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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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[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

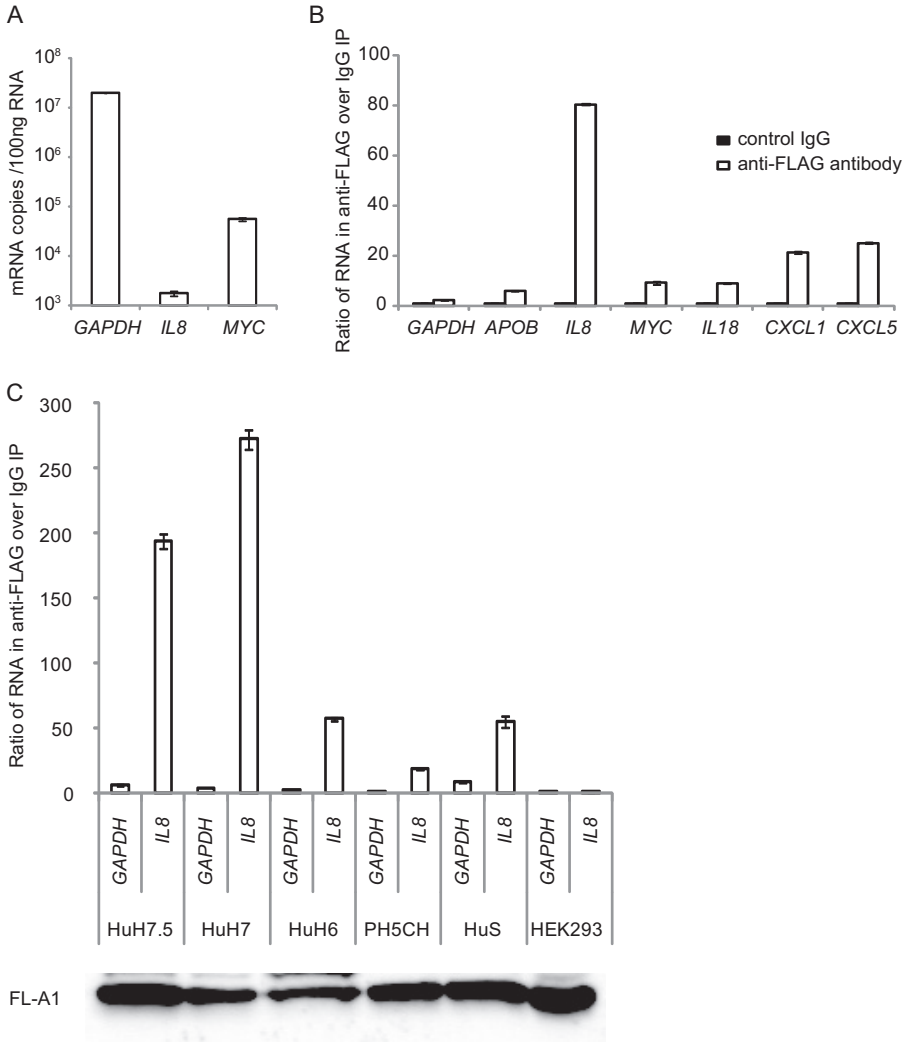


FIGURE 1. **APOBEC1 binds preferentially to *IL8* mRNA in HuH7.5 cells.** *A*, copy numbers of the *GAPDH*, *IL8*, and *MYC* mRNAs in 100 ng of total RNA extracted from HuH7.5 cells, as determined by RT-qPCR analyses. For absolute quantification, a plasmid expressing the *GAPDH*, *IL8*, or *MYC* cDNA 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*-

