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H．知的財産権の出願・登録状況
（予定を含む。）

1．特許取得
なし

2．実用新案登録
なし

3．その他
なし

厚生労働科学研究費補助金　肝炎等克服緊急対策研究事業

H26 年度　分担研究報告書

「HBV 感染を阻害する低分子化合物のスクリーニング」

長田 裕之，理研 環境資源科学研究センター，副センター長

研究要旨：微生物や植物の二次代謝産物，およびその誘導体を中心に収集した化合物ライブラリーを用いて HBV 感染阻害物質を探索する．この探索においては，HBV の感染初期過程を高感度に検出できる感染モデル系を利用し，従来の治療薬（インターフェロンや核酸アナログ）とは作用が異なる新規 HBV 治療薬の開発を目的とする．研究前期（H24-25）は微生物生合成遺伝子改変技術やフラクションライブラリー，表現型スクリーニング基盤を利用し，新規天然化合物の創製を行う．研究中期（H26-）から HBV 様擬粒子を用いたスクリーニングを実施し，阻害活性を示す化合物をバイオプローブとしたケミカルバイオロジー研究を展開する．これら一連の研究を通じて，HBV 治療に資する候補化合物の創製と新規創薬標的（HBV 受容体など）の提示を目指す．

A．研究目的

HBV 感染阻害物質の探索を実施するためには，多様性に富んだケミカルライブラリーが必要である．研究前期では，主に，①微生物生合成遺伝子改変技術やフラクションライブラリーを用いた天然化合物の収集と②化学構造や活性情報が充実したデータベースやデータマイニング技術の開発を実施し，HBV 創薬に資する化合物ライブラリー拡充を進めてきた．本年度は，NanoLuc 遺伝子を導入した発光型組換え HBV 粒子を用いたスクリーニング系の確立を目的とした．

B．研究方法

ウイルス感染率は NanoLuc 遺伝子を導入した組換え HBV 粒子（HBV/NL）とパッケージシグナルを欠いた HBV 粒子とを胆汁酸トランスポーターNTCP 過剰発現 HepG2 細胞に共感染させ，ルシフェラーゼ活性を指標に評価した．また宿主に対する毒性は細胞内 ATP レベルを指標に評価した．

評価系の確認には，理研天然化合物バンク NPDepo から提供された標準化合物ライブラリー（作用既知薬剤 80 種）を用いた．また予備スクリーニングとして，フラクションライブラリーから見つかった新規化合物を中心に NPDepo 化合物ライブラリーの抗ウイルス活性を評価した．

また前年度に引き続き，生合成遺伝子改変微生物やフラクションライブラリー，天然化合物データベース NPPlot を用いて新規天然化合物を探索した．

（倫理面への配慮）

遺伝子組み換え生物等の使用に際して，理研の定める細則や指針を遵守した．

C．研究結果

① スクリーニング系の確立

迅速かつ定量性よく活性を評価するため，ウイルス量と培養日数を検討した．その結果，ウイルス液 10 μL，8 日間感染を至適な条件として設定した．このとき，陽性対照として用いた抗 HBs 免疫グロブリンと heparin は濃度依存的な阻害効果が見られた．次に NPDepo から提供された標準化合物について抗ウイルス活性を評価した．その結果，タンパク質合成や DNA 合成阻害剤，微小管重合阻害剤，抗ウイルス物質で顕著な活性が見られた．

② 予備スクリーニング

フラクションライブラリーから見出した新規化合物を中心に，NPDepo 化合物ライブラリーの一部について予備スクリーニングを開始した．その結果，環状ペプチドやカルボリン系化合物など複数のヒッ

トを得た．またこれらの化合物については宿主に対して毒性を示さないことを確認した．

③ 天然化合物の網羅的な収集

微生物代謝産物を系統的に収集したフラクションライブラリーと天然化合物データベース NPPlot を用いた新規天然化合物探索を実施し，オキシインドール系新規代謝産物 RK-270A，B，C（Nogawa et al）など，様々な新規物質を取得した．

D．考察

本年度は HBV/NL を用いたスクリーニング系の確立を計画した．NPDepo の標準ライブラリーの阻害活性を評価した結果，HBV の生活環からヒットが予想される化合物群で活性が見られ，評価系がワークしていることを確認した．化合物ライブラリーを評価する準備が整ったので，今後，大規模スクリーニングを本格化させる．また予備的検討で得た候補化合物のうち，カルボリン系化合物については純度が低かったため，活性本体を精製し，抗ウイルス活性を再評価する．

E．結論

HBV/NL 粒子，HepG2/NTCP 細胞を用いたハイスループットスクリーニング系を確立し，大規模スクリーニングを開始する準備を整えた．

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況（予定を含む．）

1．特許取得：なし
2．実用新案登録：なし
3．その他：なし

厚生労働科学研究費補助金　肝炎等克服緊急対策研究事業

H26 年度　分担研究報告書

「HBV 感染モデル細胞系の確立」

分担研究者　落谷孝広　国立がん研究センター研究所・分子細胞治療研究分野　分野長

研究要旨：スクリーニングに用いる感染細胞の利用については、これまでの研究からヒト初代肝細胞に対する HBV 感染が認められているので、この系を用いて評価を行いつつ、既に樹立されている肝細胞の培養条件を検討し、落谷らは独自に開発した YPAC などの因子（インヒビターカクテル）を用いた感染効率の高い細胞株を得る事、あるいは肝幹細胞や成体幹細胞からの肝細胞分化技術を駆使するなどして感染効率の高い培養細胞の獲得をおこなう。平成２６年度の成果は以下の通りである：（1）成熟肝細胞から３種類の小分子シグナル阻害剤刺激下で，増殖能および成熟肝細胞・胆管上皮細胞への分化能を持つ肝幹細胞様細胞の最適培養条件、保存条件を確立した。（2）肝幹細胞様細胞のリプログラミングのメカニズム解明のため、ステム関係遺伝子群などの網羅的解析を行ない、複数のステム細胞特異的遺伝子の発現を確認したことから、ステム細胞様に変化している事が明らかとなった。

Ａ．研究目的

抗ウイルス薬開発のスクリーニングに用いる感染細胞の利用については、これまでの研究からヒト初代肝細胞に対する HBV 感染が認められているので、この系を用いて評価を行いつつ、既に樹立されている肝細胞の培養条件を検討し、分担研究者らは独自に開発した YPAC などのインヒビター（低分子化合物）を用いた感染効率の高い細胞株を得る事、あるいは肝幹細胞や成体幹細胞からの肝細胞分化技術を駆使するなどして感染効率の高い培養細胞の獲得をおこなう。

