonstrated in lane 2 of Fig. 3B, showing a radiolabeled band corresponding to the dominant translation product of ACF alone. The converse experiment, mixing radiolabeled GRY-RBP with cold, unlabeled FLAG-ACF followed by immunoprecipitation with FLAG IgG, similarly revealed a single radiolabeled band corresponding to the dominant translation product of GRY-RBP alone (Fig. 3B, lane 4). Control immunoprecipitations, performed with the radiolabeled ligand and anti-epitope IgG, but without the target protein, revealed no coprecipitation (Fig. 3B, lanes 1 and 3).

To demonstrate the interaction of ACF and GRY-RBP in a physiological context, cotransfection experiments were conducted in which epitope-tagged ACF, GRY-RBP, and apobec-1 were introduced either alone or in combinations into COS cells. Cell lysates were prepared and examined by Western blotting to demonstrate expression of the relevant protein. Cells transfected with ACF, apobec-1, or GRY-RBP cDNA alone demonstrated comparable expression of the cognate protein (Fig. 3C, lanes 2–4). Cotransfection of ACF and apobec-1 (Fig. 3C, lane 5) or of ACF and GRY-RBP (Fig. 3C, lane 6) also revealed expression of the relevant proteins in cell lysates. Immunoprecipitation of cell lysates was then conducted to demonstrate protein-protein interaction in vivo. Apobec-1 and ACF was immunoprecipitated from COS cells transfected with both cDNAs (Fig. 3D, lane 5), confirming the recent findings of Driscoll and colleagues (31). Extending these findings, COS cells transfected with ACF, and GRY-RBP demonstrated coprecipitation of GRY-RBP following immunoprecipitation of ACF (Fig. 3D, lane 6). These cumulative findings provide further evidence for a physical interaction of ACF and GRY-RBP in vivo.

The interaction of GRY-RBP, and of ACF, with apobec-1 was further examined using a yeast two-hybrid binding assay. As indicated in Fig. 3E, the data reveal strong (+ +) interaction between ACF and apobec-1 and comparable interaction between apobec-1 and GRY-RBP. Apobec-1 has been demonstrated previously to exist as a homodimer (35, 38), and its self-interaction (+++ in this assay) is shown by way of a positive control. Both C- and N-terminal deletions of apobec-1 failed to support an interaction with GRY-RBP, suggesting that these domains may be of importance in stabilizing and maintaining the interaction with additional proteins in the holoenzyme. This speculation is supported by the earlier demonstration that these very N- and C-terminal deletions in apobec-1 eliminate homodimerization, RNA binding, and apoB RNA editing activity (35). Nevertheless, it bears emphasis that other interpretations, such as an effect on apobec-1 folding, cannot be excluded.

Finally, the interaction of GRY-RBP and apobec-1 was examined by far Western blotting using immobilized GRY-RBP and 35S-labeled apobec-1, generated from in vitro translation (Fig. 3F). The ability of apobec-1 to bind to GRY-RBP in this renaturation assay further suggests that these two proteins have the capacity to interact biochemically.