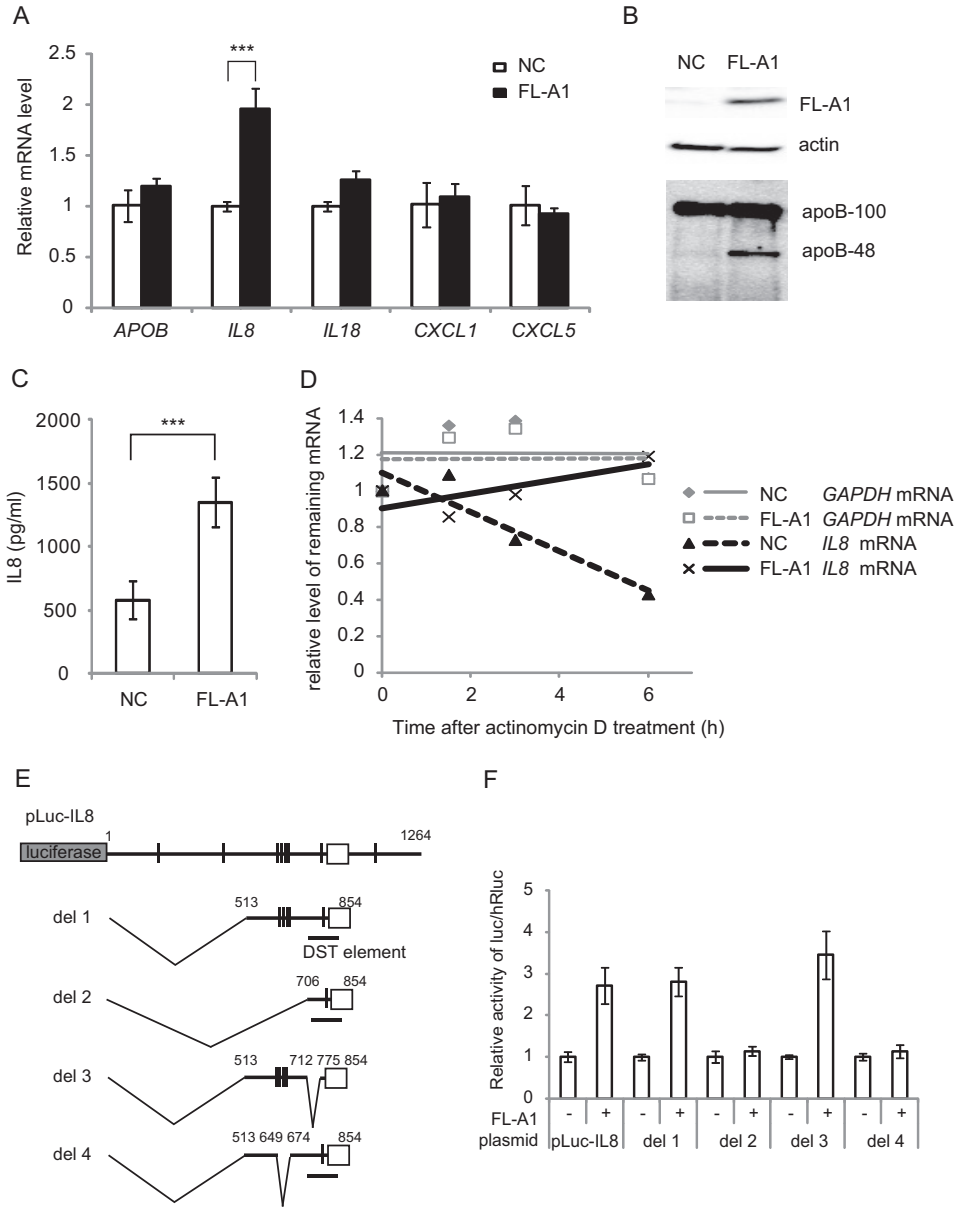


FIGURE 2. **APOBEC1 increases IL8 production at the post-transcriptional level.** *A*, the effect of transient overexpression of APOBEC1 on the levels of various mRNAs in HuH7.5 cells. The cells were seeded 16 h prior to transfection with empty plasmid (*NC*) or a plasmid overexpressing FL-A1, harvested 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*

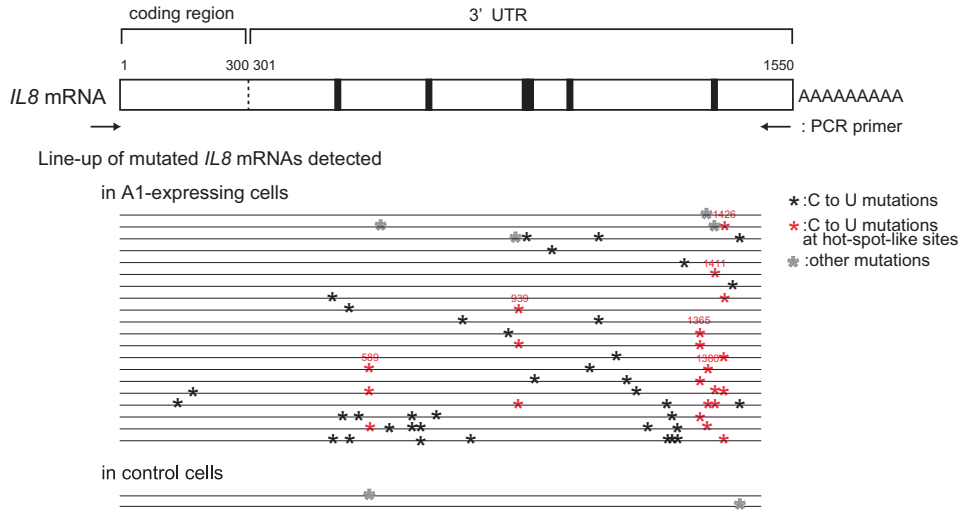


FIGURE 3. **APOBEC1 introduces C to U mutations throughout the *IL8* 3′-UTR.** The distribution of C to U and other mutations in the full-length *IL8* mRNA sequence (1,522 nucleotides), excluding the 5′ noncoding region and the 28 bp immediately upstream of the poly(A) attachment site. HuH7.5 cells were 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