Ｂ．研究方法

HBV 感染過程を再現するために最適かつ安定な肝細胞の培養系が確立されていない。分担研究者（落谷）が独自に発見した４種のシグナル伝達阻害剤カクテルである YPAC (PNAS, 2010) を応用する事で、初期感染過程を標的とした新規治療薬の開発に有用な細胞系を提供する。具体的には，この４種類の低分子化合物の組み合わせを変える事で，最も効率的に，ヒト肝細胞の長期機能維持培養を可能にするインヒビターカクテルを同定する。さらに、エピジェネティクス制御因子によるヒト肝細胞誘導を試みる。

（倫理面への配慮）

本研究で使用する細胞は，いずれもインフォームドコンセントのもとに倫理審査を得て採取され，市販されているヒト肝細胞であるため、倫理的な問題点はない。ラットの肝細胞に関しては，所内の動物倫理委員会の承認を得て，動物愛護に基づく実験を実施する。

Ｃ．研究結果

１）３種のシグナル伝達阻害剤カクテルをラット初代培養肝細胞に添加することでリプリグラミングした肝幹細胞の性質を持つ細胞の最適培養条件を設定した。その結果、DMEM をベーストした無血清の培地

で１００倍以上に増殖させる事が可能になった。さらに、通常の１０％DMSO を含む不凍結液での保存が可能であった。成熟肝細胞と胆管細胞の二方向性分化能の最適条件も設定し、それぞれ１００％に近い状態での誘導が可能となった。

２）肝幹細胞様細胞のリプログラミングのメカニズム解明のため、ステム関係遺伝子群などの網羅的解析を行ない、複数のステム細胞特異的遺伝子の発現を確認したことから、ステム細胞様に変化している事が明らかとなった。

Ｄ．考察

本年度の研究成果は 未だラットの肝細胞であるが、この肝細胞の表面に人工的にCNTP の発現を導入する事で、簡易的にHBV の感染をモニターできる細胞が可能かもしれない。今後の検討課題である。さらに、この低分子化合物によるリプログラミング手法が、ヒトの成熟肝細胞から未分化肝幹細胞の誘導にも有効であるかどうかは、今後の研究次第である。

Ｅ．結論

インヒビターによる遺伝子発現変化の誘導が，肝細胞の分化機能維持や長期培養に貢献する可能性が明らかとなった。さらに、エピジェネティクス制御因子が、肝がん細胞を正常な肝細胞様細胞にリプログラミング可能な事も明らかになった事は大きな成果である。この肝幹細胞をヒト肝細胞に応用する事で、肝細胞のリプログラミングによる作成が可能となる。

Ｆ．健康危険情報

なし

Ｇ．研究発表

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Ｈ．知的財産権の出願・登録状況

（予定を含む。）

１．特許取得　無し

２．実用新案登録　無し

3.その他

厚生労働科学研究費補助金　肝炎等克服緊急対策研究事業

H26 年度　分担研究報告書

「HBV 感染、複製機構解析のためのアデノウイルスベクターの開発」

近藤　小貴、東京大学医科学研究所、助教

研究要旨： HBV は感染宿主域が非常に限られていることからモデル動物を用いた検討は困難であるが培養細胞における HBV のゲノム複製効率は極めて低いため、ウイルス生活環の詳細な解析には高効率な評価系の確立が必須である。そこで本研究では肝細胞への遺伝子導入効率が極めて高いアデノウイルスベクター（AdV）から HBV 遺伝子を高発現する系について検討を行った。まず、プレゲノム RNA を CMV プロモーターから発現する AdV を作製し肝細胞由来 HuH-7 細胞へ感染した結果、感染 3 日で複製ゲノムの生成を確認することに成功した。また、HBV コード遺伝子 4 種類（Pol、Core、S、X）をそれぞれ発現する AdV の作製も行い、Pol 遺伝子についてはその活性を確認した。

A．研究目的

本研究では HBV 感染及び複製機構解析を効率的に行うためのツールとして AdV の作製を行う。AdV は特に肝臓細胞への遺伝子導入効率が高く、遺伝子発現に用いるプロモーターも問わないため、プレゲノム RNA 発現 AdV を作製し、HBV ゲノム複製効率が極めて低い培養細胞における効率的な複製ゲノム検出系を確立する。その検出系を用いて 4 種類の HBV コード遺伝子発現 AdV から発現する遺伝子の活性を確認し、作製した AdV を班員へ供与することで研究の推進を図る。

B．研究方法

HBV コード遺伝子及びプレゲノム RNA 発現 AdV を常法により作製した。HuH-7 細胞へ感染後 3 日目の細胞 DNA を用いた PCR 及び 6 日目の細胞 DNA を Southern blot に供してそれぞれ複製ゲノムの検出及び解析を行うことでプレゲノム発現 AdV の評価を行うとともに複製ゲノム検出系を確立した。PCR においては複製ゲノムのみが増幅するように primer を工夫した。また、HBV コード遺伝子発現 AdV の評価はそれぞれの遺伝子を欠失したプレゲノム発現 AdV と共感染することにより行った。

（倫理面への配慮）

本年度の研究については既に報告されている HBV を用いており、特に倫理面に抵触する検討は行っていない。

C．研究結果

(1) プレゲノム RNA 及び HBV がコードする Core、Pol、S、X 遺伝子を各々発現する AdV の作製を行い、それぞれ Southern blot 法、PCR 又は western blot により発現の確認、及び評価を行った。

(2) Pol 領域を一部欠失することでゲノム複製が起こらないように設計した HBV ゲノムを Pol 発現 AdV と同時に HuH-7 細胞へ導入することで Pol 発現 AdV 依存的なゲノム複製を検出したことから、AdV から発現した Pol の活性を確認した。

D．考察

本研究で作製した HBV コード遺伝子発現 AdV は効率的に単一又は複数の HBV 遺伝子を細胞へ導入することが可能であるため、ウイルス生活環における各々の遺伝子の役割を解析することや班員の研究促進に有用であると考える。また、AdV は特に肝臓由来細胞への導入効率が高いため、薬剤誘導やトランスフェクション法など一般的に用いられている手法と比べて迅速かつ定量的に HBV ゲノム複製を検

出することが可能であることから抗 HBV 薬のスクリーニング等にも有用性が高いと考える。

E．結論

本研究で作製したプレゲノム発現 AdV を用いた複製ゲノム検出系は培養細胞においても十分複製ゲノムの検出が可能であったことから、HBV コード遺伝子発現 AdV を評価するための検出系を確立することが可能であっただけでなく、ゲノム複製の研究にも応用可能であると考える。また、Pol 遺伝子発現 AdV においてはその活性を確認出来たため、今後は他のコード遺伝子発現 AdV に関しても評価を行っていくとともに班員への供給を進める。