Taken together, the results from several independent lines of
FIG. 2. Sequence alignment of GRY-RBP and ACF. A, the deduced amino acid sequence of GRY-RBP and human/chicken (h/Ch) ACF (see text) are aligned. The three RNA recognition motifs (RRMs) are indicated in shaded text. Peptides identified through mass spectroscopy are indicated by the bold line above the sequence. The putative nuclear localization motif in GRY-RBP is indicated by a broken line. B, phylogenetic analysis of GRY-RBP and ACF. The RRM domains were aligned, and a distance matrix was calculated using the PROTDIST and Phylip programs.
FIG. 3. GRY-RBP interacts with ACF and with apobec-1. A, Myc-GRY-RBP and FLAG-ACF were synthesized in vitro using a coupled TnT lysate (Promega) in the presence of [35S]methionine. Lanes 1 and 2 are representative examples of the products revealed by autoradiography of 10% denaturing SDS-PAGE. The mobility of the respective species is indicated by the arrowhead or the bold arrow with molecular weight markers indicated on the left. B, co-Immunoprecipitation of ACF and GRY-RBP. The products of 35S-radiolabeled in vitro translation of ACF (lanes 1 and 2) or GRY-RBP (lanes 3 and 4) were mixed with unlabeled Myc-tagged GRY-RBP (lane 2) or FLAG-tagged ACF (lane 4), respectively. After mixing for 30 min at 30 °C, the indicated IgG (α-Myc or α-FLAG, respectively) was added to all incubations and then was gently rotated at 4 °C for 90 min and collected on protein A-Sepharose beads. Lanes 2 and 4 indicate the coimmunoprecipitation of each species. C, COS cells were transfected with vector DNA (lane 1) or with DNA encoding ACF, apobec-1, or GRY-RBP (lanes 2–4). Other transfections were conducted in which apobec-1 and ACF or GRY-RBP and ACF were simultaneously introduced (lanes 5 and 6). After 48 h, cell lysates were prepared and extracts analyzed by Western blotting with anti-FLAG or anti-Myc IgG. D, ACF coimmunoprecipitates with GRY-RBP and with apobec-1 in transfected cells. The extracts from COS cells prepared as in C were immunoprecipitated with anti-FLAG IgG, and the immunoprecipitates were resolved by SDS-PAGE and Western-blotted with anti-Myc IgG. The data indicate coimmunoprecipitation of apobec-1 with ACF (lane 5) and of GRY-RBP with ACF (lane 6), with specificity demonstrated by the absence of corresponding bands in the other lanes. E, protein-protein interaction of GRY-RBP with apobec-1 using yeast two-hybrid assay. Apobec-1 or mutants thereof, GRY-RBP and ACF cDNAs, were cloned into the indicated yeast vectors (see under “Materials and Methods”) and interactions were determined using 0-nitrophenyl-β-D-galactopyranoside staining as previously validated (33). Descriptions of the different apobec-1 mutations are detailed (33). F, far Western blotting reveals interaction between GRY-RBP and apobec-1. Left panel, GRY-RBP (1 μg) was resolved on a 10% SDS-PAGE and stained with Coomassie Blue. Middle panel, GRY-RBP was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Immobilized GRY-RBP was submitted to 12 cycles of denaturation-renaturation and probed with 35S-labeled apobec-1. Right panel, the identity of GRY-RBP was confirmed by Western blot analysis using affinity-purified GRY-RBP IgG. In each case, a representative result is shown from replicate experiments.
evidence indicate that GRY-RBP interacts with ACF and with apobec-1.

**RNA Binding Activity of ACF and GRY-RBP**—Recombinant ACF and GRY-RBP (Fig. 4A) were used to establish the RNA binding activity of the respective proteins with an apoB template. GRY-RBP binds to apoB RNA as evidenced by the dominant UV cross-linked band (Fig. 4B). The binding of ACF to apoB RNA, previously demonstrated by Driscoll and colleagues (31), was confirmed in our hands (Fig. 4C). Additionally, incremental supplementation with ACF inhibited GRY-RBP binding to apoB (Fig. 4C). GRY-RBP additions in turn appeared to interfere with ACF binding to apoB RNA, the data suggesting that binding of GRY-RBP to apoB RNA was of lower affinity than that of ACF (compare 1000 ng of GRY-RBP in D to 500 ng of ACF in C of Fig. 4). These findings are of interest in light of the recent demonstration by Greeve and colleagues (32) that binding of ACF to apoB RNA was competitively displaced by KSRP, suggesting that other RNA-binding proteins, in addition to ACF and apobec-1, may regulate the assembly and composition of the holoediting enzyme. The findings do not allow us to distinguish whether this apparent displacement is mediated directly, through competitive binding for a shared site on the RNA, or alternatively through the binding of ACF and GRY-RBP to one another indirectly modulating RNA binding affinity. However, we will return to the functional consequences of ACF and GRY-RBP binding below.

**RNA Binding Specificity of GRY-RBP and ACF**—The RNA binding activity of ACF and GRY-RBP was further examined using homopolymeric RNAs to compete their binding to apoB RNA. Poly(U), poly(AU), poly(I), and poly(G) competed both proteins but poly(A) competed only ACF (Fig. 5A). Poly(C) failed to compete RNA binding with either ACF or GRY-RBP (Fig. 5A). These data suggest that there are differences in the specificity of RNA binding between GRY-RBP and ACF, particularly with respect to A-rich templates. Despite these differences, however, the results suggest that both proteins bind to U- and AU-rich targets. This feature would be anticipated in light of their binding to the apoB RNA template, which is ~70% AU-rich in the region flanking the edited base (14).