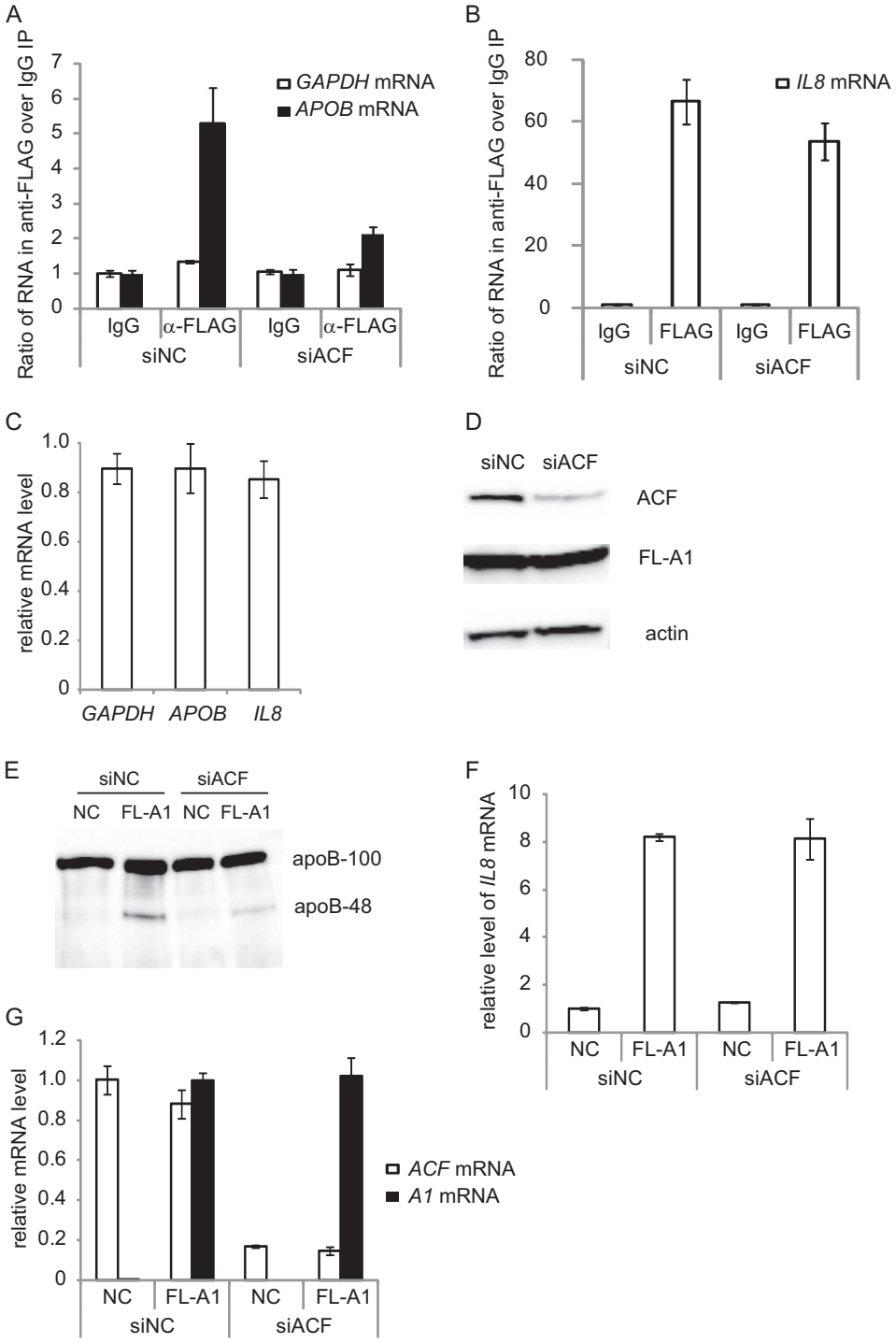


FIGURE 4. **Knockdown of ACF attenuates APOBEC1-mediated *APOB* editing but does not affect the APOBEC1-mediated increase in *IL8* mRNA expression.** *A*, the effect of knockdown of ACF on binding of APOBEC1 to *APOB* mRNA. HuH7.5 cells were reverse-transfected with a control (*si-NC*) or ACF-specific (*si-ACF*) siRNA (30 nM) and then transfected with the FL-A1 plasmid 16 h later. 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. Each immunoprecipitate was subjected to RNA extraction and RT-qPCR analyses of *APOB* and *GAPDH* (control) mRNA levels. For each mRNA, the data were normalized to the level of RNA immunoprecipitated with control IgG. Data are represented as the mean ± S.D. (*error bars*) of $n = 3$ replicate samples. *B*, the effect of knockdown of ACF on binding of APOBEC1 to *IL8* mRNA. The cDNA samples prepared as described in *A* were subjected to RT-qPCR analyses to detect *IL8* mRNA expression. The data were normalized to the level of RNA immunoprecipitated with control IgG. Data are represented as the mean ± S.D. of $n = 3$ replicate samples. *C*, RT-qPCR analyses of *GAPDH*, *APOB*, and *IL8* mRNA levels in the cells described in *A*. The relative mRNA levels in cells treated with si-ACF compared with those treated with si-NC are shown. Data are represented as the mean ± S.D. of $n = 3$ replicate samples. *D*, immunoblot analyses of ACF, FL-A1, and actin (control) in lysates prepared from the cells described in *A*. *E*, the effect of knockdown of ACF on *APOB* editing by APOBEC1. HuH7.5 cells were reverse-transfected with si-NC or si-ACF (30 nM) and then transfected with empty plasmid (pCAG-FLAG; *NC*) or the FL-A1 plasmid 16 h later. The culture medium was harvested 48 h after transfection and used for immunoblot analyses of apoB-100 and apoB-48. *F*, the effect of knockdown of ACF on the APOBEC1-mediated increase in *IL8* mRNA expression. The HuH7.5 cells described in *E* were harvested 48 h after transfection and subjected to RNA extraction and RT-qPCR analyses. The *IL8* mRNA levels were normalized to those of *GAPDH*. Data are represented as the mean ± S.D. of $n = 3$ replicate samples. *G*, RT-qPCR analyses of *ACF* and *A1* mRNA levels in the cells used in *E*. The expression level of each mRNA was normalized to that of *GAPDH*. Data are represented as the mean ± S.D. of $n = 3$ replicate samples.

TABLE 1

Proteins identified in a yeast two-hybrid analysis in which APOBEC1 was used as the bait

For hnRNPQ and hnRNPR, two isoforms were identified as candidate binding partners of APOBEC1.

Number	Protein	Isoform
1	Heterogeneous nuclear ribonucleoprotein K	Isoform a
2	Heterogeneous nuclear ribonucleoprotein Q	Isoform 2
	Heterogeneous nuclear ribonucleoprotein Q	Isoform 4
3	Heterogeneous nuclear ribonucleoprotien R	Isoform 3
	Heterogeneous nuclear ribonucleoprotien R	Isoform 4
4	Heterogeneous nuclear ribonucleoprotein U-like protein 2	
5	RNA-binding protein Raly	Isoform 1
6	RNA-binding protein Fox-1 homolog 3	
7	G protein-coupled receptor-associated sorting protein 1	
8	KH domain-containing, RNA binding, signal transduction-associated protein 1	
9	Extracellular matrix protein 1	Isoform 2
10	Amino-terminal enhancer of split	Isoform a
11	Thioredoxin domain-containing protein 11	

(Fig. 4*D*). Knockdown of ACF attenuated FL-A1-mediated apoB-48 production but did not affect the FL-A1-mediated increase in *IL8* mRNA expression (Fig. 4, *E* and *F*). In this experiment, knockdown of ACF did not affect the *FL-A1* mRNA level, and overexpression of FL-A1 did not affect *ACF* mRNA expression (Fig. 4*G*). These results suggest that, unlike *APOB* editing, the APOBEC1-mediated increase in *IL8* mRNA expression does not require ACF.

Next, a yeast two-hybrid analysis was performed to identify possible other complementing proteins that promote binding of APOBEC1 to *IL8* mRNA. In this experiment, a human brain cDNA library was used as prey, and APOBEC1 was used as bait. Of the 21 colonies isolated, five encoded hnRNPR isoform 4, three encoded hnRNPQ isoform 2, three encoded KH domain-containing RNA-binding signal transduction-associated protein 1, and the remainder encoded 10 different proteins. Eleven different proteins were identified as potential binding partners of APOBEC1 (Table 1). Two isoforms of hnRNPQ (hnRNPQ2 and -4) and two isoforms of hnRNPR (hnRNPR3 and -4), were included in the list of candidates. Another candidate, RNA-binding protein Fox-1 homolog 3, was not expressed in HuH7.5 cells (data not shown).