F．健康危険情報

なし

G．研究発表

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H．知的財産権の出願・登録状況
（予定を含む。）
なし

Ⅲ　研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表レイアウト

雑誌

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Ⅳ　研究成果の刊行物・別刷り

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The RNA-editing Enzyme APOBEC1 Requires Heterogeneous Nuclear Ribonucleoprotein Q Isoform 6 for Efficient Interaction with Interleukin-8 mRNA*[S]

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Background: APOBEC1 stabilizes target mRNAs by suppressing nonsense- or AU-rich element-mediated decay; however, the mechanisms regulating target selection are unknown.
Results: In the presence of hnRNPQ isoform 6, APOBEC1 stabilizes interleukin-8 mRNA independently of APOBEC1 complementation factor.
Conclusion: APOBEC1 utilizes a complementing protein to select target mRNAs.
Significance: These data shed light on the selective regulation of APOBEC1 target genes.

Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1 (APOBEC1) is an intestine-specific RNA-binding protein. However, inflammation or exposure to DNA-damaging agents can induce ectopic APOBEC1 expression, which can result in hepatocellular hyperplasia in animal models. To identify its RNA targets, FLAG-tagged APOBEC1 was immunoprecipitated from transfected HuH7.5 hepatocellular carcinoma cells and analyzed using DNA microarrays. The interleukin-8 (*IL8*) mRNA was the most abundant co-precipitated RNA. Exogenous APOBEC1 expression increased IL8 production by extending the half-life of the *IL8* mRNA. A cluster of AU-rich elements in the 3′-UTR of *IL8* was essential to the APOBEC1-mediated increase in IL8 production. Notably, *IL8* mRNA did not co-immunoprecipitate with APOBEC1 from lysates of other cell types at appreciable levels; therefore, other factors may enhance the association between APOBEC1 and *IL8* mRNA in a cell type-specific manner. A yeast two-hybrid analysis and siRNA screen were used to identify proteins that enhance the interaction between APOBEC1 and *IL8* mRNA. Heterogeneous nuclear ribonucleoprotein Q (hnRNPQ) was essential to the APOBEC1/*IL8* mRNA association in HuH7.5 cells. Of the seven hnRNPQ isoforms, only hnRNPQ6 enabled APOBEC1 to bind to *IL8* mRNA when overexpressed in HEK293 cells, which expressed the lowest level of endogenous hnRNPQ6 among the cell types examined. The results of a reporter assay using a luciferase gene fused to the *IL8* 3′-UTR were consistent with the hypothesis that hnRNPQ6 is required for APOBEC1-enhanced IL8 production. Collectively, these data indicate that hnRNPQ6 promotes the interaction of APOBEC1 with *IL8* mRNA and the subsequent increase in IL8 production.

Apolipoprotein B (apoB)[2] mRNA-editing enzyme, catalytic polypeptide 1 (APOBEC1) is a cytidine deaminase that converts a specific cytidine residue in the apoB mRNA (*APOB*) to uridine. This deamination generates an in-frame premature stop codon that results in the production of apoB-48, the short isoform of apoB. The full-length (apoB-100) and short (apoB-48) isoforms of apoB are lipoproteins that mediate lipid transfer through the bloodstream. In many mammals, including humans, apoB is expressed in the small intestine and liver, whereas APOBEC1 is expressed only in the small intestine. However, in mice, APOBEC1 is expressed in both the small intestine and liver, and the level of high density lipoprotein, which is inversely associated with the development of coronary disease, is higher in mice than in humans (1). Furthermore, APOBEC1-deficient mice have reduced high density lipoprotein levels (2).

APOBEC1-mediated editing of *APOB* requires a *cis*-acting and a *trans*-acting element; the *cis*-acting element is located in the mooring sequence downstream of the editing site in the *APOB* mRNA (3, 4), whereas the *trans*-acting element is APOBEC1 complementation factor (ACF) (5, 6). APOBEC1 forms a complex with ACF and other proteins that positively or negatively regulate *APOB* editing. In this manner, APOBEC1-mediated editing of *APOB* is strictly controlled. Furthermore, ACF protects the APOBEC1-edited *APOB* mRNA isoform, which

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[S] This article contains supplemental Table S1.
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[2] The abbreviations used are: apoB/APOB, apolipoprotein B; ACF, APOBEC complementation factor; APOBEC1, apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1; ARE, AU-rich element; DST, downstream; FL-A1, FLAG-tagged APOBEC1; FL-Q6, FLAG-tagged hnRNPQ6; hnRNP, heterogeneous ribonucleoprotein; IP, immunoprecipitation; NC, negative control; ARE, AU-rich element; qPCR quantitative PCR; RIPA, radioimmune precipitation assay.

contains a premature stop codon, from nonsense-mediated decay (7).

Ectopic expression of *APOBEC1* occurs in hepatocellular carcinoma (8), lung carcinoma (9), carcinoma *in situ* cells of the adult testis (10), and contused rat spinal cords (11). A consensus p53 response element in the *APOBEC1* promoter can drive expression of the gene in non-intestinal cell types. For example, exogenous expression and doxorubicin-mediated induction of p53 up-regulates *APOBEC1* expression in H1299 lung carcinoma cells and HepG2 hepatocyte carcinoma cells, respectively (12). Notably, forced liver-specific overexpression of transgenic APOBEC1 results in hepatocellular carcinoma and hyperplasia in mice and rabbits (13), and the mRNA encoding novel APOBEC1 target 1 undergoes cytidine to uracil (C to U) RNA editing in the livers of these animals (14), indicating that the aberrant APOBEC1-driven editing of hepatic mRNAs may be involved in tumorigenesis. Similarly, aberrant APOBEC1-driven editing of the mRNA encoding neurofibromin 1 may promote the formation of neurofibromatous tumors (15). By contrast, Greeve *et al.* (8) have suggested that most types of carcinoma, including hepatocellular carcinoma, are not associated with aberrant editing of the mRNAs encoding APOB, novel APOBEC1 target 1, or neurofibromin 1. However, more recently, APOBEC1-driven mRNA editing has been shown to be associated with lung adenocarcinoma (9); therefore, the role of aberrant APOBEC1-driven mRNA editing in tumorigenesis requires further clarification.

In addition to its role in *APOB* mRNA editing, APOBEC1 can stabilize mRNAs that have one or more AU-rich elements (AREs) in their 3′-UTR (16) and can deaminate 5-hydroxymethylcytosine to 5-hydroxymethyluracil, which is one of several steps in the demethylation process of methylated DNA (17).