To refine the binding specificity, the recombinant proteins were used in a binding assay with apoB RNAs, into which various scrambling mutations have been introduced. These apoB mutants, each of identical length and spanning the minimal editing cassette, were created by changing 6-nucleotide sections to the complementary sequence, as previously described (Fig. 5B) (13, 17). The results of these experiments suggest that GRY-RBP binds with low specificity throughout the apoB template, as evidenced by the comparable reduction in binding with all the scrambled mutations, compared with that observed with a nonspecific, actin RNA (Fig. 5B). On the other hand, ACF appears to bind preferentially to two regions within the minimal apoB RNA, as evidenced by the abrogation of competition with mutants C and D (Fig. 5B). One site is upstream of the edited base (nt 6660–6665) and another is downstream (nt 6667–6673), the latter spanning the proximal end of the mooring sequence and partially overlapping the binding site recently identified for apobec-1 (16, 17, 20). These results are similar to those obtained using crude rat enterocyte S100 extracts where binding activity of p60 was localized to a region spanning nts 6671–6674 (25). The current data also extend the results of Driscoll and colleagues (29, 31) who demonstrated that ACF failed to bind to a 280-nt baboon apoB RNA containing three mutations within the mooring sequence (6671, 6675, and 6678). The cumulative interpretation of these earlier findings, along with the current data, suggests that regions immediately flanking the edited base may represent binding sites for ACF. Nevertheless, fine mapping of the binding site of
ACF and its role in the cooperative assembly of the apoB RNA editing enzyme will require further study.

**Regulation of C to U RNA Editing Activity through ACF-GRY-RBP Interaction**—ACF and apobec-1 were used in an *in vitro* editing assay to confirm the demonstration that these two proteins represent the minimal editing enzyme complex for C to U deamination of apoB RNA. The data reveal a linear increase in C to U editing activity in an assay using 250 ng of GST-apobec-1 and 1–40 ng of ACF (Fig. 6A). Optimal editing activity (>80% C to U conversion) was noted in assays containing ~40 ng of ACF and ~250 ng of GST-apobec-1 (Fig. 6A). This corresponds to a predicted molar ratio of apobec-1:ACF of 3:1, assuming that all the available protein exists in a functionally active complex. As noted previously in assays utilizing chicken intestinal S100 extracts, further addition of complementation activity (in the present studies, authentic recombinant ACF) led to a progressive decline in editing activity (Fig. 6A). These findings emphasize the crucial stoichiometry of apobec-1 and ACF in this assay and lend indirect support to the hypothesis that other proteins may participate in a regulatory capacity in vivo. Although antisera against GRY-RBP, ACF, and apobec-1 are available, the abundance of ACF and GRY-RBP is below detection levels by Western blotting of fractionated cell or tissue extracts (data not shown, but in agreement with Driscoll et al. (29)), precluding estimates of their relative proportions within a functional editing enzyme. Accordingly, the precise molar concentrations of apobec-1, ACF, and potentially GRY-RBP within the holoenzyme remain to be ascertained directly.

The addition of increasing amounts of recombinant GRY-RBP to assays containing ACF and apobec-1 demonstrated progressive inhibition of RNA editing, with complete abrogation of C to U deamination of apoB RNA noted at the highest amounts tested (Fig. 6B). By contrast, assays containing apobec-1 and up to 100 ng of GRY-RBP failed to demonstrate evidence of C to U editing activity, indicating that GRY-RBP itself lacks the ability to complement apobec-1 (Fig. 6B). Assays were then conducted using a range of input RNA in the presence of apobec-1 and ACF and with increasing amounts of GRY-RBP. C to U conversion demonstrated saturable kinetics with an apparent *K*_m* of 7 ± 1.1 nM (Fig. 6C). The presence of increasing amounts of GRY-RBP (10 and 100 nM) altered the *K*_m for this reaction to 24 and 40 nM, respectively. Lineweaver-Burk plots of the data suggest that this is the result of competitive inhibition (Fig. 6C, inset). To examine further the mechanism of this inhibition, experiments were conducted in which assays, containing amounts of ACF and apobec-1 sufficient to yield ~25–50% editing, were modified through addition of GRY-RBP and then rescued with the addition of ACF, apobec-1, or both. The results of a representative series of such experiments indicate that the addition of ACF (Fig. 6D) but not apobec-1 (data not shown) rescues the inhibition produced by GRY-RBP. Furthermore, supplemental apobec-1 failed to produce incremental effects in assays rescued with ACF (data not shown), suggesting that GRY-RBP exerts its inhibitory effects through binding to and sequestering ACF. This suggestion may also account for the effects noted on apoB RNA binding, alluded to above (Fig. 4).