Knockdown of hnRNPQ Reduces Binding of APOBEC1 to IL8 mRNA in HuH7.5 Cells—To determine whether any of the 11 candidate binding partners of APOBEC1 enhance or facilitate the interaction between APOBEC1 and *IL8* mRNA, we examined the effect of siRNA-mediated knockdown of each protein on co-IP of FL-A1 and *IL8* mRNA from HuH7.5 cell extracts. For two candidates (hnRNPQ and hnRNPR), siRNAs that were complementary to both isoforms identified as candidate binding partners of APOBEC1 were used. The siRNA targeting hnRNPQ (si-hnRNPQ) was the only siRNA that affected co-IP of FL-A1 and *IL8* mRNA (Fig. 5*A*) (data not shown). Transfection of HuH7.5 cells with si-hnRNPQ caused a 70% decrease in *hnRNPQ* mRNA levels (Fig. 5*B*) but did not affect FL-A1 protein expression (Fig. 5*C*). Furthermore, knockdown of hnRNPQ also reduced *IL8* mRNA levels slightly (Fig. 5*D*). These findings indicate that hnRNPQ contributes to the association between *IL8* mRNA and ectopically expressed FL-A1 in HuH7.5 cells.

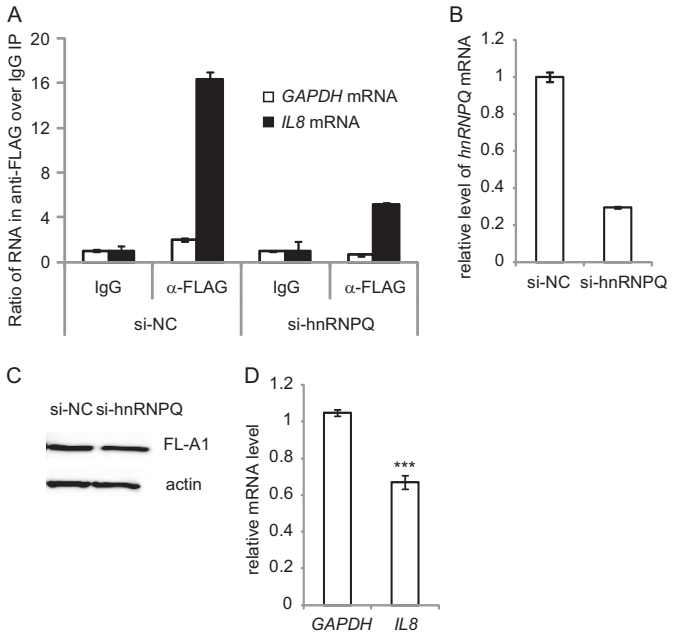
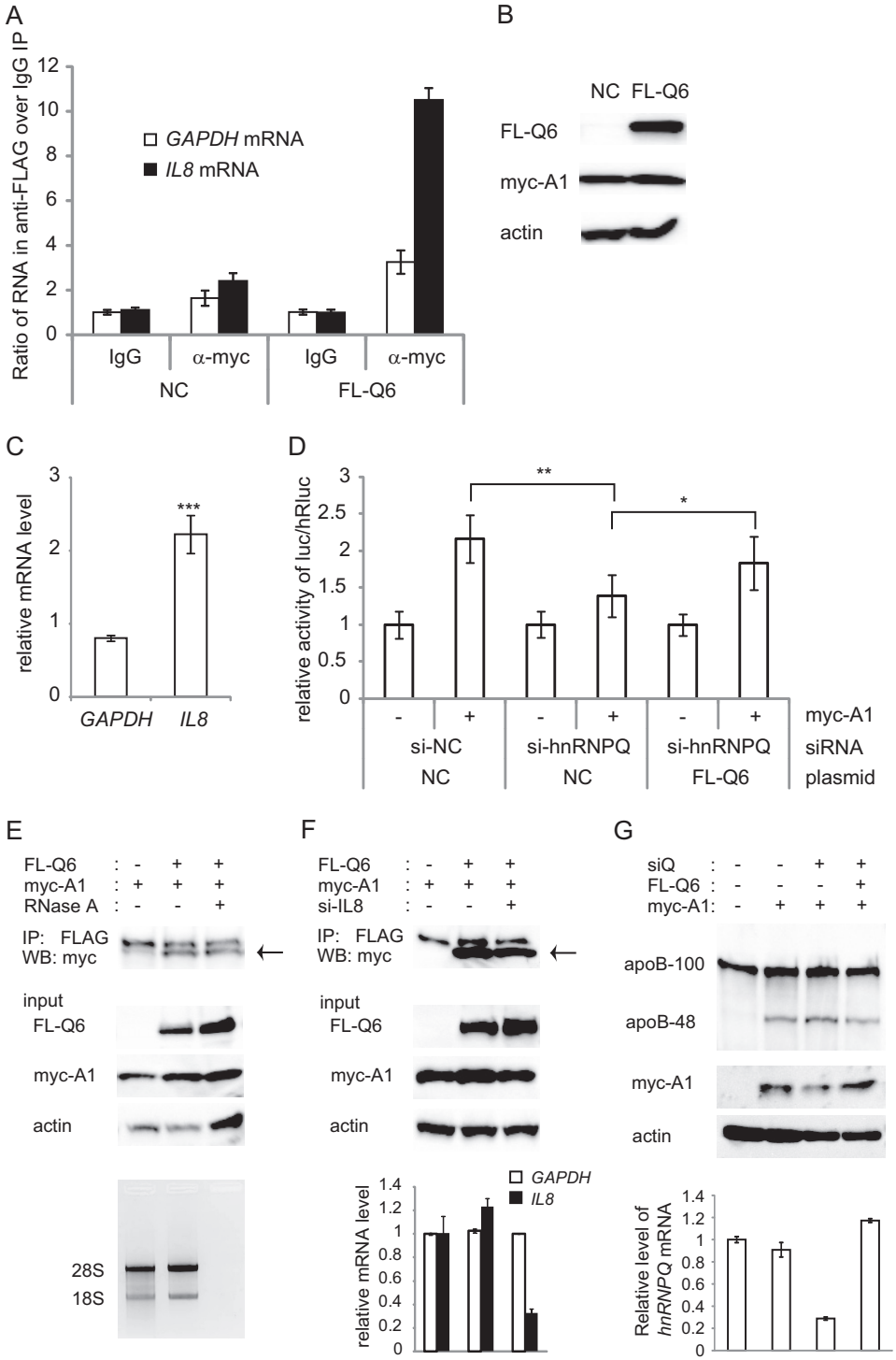