Because APOBEC1 can bind RNA, in this study, FLAG-tagged APOBEC1 (FL-A1) was expressed in the HuH7.5 hepatocellular carcinoma cell line and used to co-immunoprecipitate its target RNAs, which were identified via a microarray analysis. The mRNA encoding interleukin-8 (IL8) was identified as a direct binding target of APOBEC1. Recombinant APOBEC1 is known to interact directly with some synthetic mRNAs, such as *MYC*, *COX2*, and *IL8* (16, 18); hence, the association of APOBEC1 with the *IL8* mRNA observed here was somewhat predictable. Given the relatively high level of *MYC* expression in HuH7.5 cells, the association between *MYC* mRNA and FL-A1 was weak. Moreover, FL-A1 did not associate with *IL8* mRNA in any of the other cell types examined. Further exploration of the mechanisms underlying the interaction between APOBEC1 and *IL8* mRNA revealed that another factor, heterogeneous nuclear ribonucleoprotein Q isoform 6 (hnRNPQ6), enhanced this interaction.

EXPERIMENTAL PROCEDURES

Cell Culture—HuH7.5, HuH7, HuH6, PH5CH, and HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. HuS cells were cultured as described previously (19).

Constructs—Human *APOBEC1* was cloned into the EcoRI-NotI site of the pCAG-FLAG or pCAG-Myc vector. Human *hnRNPQ6* was cloned into the BamHI-NotI site of the pcDNA3-FLAG vector. The plasmids were constructed using the In-Fusion HD Cloning Kit (Clontech). The *GAPDH*, *IL8*, and *MYC* sequences were cloned into the pGEM-T Easy vector (Promega). The resulting plasmids were used as standards for absolute quantification.

Transfection—The pCAG-FLAG-APOBEC1, pCAG-Myc-APOBEC1, and pcDNA3-FLAG-hnRNPQ6 plasmids were transfected into cells using TransIT-LT1 reagent (Mirus Bio). All of the experiments were based on the transient transfection system. As negative controls, cells were transfected with the empty pCAG-FLAG, pCAG-Myc, or pcDNA3-FLAG vector.

RNA Extraction and Quantitative RT-PCR—The RNeasy Mini Kit (Qiagen) was used to extract RNA. Complementary DNA was prepared by incubating the RNA samples with SuperScript III RT (Invitrogen) and oligo(dT) primers (for whole cell RNA samples) or random primers (for RNA samples collected from immunoprecipitates). The 7500 Fast Real Time PCR System (Applied Biosystems) was used for all quantitative PCR (qPCR) analyses. The primer sequences were as follows: *IL8* forward, CTGTTAAATCTGGCAACCCTAGTCT; *IL8* reverse, CAAGGCACAGTGGAACAAGGA; *GAPDH* forward, CCATGCCATCACTGCCACCC; *GAPDH* reverse, GCCAGTGAGCTTCCCGTTCAG; *MYC* forward; AGGGTCAAGTTGGACAGTGTCA; *MYC* reverse, TGGTGCATTTTCGGTTGTTG; *APOB* forward, CTGTCAGCGCAACCTATGAG; *APOB* reverse, TCTGCCGATTATATTTGAATGTCA; *IL18* forward, CCAACGCTGGCTGCTAAAGT; *IL18* reverse, CCTCTTCCCGAAGCTGTGTAGA; *CXCL1* forward, CCACTGCGCCCAAACC; *CXCL1* reverse, GCAAGCTTTCCGCCCATT; *CXCL5* forward, CAGACCACGCAAGGAGTTCA; *CXCL5* reverse, GGGCCTATGGCGAACACTT; *hnRNPQ* forward, TGCCTTTTTATGTGGAGTCATGA; *hnRNPQ* reverse, AATCTGCTACTTTGGTCCCTTGTT; *ACF* forward, CCATGGCGAGGAGGAAACT; *ACF* reverse, TGCAATACCATGTCCCCATAAC; *APOBEC1* forward, GACCCCAGAGAACTTCGTAAAGAG; *APOBEC1* reverse, CGGCTCATGCCCCACTT.

Immunoprecipitation (IP)—Cell lysates were incubated with IgG rabbit (Santa Cruz Biotechnology, Inc.), an anti-FLAG antibody (Sigma), or an anti-Myc antibody (Sigma) for 2 h at 4 °C. Protein G-Sepharose (GE Healthcare) was then added, and the immunocomplexes were collected by centrifugation. The pellets were used for RNA extraction.

Western Blotting—Western blots were probed with an anti-FLAG antibody (Sigma), anti-Myc antibody (Sigma), anti-apoB antibody (Biodesign International), anti-ACF antibody (Abnova), or anti-actin antibody (AC-40; Sigma). Detection was carried out using ECL Plus reagent (GE Healthcare).

Luciferase Assay—The pGL4.10 and pGL4.75 vectors (Promega) were used for analyses of the 3′-UTR of the *IL8* mRNA. Briefly, the CMV promoter from pGL4.75 (BspMI-HindIII) was inserted into pGL4.10 to construct pLuc2/CMV-SV40 poly(A). The SV40 poly(A) sequence was then substituted with the 3′-UTR of the *IL8* mRNA to generate pLuc2-IL8. The deletion mutants were generated from the wild-type construct using the KOD-Plus-Mutagenesis Kit (Toyobo). The pGL4.74[hRluc/TK] construct (Promega) was used as an internal control. The constructs were transfected into cells using TransIT-LT1 re-

agent (Mirus Bio). Lipofectamine 2000 reagent (Invitrogen) was used to co-transfect the cells with siRNAs. The mixtures of constructs and/or siRNAs and reagents were plated before the addition of the cells (reverse transfection method). At 48 h post-transfection, the cells were lysed in 100 μl of 1× passive lysis buffer (Promega), and the samples were processed using the Dual-Luciferase reporter assay system (Promega), according to the manufacturer's instructions. The firefly and *Renilla* luciferase signals were determined using a GloMax 20/20 luminometer (Promega). To control for off-target effects of the expression plasmids or the transfection procedure, the firefly luciferase signal was normalized to that of *Renilla* luciferase.

ELISA—A chemiluminescent ELISA system (Thermo Scientific) was used to measure IL8 levels in the culture medium, according to the manufacturer's instructions.

Knockdown of ACF or hnRNPQ—Lipofectamine RNAiMAX reagent (Invitrogen) was used to transfect cells with the On-TARGET plus human A1CF (ACF) or human SYNCRIP (hnRNPQ) siRNAs (Thermo Scientific).