**Effects of Other ApoB RNA-binding Proteins on C to U Editing Activity**—The demonstration that GRY-RBP binds to ACF and to apoB RNA and also inhibits C to U RNA editing raised the possibility that other apoB RNA-binding proteins may also modulate this process, as previously demonstrated with hnRNP C and D (26, 39). However, as demonstrated in Fig. 7, pyrimidine tract-binding protein (PTB), hnRNP-A, and hnRNP-F, all bind to apoB RNA (Fig. 7, upper panel), yet none produce significant inhibition of apoB RNA editing (Fig. 7, lower panel). These results imply that the interaction of GRY-RBP with apoB RNA and the consequent inhibition of C to U

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**Fig. 5.** ApoB RNA binding activity of GRY-RBP and ACF, competition with homopolymeric RNA and apoB RNA mutants. A. 250 ng of unlabeled homopolymeric RNA was added to binding reactions containing radiolabeled RB105 and 200 ng of either GRY-RBP (left panel) or ACF (right panel). After treatment with RNase T1 and UV irradiation, the cross-linked products were analyzed on a 10% SDS-PAGE. Molecular weight markers are indicated at the left of each gel. B, competition with mutant apoB RNAs. Upper panel, cross-linking was carried out with a 55-nt apoB RNA flanking the edited base, in the absence (−) or presence of competitor RNA; 55-nt wild-type RNA (WT), scanning mutants B–I, representing 6 nucleotide sections immediately upstream (B and C) or downstream (D–I), of the edited base (shown in lower panel). As nonspecific control, an actin cRNA was used at equivalent concentrations. Lower panel, the cross-linked material was stained with Coomassie Blue to demonstrate equivalent amounts of protein in each lane. These results are representative of triplicate experiments.
editing is not a general phenomenon associated with AU-rich RNA-binding proteins.

Antisense Inhibition of GRY-RBP Expression, Effects on ApoB mRNA Editing—To demonstrate a potential physiological role for GRY-RBP in regulating apoB RNA editing, we undertook antisense oligonucleotide treatment of rat hepatoma McA cells to decrease expression of GRY-RBP. McA cells were selected since they express the requisite trans-acting proteins to edit endogenous apoB mRNA. Our prediction, based upon the findings reported above, was that decreased expression of GRY-RBP might result in increased editing activity. Control, untransfected cells demonstrated ~15% C to U editing of endogenous apoB mRNA (Fig. 8, lane 1), a range frequently encountered with this cell line (24). Cells transfected with an antisense oligonucleotide to GRY-RBP, by contrast, demonstrated a 2-fold increase in endogenous apoB RNA editing (~50%, Fig. 8, lanes 2–4), whereas a scrambled oligonucleotide was without effect. The results are summarized in Fig. 8B and support our prediction that GRY-RBP may play a physiological role in the regulation of apoB RNA editing in vivo. Further study will be required to address the regulatory mechanisms involved.

Colocalization of ACF, Apobec-1, and GRY-RBP in Trans-
Identification of GRY-RBP as an ApoB RNA-binding Protein

**FIG. 7.** Other apoB RNA-binding proteins fail to modulate C to U editing. Upper panel, UV cross-linking assays were conducted with recombinant hnRNP A1, F, and PTB together with a radiolabeled apoB RNA (see under “Materials and Methods”). The bound transcript was resolved by denaturing SDS-PAGE. Lower panel, C to U editing assays were conducted with recombinant apobec-1 and ACF (lane 3) to which was added 2 μg recombinant hnRNP A1 (lanes 4 and 5), F (lanes 6 and 7), or PTB (lanes 8 and 9). There was no detectable difference in the extent of C to U editing in any of the incubations containing ACF and apobec-1. A representative assay is shown.

**FIG. 8.** Antisense oligonucleotide inhibition of GRY-RBP expression increases apoB RNA editing in McA rat hepatoma cells. A, McA hepatoma cells were incubated with 5 μM antisense GRY-RBP (α-GRY-RBP, lanes 2–4) or a scrambled oligonucleotide (lanes 5–7) and RNA analyzed by primer extension. The products were resolved by PAGE and fluorography; p, primer; C, unedited apoB RNA; U, edited apoB RNA. B, apoB RNA editing was quantitated by phosphorimaging of the gel and expressed as % U. Antisense GRY-RBP treated cells showed a significant increase in apoB RNA editing.