FIGURE 5. **Knockdown of hnRNPQ attenuates binding of APOBEC1 to *IL8* mRNA in HuH7.5 cells.** *A*, the decrease in binding of APOBEC1 to *IL8* mRNA following knockdown of hnRNPQ. HuH7.5 cells were reverse-transfected with a control (*si-NC*) or hnRNPQ-specific (*si-hnRNPQ*) siRNA (30 nM) and then transfected with the FL-A1 plasmid 16 h later. 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. Each immunoprecipitate was subjected to RNA extraction and RT-qPCR. 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. (*error bars*) of $n = 3$ replicate samples. *B*, RT-qPCR analyses of the effect of transfection of HuH7.5 cells with a hnRNPQ-specific siRNA on *hnRNPQ* mRNA expression. Knockdown efficiency was evaluated using one-tenth of the cells used in the IP experiments described in *A*. The expression level of *hnRNPQ* mRNA was normalized to that of *GAPDH*. Data are represented as the mean ± S.D. of $n = 3$ replicate samples. C, immunoblot analyses of FL-A1 and actin (control) in a portion of the lysates used in the IP experiments described in *A*. *D*, RT-qPCR analyses of *IL8* and *GAPDH* (control) mRNA levels in the cells used in the IP experiments described in *A*. The relative mRNA levels in cells treated with si-hnRNPQ compared with those treated with si-NC are shown. Data are represented as the mean ± S.D. of $n = 3$ replicate samples. ***, $p < 0.005$ by Student's *t* test.

The Association between APOBEC1 and IL8 mRNA Is Promoted by hnRNPQ6—As mentioned above, an interaction between APOBEC1 and *IL8* mRNA was not observed in HEK293 cells; therefore, we investigated whether overexpression of hnRNPQ in this cell line could lead to the promotion of this association. As mentioned above, of the seven hnRNPQ isoforms (hnRNPQ1–7), two (hnRNPQ2 and hnRNPQ4) were isolated in the yeast two-hybrid screen (Table 1). Initially, the effects of overexpression of FLAG-tagged hnRNPQ1 (the canonical form), FLAG-tagged hnRNPQ2, or FLAG-tagged hnRNPQ4 on the interaction between Myc-tagged APOBEC1 (Myc-A1) and *IL8* mRNA in HEK293 cells were examined. However, overexpression of these hnRNPQ isoforms did not facilitate this association (data not shown). Similarly, overexpression of FLAG-tagged hnRNPQ3 or FLAG-tagged hnRNPQ7 did not promote an interaction between APOBEC1 and *IL8* mRNA (data not shown). However, overexpression of FLAG-tagged hnRNPQ6 (FL-Q6) led to an association between ectopically expressed Myc-A1 and *IL8* mRNA in HEK293 cells (Fig. 6*A*). Overexpression of FL-Q6 did not affect Myc-A1 pro-

tein expression (Fig. 6*B*) but did enhance *IL8* mRNA expression (Fig. 6*C*). As mentioned above, TNFα-mediated up-regulation of *IL8* expression did not affect co-IP of Myc-A1 and *IL8* mRNA from HEK293 extracts; therefore, elevated *IL8* expression alone could not explain the FL-Q6-mediated association of Myc-A1 with *IL8* mRNA in this cell line. Instead, overexpression of FL-Q6 in HEK293 cells may have promoted or stabilized the association between Myc-A1 and *IL8* mRNA, thereby increasing the stability of the *IL8* mRNA. In support of this theory, siRNA-mediated knockdown of hnRNPQ in HuH7.5 cells attenuated the Myc-A1-dependent increase in the activity of the pLuc-IL8 plasmid, which contained the full-length *IL8* 3′-UTR downstream of a luciferase coding sequence, and overexpression of exogenous FL-Q6 in the hnRNPQ-specific siRNA-treated cells recovered this defect (Fig. 6*D*). These results indicate that hnRNPQ was required for the FL-A1-dependent increase in *IL8* mRNA expression and that hnRNPQ6 led APOBEC1 to stabilize the *IL8* mRNA by binding to its 3′-UTR.

A protein-protein co-IP experiment demonstrated an association between Myc-A1 and FL-Q6 in HEK293 cells (Fig. 6, *E*



and *F*). This association was not disrupted by RNase treatment (Fig. 6*E*). Moreover, *IL8* mRNA depletion by IL8-specific siRNA did not affect their association (Fig. 6*F*), suggesting that the association between APOBEC1 and hnRNPQ6 was independent of *IL8* mRNA. Taken together, these findings are consistent with the hypothesis that the association between APOBEC1 and hnRNPQ6 promotes binding of APOBEC1 to *IL8* mRNA.

As mentioned above, transient expression of APOBEC1 led to the production of apoB-48 in HuH7.5 cells (Fig. 2*B*). Neither knockdown nor knockdown and subsequent rescue of hnRNPQ6 affected APOBEC1-mediated apoB-48 production in HuH7.5 cells (Fig. 6*G*), suggesting that hnRNPQ6 may not be involved in *APOB* mRNA editing.