Semiquantification of hnRNPQ mRNA—Complementary DNA was amplified using PrimeSTAR Max DNA Polymerase (Takara) according to the manufacturer's instructions. The sequences of the first round PCR primers were as follows: forward, ATGGCTACAGAACATGTTAATGG; reverse CATTGTAACAGGTCAGGACCG. The sequences of the second round PCR primers were as follows: forward, ATCCTGATCCTGAGGTTATGG; reverse, CATTGTAACAGGTCAGGACCG. As a control, the qPCR primers described above were used to detect *GAPDH*.

Microarray Analysis—HuH7.5, HuH6, PH5CH, HuS, and HEK293 cells were seeded 16 h prior to transfection with the FL-A1 plasmid. The cells were harvested 48 h after transfection and lysed in RIPA buffer. Each lysate was divided into two fractions, one of which was reacted with control IgG and the other with an anti-FLAG antibody. RNA was extracted from the immunoprecipitates and subjected to microarray analysis using the Human Oligo chip 25k (Toray Industries).

Yeast Two-hybrid Analysis—Human brain cDNA was used as prey and APOBEC1 was used as the bait.

RESULTS

Ectopically Expressed APOBEC1 Binds Preferentially to IL8 mRNA in HuH7.5 Cell Lysates—Previous biochemical analyses showed that recombinant APOBEC1 binds to synthetic 3′-UTRs derived from the *MYC* and *IL8* mRNAs (16, 18). To identify additional mRNAs that bind to APOBEC1 in the presence of other cellular proteins, extracts from HuH7.5 cells transiently expressing FL-A1 were immunoprecipitated with an anti-FLAG antibody or IgG as a control and then analyzed using DNA microarrays. For 2,786 of the 25,000 genes examined, the signal intensity in the FL-A1 co-precipitate was significantly higher than that in the IgG co-precipitate, which was set to 1. Of these 2,786 RNAs, 68 had signal ratios (FL-A1/IgG) higher than that of the *APOB* mRNA (6.01), a known RNA target of APOBEC1 (supplemental Table S1). Among these 68 RNAs, which included 65 mRNAs, 1 noncoding RNA, and 2 processed pseudogene transcripts, 94% represented mRNAs with at least one core ARE sequence (AUUUA) in the 3′-UTR, and 30% were related to inflammatory responses, transcriptional regulation, cell cycle regulation, apoptosis, and signaling. The *IL8* mRNA exhibited the highest signal ratio (58.31) among all transcripts examined, but the signal ratio of the *MYC* mRNA (1.04) was not elevated significantly, despite its robust expression in HuH7.5 cells (supplemental Table S1 and Fig. 1*A*). Taken together, these findings indicate that APOBEC1 binds preferentially to *IL8* mRNA in HuH7.5 cells.

Next, qPCR analyses were performed to validate the microarray results. In the microarray analysis, the expression level of the *GAPDH* mRNA in the FL-A1 co-precipitate was not significantly higher than that in the IgG co-precipitate (supplemental Table S1); therefore, *GAPDH* and *APOB* were used as negative and positive qPCR controls, respectively. For each transcript examined, the mRNA level in the FL-A1 co-precipitate was normalized to that in the control IgG co-precipitate. In agreement with the microarray data, the relative *IL8* mRNA level in the FL-A1 co-precipitate was much higher than that of any other mRNA examined (Fig. 1*B*). Furthermore, the relative *MYC* mRNA level in the FL-A1 co-precipitate was lower than that of the *IL8* mRNA, although *MYC* was expressed at a much higher level than *IL8* in HuH7.5 cells (Fig. 1, *A* and *B*). The expression levels of the *CXCL1* and *CXCL5* mRNAs, which encode cytokines and were among the 68 RNAs with the highest microarray signal ratios (supplemental Table S1), were also examined by qPCR. In these experiments, *IL18* mRNA was selected as a negative control to represent cytokine-encoding mRNAs. The relative *CXCL1* and *CXCL5* mRNA levels in the FL-A1 co-precipitate were higher than that of *IL18* mRNA (Fig. 1*B*). Overall, the results of the qPCR analyses validated the microarray data and confirmed that APOBEC1 associates preferentially with *IL8* mRNA in HuH7.5 cells.

To determine whether it associates preferentially with *IL8* mRNA in other cell types, APOBEC1 (FL-A1) was transiently expressed in HuH6 (a human hepatoblastoma-derived line), PH5CH (human non-neoplastic-derived hepatocytes), HuS (human immortalized hepatocytes) (19), and HEK293 cells. The cell extracts were immunoprecipitated with an anti-FLAG antibody or IgG as a control and then analyzed using DNA microarrays. The interaction between FL-A1 and *IL8* mRNA was much stronger in HuH7.5 cells than in HuH6, PH5CH, or HuS cells (supplemental Table S1). Notably, the level of the *IL8* mRNA was below the limit of detection in HEK293 cells (supplemental Table S1). These results were confirmed by RT-qPCR analyses of the cell lysates (Fig. 1*C*). Similar qPCR experiments were also performed in the HuH7 cell line, which is the parental cell line of HuH7.5. A similar level of binding of APOBEC1 to *IL8* mRNA to that observed in HuH7.5 cells was also observed in HuH7 cells (Fig. 1*C*).

APOBEC1 Extends the Half-life of IL8 mRNA—Recombinant APOBEC1 produced in *Escherichia coli* binds to the 3′-UTR of synthetic *MYC* mRNA, and overexpression of APOBEC1 stabilizes the *MYC* mRNA in mouse cells (16). To determine whether the association between APOBEC1 and *IL8* mRNA in intact cells leads to increased IL8 production, the effect of overexpression of FL-A1 on *IL8* mRNA levels in HuH7.5 cells was examined. Transfection of the cells with an expression plasmid harboring FL-A1 increased the endogenous *IL8* mRNA level