**fected Cells—**To characterize further the interaction of ACF, GRY-RBP, and apobec-1 within the cell, epitope-tagged proteins were introduced into HepG2 and McA cells and localized using immunofluorescence microscopy. Transfection of epitope-tagged proteins permitted the detection of exogenous protein in cells that express endogenous ACF, with (McA) or without (HepG2) coexpression of endogenous apobec-1. Cotransfection of ACF and GRY-RBP suggests a nuclear localization of both proteins in HepG2 and McA cells (Fig. 9, upper left panel). Cotransfection of GRY-RBP and apobec-1 suggests that GRY-RBP is again found in a nuclear distribution, whereas apobec-1 is distributed in a predominantly nuclear localization pattern also but with evidence of cytoplasmic staining (Fig. 9, upper right panel). Cotransfection of ACF and apobec-1 indicates a predominantly nuclear staining pattern of ACF, again with evidence of cytoplasmic staining of apobec-1 along with the intense nuclear staining pattern (Fig. 9, lower panel). It must be emphasized, however, that the interpretation of these results is based upon the distribution of epitope-tagged proteins within cells that express a small but presumably functional pool of endogenous ACF, GRY-RBP, and for McA cells, apobec-1. In all cases, the size of this pool is unknown, since these proteins defy quantitation using conventional immunohistochemical approaches (data not shown). Accordingly, resolution of the crucial question of whether these findings reflect the pattern of endogenous proteins must await the development of more sensitive methodology and reagents.

To refine the colocalization data hinted at above, confocal microscopy was undertaken in COS-7 cells, which express very low levels of ACF, GRY-RBP, and undetectable levels of apobec-1 (data not shown). Apobec-1 was found predominantly in the nucleus, although some staining was noted within the cytoplasm (Fig. 10A). In both locations, apobec-1 staining colocalized with ACF (Fig. 10, A–C). Similarly, cotransfection of apobec-1 and GRY-RBP demonstrated a predominantly nuclear localization of both proteins, with diffuse cytoplasmic staining also evident (Fig. 10, D–G). By contrast, cotransfection of ACF and GRY-RBP demonstrated almost exclusively nuclear staining, with both proteins again colocalizing in the confocal, merged image (Fig. 10, H–K). Taken together, the imaging results support the proposal that ACF and GRY-RBP are colocalized nuclear proteins and demonstrate that apobec-1 associates in vivo with both GRY-RBP and with ACF.

The pattern of apobec-1 distribution (predominantly nuclear with some cytoplasmic staining) is consistent with the working model of C to U editing of apoB RNA as a nuclear event and raises the additional possibility that apobec-1 may shuttle from the cytoplasm to nuclear pool. Such speculation requires formal proof but is consistent with recent data demonstrating a role for apobec-1 in regulating the stability of AU-rich transcripts through its binding activity, a function presumed to imply a cytoplasmic location (15). In addition, recent results from Smith and colleagues (40) imply the possibility that apobec-1 may edit apoB RNA in the cytoplasm of rat hepatoma cells, again consistent with its localization in this compartment rather than the nucleus, as had been earlier concluded (37). However, the current findings are somewhat at variance with those of Smith and colleagues (40) in regard to the localization of ACF. Their findings suggest both a cytoplasmic and nuclear distribution as evidenced by standard immunofluorescence microscopy, whereas our data, using confocal microscopy, suggest...
that ACF exhibits a predominantly nuclear localization (Figs. 8 and 9). Further examination of this apparent compartmentalization is clearly warranted.

In summary, the findings of this report indicate that the S100 fraction of chicken enterocyte extracts, a source established to be enriched for apoB editing complementation activity, contains ACF as well as a related protein, GRY-RBP. Both proteins bind to apoB RNA and also bind to one another. GRY-RBP and ACF both exhibit binding activity for apoB RNA, the binding site for ACF localizing to a region flanking the edited base. In addition, these proteins colocalize with one another in the nucleus of transfected cells, and each appears to colocalize with apobec-1. These data, taken together with the finding that addition of GRY-RBP to the minimal editing reaction components (apobec-1 plus ACF) produces a competitive inhibition of C to U editing and the demonstration that antisense inhibition of GRY-RBP expression increases apoB RNA editing in rat hepatoma cells, suggest that GRY-RBP may play a role in the regulation of apoB RNA editing. One possible interpretation is that this regulation may be exerted through a complex interaction that reflects the role of additional components that compose the holoediting enzyme. The mechanism of
interaction of these and other candidate genes will be the focus of future reports.

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Identification of GRY-RBP as an Apolipoprotein B RNA-binding Protein That Interacts with Both Apobec-1 and Apobec-1 Complementation Factor to Modulate C to U Editing

Valerie Blanc, Naveenan Navaratnam, Jeffrey O. Henderson, Shrikant Anant, Susan Kennedy, Adam Jarmuz, James Scott and Nicholas O. Davidson

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