Endogenous Expression of hnRNPQ6 Enables APOBEC1 to Increase IL8 Production—The finding that hnRNPQ6 is a complementing protein that facilitates the association between APOBEC1 and *IL8* mRNA led us to examine whether hnRNPQ6 expression levels were positively correlated with the amount of FL-A1/*IL8* mRNA in co-immunoprecipitates prepared from HuH7.5 and HEK293 cell extracts. Because of their sequence similarities, it is difficult to distinguish between the mRNAs encoding the different hnRNPQ isoforms with a single round of qPCR; therefore, two rounds of PCR were performed. For the first round, primers that would amplify *hnRNPQ3*, *hnRNPQ5*, and *hnRNPQ6* (expected sizes: 1584, 1686, and 1686 bp, respectively) were used. A second round of PCR was performed to amplify *hnRNPQ5* and *hnRNPQ6* (expected size: 704 bp) and distinguish these isoforms from *hnRNPQ3*. None of the hnRNPQ isoforms examined were detected in HEK293 cells (Fig. 7*A*). By contrast, cloning and sequence validation of the second-round PCR products from HuH7.5 cells showed that both *hnRNPQ5* and *hnRNPQ6* were expressed in this cell line and that they were expressed at a 1:15 ratio of *hnRNPQ5* to *hnRNPQ6*. These results suggest that hnRNPQ6 is the authentic complementing protein that mediates the association between APOBEC1 and *IL8* mRNA. Moreover, the lower level of association between FL-A1 and *IL8* mRNA observed in HEK293 cells may be due to the lack of hnRNPQ6 in this cell line.

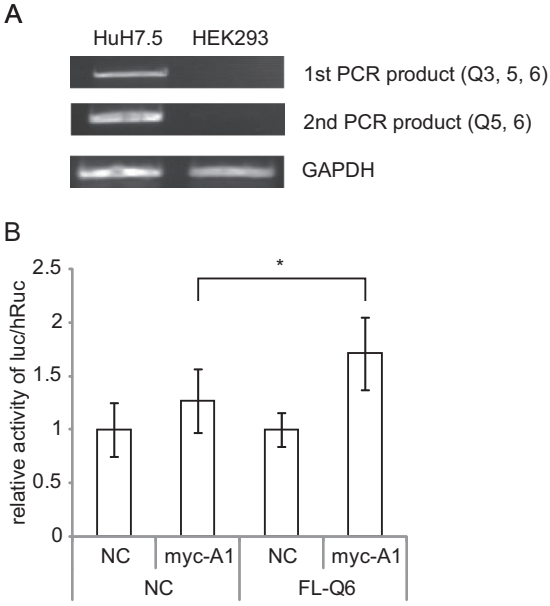


FIGURE 7. **The complementing activity of hnRNPQ6 enables APOBEC1 to regulate IL8 production in HEK293 cells.** *A*, PCR analyses of the expression levels of *hnRNPQ* and *GAPDH* (control) mRNAs in HuH7.5 and HEK293 cells. The first round of PCR was performed using primers designed to amplify *hnRNPQ3*, *hnRNPQ5*, and *hnRNPQ6*; the second round of PCR was performed using primers specific to *hnRNPQ5* and *hnRNPQ6* only. *B*, the effects of overexpression of APOBEC1 (*myc-A1*) and hnRNPQ6 (*FL-Q6*) on the activity of the pLuc-IL8 reporter in HEK293 cells. The cells were reverse-transfected with pLuc-IL8, empty plasmid (pCAG-Myc; *NC*), or a Myc-A1-expressing plasmid and empty plasmid (pcDNA3; *NC*) or an FL-Q6-expressing plasmid. Luciferase activity was examined 48 h after transfection. The activity of the pLuc-IL8 construct was normalized to that of the control pGL4.74[hRluc/TK] construct and the level in the empty plasmid (pCAG-Myc)-transfected cells. Data are represented as the mean ± S.D. of $n = 4$ independent experiments. *, $p < 0.05$ by Student's *t* test.

FIGURE 6. **Overexpression of hnRNPQ6 promotes binding of APOBEC1 to *IL8* mRNA in HEK293 cells.** *A*, the increase in binding of APOBEC1 (*myc-A1*) to *IL8* mRNA following overexpression of FLAG-tagged hnRNPQ6 in HEK293 cells. The cells were co-transfected with the Myc-A1 plasmid and empty plasmid (*NC*) or FL-Q6 plasmid 16 h after seeding and then 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-Myc antibody. Each immunoprecipitate was subjected to RNA extraction and RT-qPCR. 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. (*error bars*) of $n = 3$ replicate samples. *B*, immunoblot analyses of FL-Q6, Myc-A1 and actin (control) in lysates prepared from the cells described in *A*. *C*, RT-qPCR analyses of *IL8* and *GAPDH* (control) mRNA levels in the cells described in *A*. The relative mRNA levels in cells expressing FL-Q6 compared with those in control (*NC*) cells are shown. Data are represented as the mean ± S.D. of $n = 3$ replicate samples. ***, $p < 0.005$ by Student's *t* tests. *D*, the effects of knockdown and rescue of hnRNPQ6 expression on the activity of the pLuc-IL8 reporter plasmid. HuH7.5 cells were reverse-transfected with pLuc-IL8, a Myc-A1-expressing (+) or empty control (−) plasmid, a negative control (si-NC) or hnRNPQ-specific (si-hnRNPQ) siRNA (30 nM), and an FL-Q6-expressing or empty control (*NC*) plasmid. Luciferase activity was examined 48 h after transfection. The activity of the pLuc-IL8 construct was normalized to that of the control pGL4.74[hRluc/TK] construct and the level of the empty plasmid (−)-transfected cells. *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test. Data are represented as the mean ± S.D. of $n = 4$ independent experiments. *E*, IP and subsequent immunoblot analyses (*WB*) of HEK293 cells that were co-transfected with a Myc-A1-expressing plasmid and empty plasmid (pcDNA3-FLAG) or an FL-Q6-expressing plasmid. The cells were seeded 16 h prior to transfection, harvested 48 h after transfection, and then lysed in RIPA buffer. The cell lysates were incubated with or without RNase A (100 μg/ml) at room temperature for 10 min, immunoprecipitated with anti-FLAG antibody, and then probed with an anti-Myc antibody. The *arrow* indicates Myc-A1. A portion of each lysate prepared for IP was used as the input for immunoblot analyses of Myc-A1, FL-Q6, and actin (control) levels. RNA was also extracted from each lysate and separated by agarose gel electrophoresis with ethidium bromide to examine the RNA status by RNase treatment. *F*, the effects of *IL8* mRNA depletion on the association between APOBEC1 and hnRNPQ6. HEK293 cells were reverse-transfected with a control or IL8-specific (*si-IL8*) siRNA (30 nM) and then transfected with empty plasmid (pcDNA3-FLAG) or an FL-Q6-expressing plasmid and Myc-A1-expressing plasmid 16 h later. The cells were harvested 48 h after transfection and lysed in RIPA buffer. The cell lysates were immunoprecipitated with anti-FLAG antibody and then probed with an anti-Myc antibody. The *arrow* indicates Myc-A1. A portion of each lysate prepared for IP was used as the input for immunoblot analyses of Myc-A1, FL-Q6, and actin (control) levels. RNA was also extracted from each lysate to examine the expression level of *IL8* mRNA and *GAPDH* mRNA (control). Data are represented as the mean ± S.D. of $n = 3$ replicate samples. *G*, the effect of knockdown and subsequent rescue of hnRNPQ expression on *APOB* editing by APOBEC1. HuH7.5 cells were reverse-transfected with a control or hnRNPQ-specific (si-Q) siRNA (30 nM) and then co-transfected with the indicated combinations of empty plasmid (pCAG-Myc or pcDNA3-FLAG), FL-Q6-expressing plasmid, or Myc-A1-expressing plasmid 16 h later. The culture medium and cells were harvested 48 h after transfection and used for immunoblot analyses of apoB-100 and apoB-48 (culture medium), and Myc-A1 and actin (cells). RNA was extracted from a portion of the cells and subjected to RT-qPCR analyses of *hnRNPQ* mRNA expression. The *hnRNPQ* mRNA level was normalized to that of *GAPDH*. Data are represented as the mean ± S.D. of $n = 3$ replicate samples.