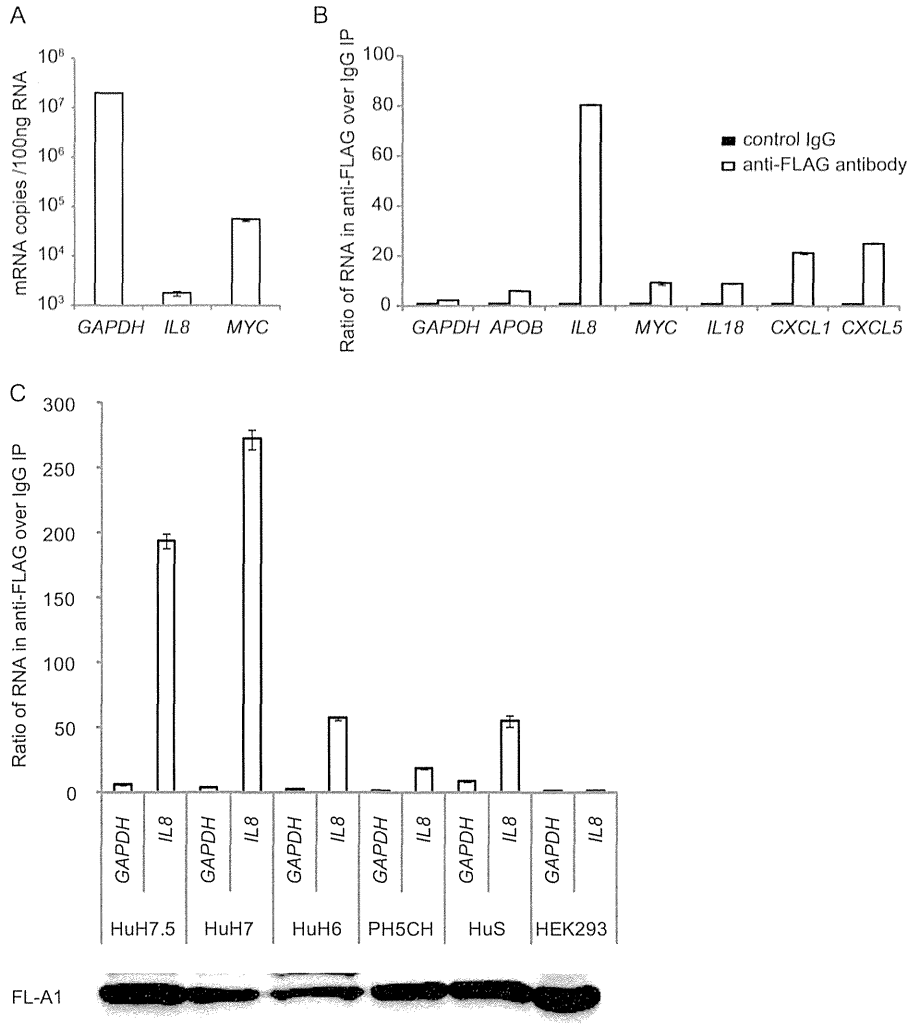

FIGURE 1. **APOBEC1 binds preferentially to *IL8* mRNA in HuH7.5 cells.** *A*, copy numbers of the *GAPDH*, *IL8*, and *MYC* mRNAs in 100 ng of total RNA extracted from HuH7.5 cells, as determined by RT-qPCR analyses. For absolute quantification, a plasmid expressing the *GAPDH*, *IL8*, or *MYC* cDNA was used as a standard. Data are represented as the mean ± S.D. (*error bars*) of $n = 3$ replicate samples. *B*, the interactions of APOBEC1 with different mRNAs in HuH7.5 cells. The cells were seeded 16 h prior to transfection with the FL-A1 plasmid, harvested 48 h after transfection, and lysed in RIPA buffer. Each lysate was divided into two fractions, one of which was reacted with control IgG and the other with an anti-FLAG antibody. Each immunoprecipitate was subjected to RNA extraction and RT-qPCR analysis. For each mRNA, the amount in the anti-FLAG immunoprecipitate was normalized to that in the IgG immunoprecipitate. Data are represented as the mean ± S.D. of $n = 3$ replicate samples. *C*, binding of APOBEC1 to *IL8* mRNA in different cell lines, as determined by RT-qPCR analyses. The indicated cell lines were treated as described for *B*. In parallel, Western blotting was used to examine the level of FL-A1 protein in a portion of each cell lysate. For each mRNA, the amount in the anti-FLAG immunoprecipitate was normalized to that in the IgG immunoprecipitate. Data are represented as the mean ± S.D. of $n = 3$ replicate samples.

significantly but had no effect on the mRNA level of *APOB*, a well known target of APOBEC1, or the levels of the *IL18*, *CXCL1*, and *CXCL5* mRNAs (Fig. 2*A*). However, overexpression of FL-A1 enhanced the production of apoB-48 (Fig. 2*B*), indicating that the FLAG-tagged protein retained the editing ability of native APOBEC1.

Notably, IL8 was more abundant in the culture medium of cells overexpressing FL-A1 than in that of the control cells (Fig. 2*C*). To determine whether the increased production of IL8 protein and mRNA mediated by overexpression of APOBEC1 was due to an effect at the transcriptional or post-transcriptional level, control or FL-A1-expressing HuH7.5 cells were treated with the transcriptional inhibitor actinomycin D for 0–6 h and then analyzed by qRT-PCR. Degradation of the *IL8* mRNA was delayed in cells that overexpressed FL-A1, whereas degradation of the *GAPDH* mRNA was not affected (Fig. 2*D*). This result suggests that binding of FL-A1 extends the half-life of *IL8* mRNA, leading to increased IL8 production.

Recombinant APOBEC1 binds to the synthetic full-length 3′-UTR of the *IL8* mRNA (18), but it is unclear which part of the *IL8* 3′UTR is important for this interaction and whether APOBEC1 increases IL8 production via binding to this region of the mRNA. To investigate these issues, HuH7.5 cells were co-transfected with an empty or FL-A1-harboring expression plasmid and a reporter plasmid containing the full-length *IL8* 3′-UTR downstream of a luciferase coding sequence (pLuc-IL8) (Fig. 2*E*). Luciferase activity was higher in the FL-A1-expressing cells than the control cells (Fig. 2*F*). Next, a reporter plasmid containing only a part of the *IL8* 3′-UTR (nucleotides 513–854) (del 1) was constructed. This region contains two types of *cis*-elements, namely a cluster of AREs and a downstream (DST) element (20). To evaluate whether one or both of these *cis*-