Overexpression of Myc-A1 alone did not affect the activity of the pLuc-IL8 reporter in HEK293 cells, but co-expression of FL-Q6 and Myc-A1 increased its activity significantly (Fig. 7*B*). Moreover, in HuH7.5 cells, which have much higher expression levels of hnRNPQ6 than HEK293 cells (Fig. 7*A*), knockdown of hnRNPQ attenuated the Myc-A1-mediated up-regulation of pLuc-IL8 activity, and rescue of hnRNPQ6 expression in the knockdown cells restored this defect (Fig. 6*D*).

APOBEC1 Facilitates the Association between hnRNPQ6 and IL8 mRNA—The results described above suggested that an association between hnRNPQ6 and APOBEC1 enables APOBEC1 to up-regulate IL8 production by binding to and stabilizing *IL8* mRNA. To confirm the involvement of hnRNPQ6 in the association between APOBEC1 and *IL8* mRNA further, an anti-FLAG antibody (or IgG as a control) was used to immunoprecipitate FL-Q6 and its associated RNAs from extracts of HEK293 cells overexpressing FL-Q6 alone or FL-Q6 and Myc-A1. The immunoprecipitates were subjected to RNA extraction and subsequent RT-qPCR analyses. In the absence of Myc-A1, the anti-FLAG immunoprecipitates contained slightly more *IL8* mRNA than the IgG immunoprecipitates. By contrast, in the presence of Myc-A1, the amount of *IL8* mRNA in the anti-FLAG immunoprecipitates was 30-fold higher than that in the IgG immunoprecipitates (Fig. 8*A*), suggesting that APOBEC1 enhances the efficiency of the interaction between hnRNPQ6 and *IL8* mRNA. Another possibility is that APOBEC1 serves as a chaperone that stabilizes hnRNPQ6 and is obligatory for its folding and ability to bind to *IL8* mRNA. The expression level of FL-Q6 was unaffected by overexpression of Myc-A1 (Fig. 8*B*). The *IL8* mRNA level was 2.7-fold higher in HEK293 cells that expressed both Myc-A1 and FL-Q6 than in those that expressed FL-Q6 only (Fig. 8*C*); this up-regulation may be due to binding of Myc-A1 to *IL8* mRNA. The results of analyses of the pLuc-IL8 reporter activity in HEK293 cells were concordant with the ability of Myc-A1 to bind to *IL8* mRNA in the presence of FL-Q6 (Fig. 7*B*). Therefore, FL-Q6 may be required for the association between Myc-A1 and *IL8* mRNA in these assays. These findings indicate that hnRNPQ6 may be essential to the APOBEC1/*IL8* mRNA association in some cell types.

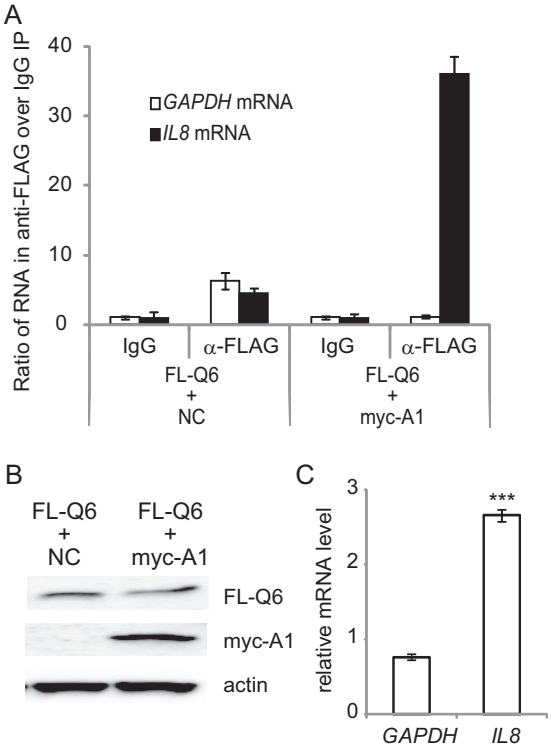


FIGURE 8. **APOBEC1 promotes the interaction of hnRNPQ6 with *IL8* mRNA, and the tripartite association leads to an increase in *IL8* mRNA expression.** *A*, associations between *IL8* mRNA and FL-Q6 in HEK293 cells overexpressing Myc-A1. The cells were seeded 16 h prior to transfection, co-transfected with FL-Q6 and empty plasmid (NC) or a Myc-A1-expressing plasmid, and then 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. 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. *B*, immunoblot analyses of FL-Q6, Myc-A1, and actin (control) in a portion of the lysates prepared for the IP experiments described in *A*. *C*, RT-qPCR analyses of *IL8* and *GAPDH* (control) mRNA levels in the cells used in the experiment described in *A*. The mRNA levels in the cells expressing both Myc-A1 and FL-Q6 were normalized to those in the cells expressing FL-Q6 only. Data are represented as the mean ± S.D. of $n = 3$ replicate samples. ***, $p < 0.005$ by Student's *t* test.