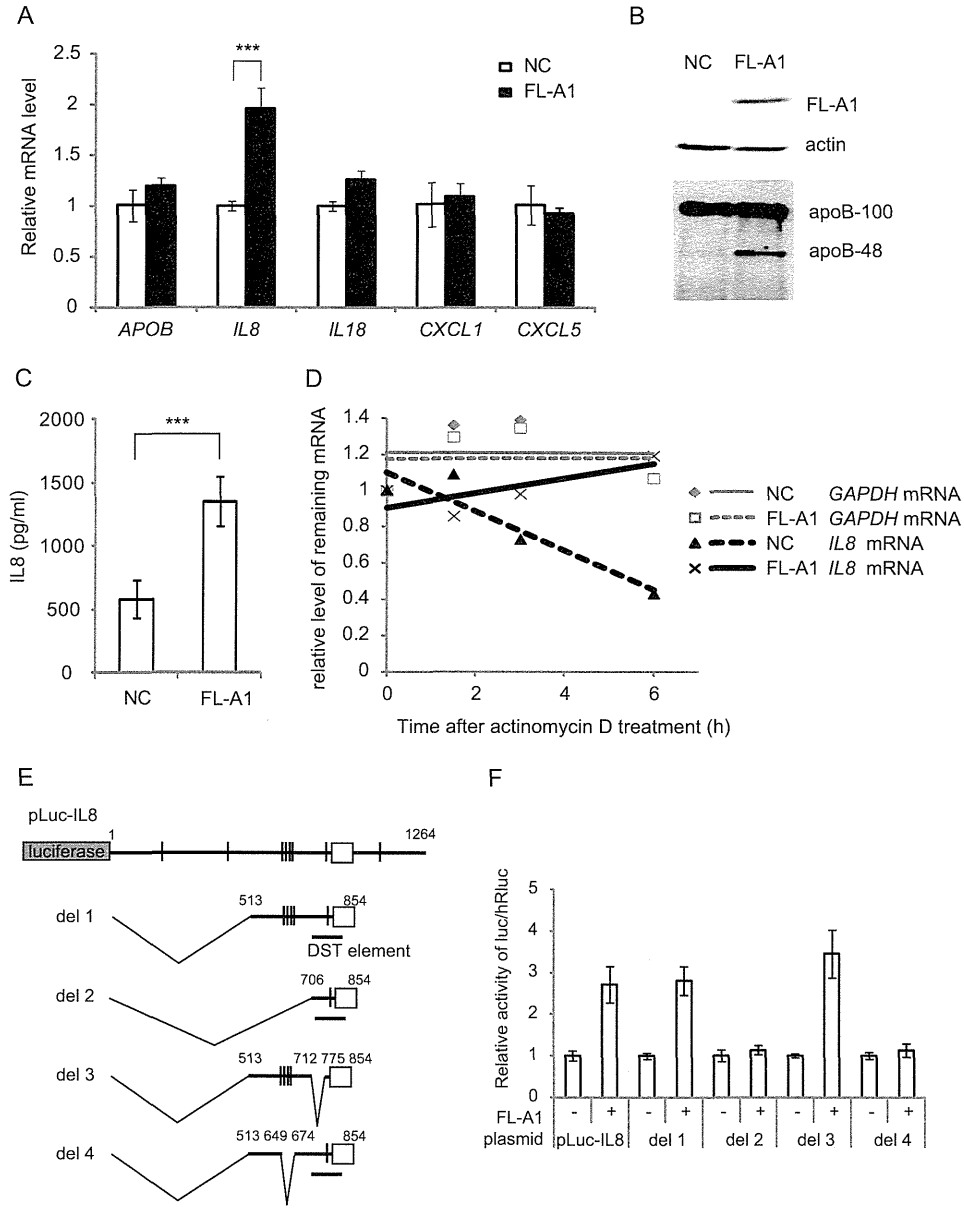

FIGURE 2. **APOBEC1 increases IL8 production at the post-transcriptional level.** *A*, the effect of transient overexpression of APOBEC1 on the levels of various mRNAs in HuH7.5 cells. The cells were seeded 16 h prior to transfection with empty plasmid (*NC*) or a plasmid overexpressing FL-A1, harvested 48 h after transfection, and then subjected to RNA extraction and RT-qPCR analyses. The level of each mRNA in FL-A1-expressing cells was normalized to that of *GAPDH* and the level in the NC cells. Data are represented as the mean ± S.D. (*error bars*) of $n = 3$ independent experiments. ***, $p < 0.005$ by Student's *t* test. *B*, immunoblot analyses of apoB-100, apoB-48, FL-A1, and actin (control) in control and APOBEC1-expressing HuH7.5 cells. The cells were seeded 16 h prior to transfection with empty plasmid (*NC*) or the FL-A1 plasmid. The culture media (*apoB-100* and *apoB-48*) and cells (*FL-A1* and *actin*) were harvested 48 h after transfection and analyzed by immunoblotting. *C*, the levels of IL8 in the culture media of control and APOBEC1-expressing HuH7.5 cells, as determined by ELISAs. The cells were seeded 16 h prior to transfection with empty plasmid (*NC*) or the FL-A1 plasmid, and then washed and supplemented with fresh medium 24 h after transfection. The culture medium was collected 48 h after transfection. Data are represented as the mean ± S.D. of $n = 3$ independent experiments. ***, $p < 0.005$ by Student's *t* test. *D*, the stabilities of the *IL8* and *GAPDH* (control) mRNAs in HuH7.5 cells. The cells were seeded 16 h prior to transfection with empty plasmid (*NC*) or the FL-A1 plasmid. At 48 h post-transfection, the cells were incubated with actinomycin D (5 μg/ml) for 0–6 h and then harvested for RNA extraction and RT-qPCR analyses. *E*, a schematic illustration of the luciferase reporter plasmids (*pLuc-IL8*) containing the indicated regions of the *IL8* 3′-UTR downstream of a luciferase gene. The *vertical lines* represent AUUUA (*ARE*) sequences. The *open box* represents the poly(A) signal. The *horizontal bar below* the gene represents the DST element. *F*, the effects of overexpression of APOBEC1 on the activities of the luciferase reporter plasmids shown in *E*. HuH7.5 cells were reverse-transfected with the indicated reporter plasmid and empty plasmid (−) or the FL-A1 plasmid (+) and then harvested 48 h after transfection. The activities of the pLuc-IL8 constructs were normalized to those of the pGL4.74[hRluc/TK] construct and the level in the empty plasmid (−)-transfected cells. Data are represented as the mean ± S.D. of $n = 4$ independent experiments.

element types are responsible for FL-A1-mediated regulation of *IL8* expression, the following regions of the *IL8* 3′-UTR were removed from the del 1 luciferase reporter construct: (i) nucleotides 513–705 (del 2), containing a cluster of AREs; (ii) nucleotides 713–774 (del 3), containing part of the DST element; and (iii) nucleotides 650–673 (del 4), containing all of the AREs in the *IL8* 3′-UTR (Fig. 2*E*). Disruption of the DST element (del 3) did not affect the FL-A1-dependent increase in luciferase activity, but deletion of the AREs (del 2 and del 4) abolished this increase (Fig. 2*F*). Therefore, the cluster of AREs in the *IL8*

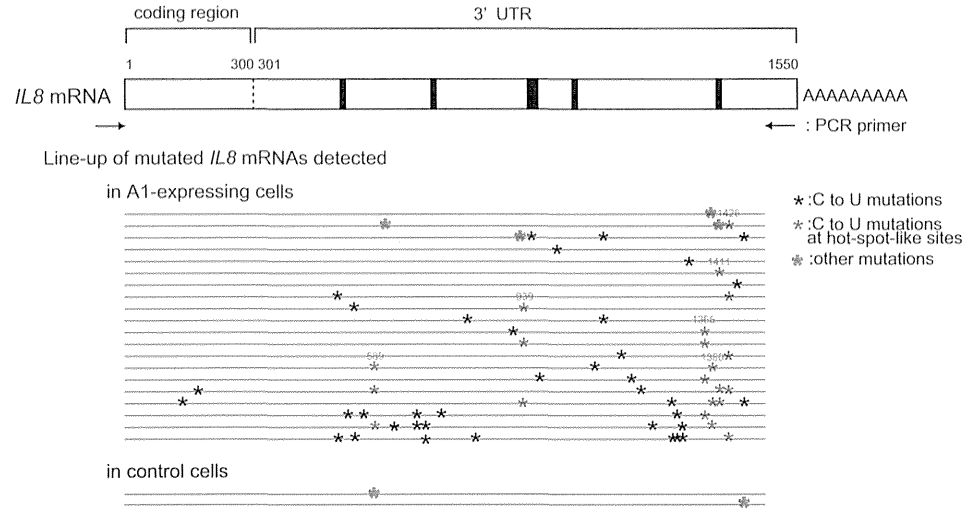

FIGURE 3. **APOBEC1 introduces C to U mutations throughout the *IL8* 3′-UTR.** The distribution of C to U and other mutations in the full-length *IL8* mRNA sequence (1,522 nucleotides), excluding the 5′ noncoding region and the 28 bp immediately upstream of the poly(A) attachment site. HuH7.5 cells were seeded 16 h prior to transfection with empty plasmid (*NC*) or the FL-A1 plasmid. The cells were harvested 48 h after transfection and subjected to RNA extraction and RT. The *IL8* mRNA sequence, including the entire coding region and 3′-UTR, was amplified by PCR using PrimeSTAR MAX DNA polymerase and then cloned into the pGEM vector. A total of 50 clones were sequenced. The nucleotides are *numbered* relative to the start of the coding region. In the *upper image*, the *dotted line* separates the coding region (nucleotides 1–300) and the 3′-UTR (nucleotides 301–1550), the *vertical lines* represent AREs, and the *arrows* represent the positions of the primers used to amplify the *IL8* mRNA. The *vertically aligned asterisks* indicate mutations that occurred at the same nucleotide position. The *red asterisks* indicate hot spotlike sites with more than three mutations (nucleotides 589, 939, 1365, 1380, 1411, and 1426).

3′-UTR may be responsible for APOBEC1-dependent increases in IL8 production.

In mouse enterocytes, APOBEC1 converts C to U at specific sites in the 3′-UTRs of various mRNAs (21); therefore, the ability of APOBEC1 to extend the half-life of the *IL8* mRNA may be related to its editing role. To examine this possibility, the full-length sequence of the *IL8* mRNA (1522 nucleotides), including the 300-nucleotide coding region and excluding the 5′ noncoding region and the 28 bp immediately upstream of the poly(A) attachment site, was scanned for mutations in control and FL-A1-expressing HuH7.5 cells (Fig. 3). The nucleotide sequences of each of the 50 independent clones of *IL8* mRNA from the control cells and FL-A1-expressing cells were determined. No C to U mutations in the *IL8* mRNA were identified in the control cells, whereas 19 FL-A1-expressing clones had C to U mutations that were scattered mainly throughout the 3′-UTR of *IL8* (Fig. 3), although some synonymous C to U mutations were also found in the coding region of *IL8*. Of these 19 clones, two had both C to U conversions and other mutations (1-bp deletion (A), U to G mutation, and A to U mutation). In addition, two control clones and one FL-A1-expressing clone had other mutations only (control: 17-bp deletion or 1-bp (U) insertion in the 3′-UTR; FL-A1-expressing: 11-bp deletion in the 3′-UTR). Overall, these data suggest that APOBEC1 expression is involved in the generation of C to U mutations in *IL8* mRNA. Given that the C to U mutations were not observed at a specific site in the independent clones, it is likely that the observed extension of the *IL8* mRNA half-life and increased production of IL8 in cells overexpressing FL-A1 were not dependent on the editing function of APOBEC1. However, several hot spots of C to U mutations were identified at nucleotides 589, 939, 1365, 1380, 1411, and 1426, suggesting that mutations at these specific sites may affect the stability and/or translational efficiency of the *IL8* mRNA. Mutations other than C to U conversions were also found in two control clones and three FL-A1-expressing clones (Fig. 3), suggesting that these mutations did not occur as a result of overexpression of APOBEC1.

Identification of Binding Partners of APOBEC1 via a Yeast Two-hybrid Analysis—The results described above demonstrated that APOBEC1 binds preferentially to and increases the stability of *IL8* mRNA in HuH7.5 cells; however, this preferential binding was not observed in HuH6, PH5CH, HuS, or HEK293 cells (supplemental Table S1 and Fig. 1*C*). HuH7.5 cells express lower levels of *IL8* mRNA than HuH6, PH5CH, and HuS cells but higher levels than HEK293 cells; however, TNFα-driven transcriptional up-regulation of *IL8* did not increase binding of FL-A1 to *IL8* mRNA in HEK293 cells (data not shown). Thus, the preferential association between APOBEC1 and *IL8* mRNA cannot be explained entirely by elevated *IL8* expression in HuH7.5 cells.

APOBEC1-mediated *APOB* editing requires ACF (5, 6), which prompted us to speculate that APOBEC1 may also require a complementing protein to bind to *IL8* mRNA and that this protein may not be expressed in HEK293 cells. To determine whether ACF functions as a complementing factor for APOBEC1-mediated *IL8* stabilization, HuH7.5 cells were reverse-transfected with a control or ACF-specific siRNA and then transfected with an FL-A1-expressing plasmid 16 h after introduction of the siRNA. As expected, knockdown of ACF reduced the amount of *APOB* mRNA that was co-immunoprecipitated from FL-A1-expressing cells with an anti-FLAG antibody (Fig. 4*A*), whereas the amount of co-immunoprecipitated *IL8* mRNA remained unchanged (Fig. 4*B*). Knockdown of ACF also had no effect on the expression levels of the *GADPH*, *APOB*, or *IL8* mRNAs (Fig. 4*C*). As expected, ACF protein expression was down-regulated in the cells treated with the ACF-specific siRNA, but FL-A1 protein expression was comparable in the control and ACF-specific siRNA-treated cells