DISCUSSION

Here, APOBEC1 was associated preferentially with *IL8* mRNA in HuH7.5 cells, and this association was independent of ACF and dependent on hnRNPQ6. Knockdown and overexpression experiments performed in cells demonstrated that APOBEC1 requires hnRNPQ6 to bind to *IL8* mRNA (Figs. 5*A* and 6*A*). There was also a correlation between the APOBEC1/*IL8* mRNA association and *IL8* mRNA level (Figs. 5*D* and 6*C*). The increase in IL8 expression in APOBEC1-expressing HuH7.5 cells was not attributable to transcriptional up-regulation of *IL8* mRNA (data not shown); rather, this increase probably resulted from an extended life span of the *IL8* mRNA caused by binding of APOBEC1/hnRNPQ6. Furthermore, the increased IL8 expression was independent of APOBEC1-mediated RNA editing but required a cluster of AREs in the 3′-UTR of the *IL8* mRNA. Therefore, association with hnRNPQ6/APOBEC1 might affect ARE-mediated decay of *IL8* mRNA. The extended *IL8* mRNA half-life may have allowed for augmented translational elongation or may have increased translational efficiency of *IL8* via an unknown mechanism.

More than 90% of the top 68 RNAs identified in the microarray analysis were mRNAs with at least one AUUUA (ARE) element in their 3′-UTR, indicating that APOBEC1 can bind to various mRNAs that harbor an ARE in this region. In addition, one noncoding RNA (LOC100289230) and two processed pseudogene transcripts (RP11-424A16.1 and AL354718.10) were identified as APOBEC1 binding partners (supplemental Table S1); therefore, APOBEC1 might bind to other types of RNAs in addition to mRNAs.

The microarray analyses identified *CXCL1*, *CXCL2*, *CXCL5*, and *CXCL6* as potential binding targets of APOBEC1 (supplemental Table S1). These mRNAs encode members of the CXC chemokine family, which includes IL8. CXCL1, CXCL2, and IL8 are members of the growth-related oncogene family that contributes to immune infiltration and tumor growth. Overexpression of APOBEC1 in HuH7.5 cells increased the *CXCL2*

and *IL8* mRNA levels but did not affect the *CXCL1*, *CXCL5*, or *CXCL6* mRNA levels (Fig. 2*A*) (data not shown). This result suggests that binding of APOBEC1 does not always result in an increase in the expression level of a target mRNA. APOBEC1 edits the 3′-UTRs of mRNAs, suggesting that it introduces or abolishes target sequences for microRNAs to enable the modification of post-transcriptional processes, including translational efficiency (21). Although we did not identify an APOBEC1-dependent editing event at a specific site in the *IL8* mRNA, it would be worthwhile analyzing other APOBEC1-bound RNAs for editing events.

The yeast two-hybrid analysis revealed that both hnRNPQ2 and hnRNPQ4 can associate with APOBEC1 (Table 1). However, interaction with these proteins was not required for binding of APOBEC1 to *IL8* mRNA. Notably, hnRNPQ1 (also known as GRY-RBP or SYNCRIP) (22) and hnRNPQ6 associate with APOBEC1 to regulate APOBEC1-mediated *APOB* mRNA editing and IL8 production, respectively. Therefore, hnRNPQ2, hnRNP4, or both may be involved in other APOBEC1-dependent functions. The other hnRNPQ isoforms have at least one domain required for association with APOBEC1, namely an N-terminal acidic domain (23) or C-terminal domain (24); therefore, any of these isoforms may associate with APOBEC1 to regulate its function.

In the case of *APOB* mRNA editing, APOBEC1 has various binding partners that act as a complementing factor (ACF) or regulator (such as APOBEC1-binding proteins 1 and 2, hnRNPC1, CUGBP2, Bcl2-associated athanogene-4, and hnRNPQ1) (5, 6, 22, 25–28). Under certain conditions, recombinant APOBEC1 binds directly to the synthetic 3′-UTRs of the *IL8* and *MYC* mRNAs (18). However, the level of binding of APOBEC1 to *IL8* in HuH7.5 cells was higher than the level of binding to *MYC*, although *MYC* is expressed at a relatively high level in these cells (Fig. 1, *A* and *B*). This finding may indicate that hnRNPQ6 does not function as a positive complementary factor that promotes the association of APOBEC1 and *MYC* mRNA. Alternatively, an inhibitory factor may inhibit binding of APOBEC1 to *MYC* mRNA even in the presence of hnRNPQ6. HuH7.5, HuH6, HuS, and PH5CH cells express hnRNPQ6 at comparable levels (data not shown). The HuH6, Hus, and PH5CH cell lines have higher *IL8* mRNA levels than HuH7.5 cells (data not shown); however, the level of binding of APOBEC1 to *IL8* mRNA was highest in HuH7.5 cells (supplemental Table S1 and Fig. 1*C*). These observations indicate that other factors may regulate the association between APOBEC1 and *IL8* mRNA in a cell type-dependent manner.

APOBEC1 mRNA expression is evident in several types of human carcinoma, but an mRNA editing event indicative of its expression is not always obvious (8). Therefore, APOBEC1 may promote carcinogenesis through functions other than RNA editing. In particular, APOBEC1-mediated increases in IL8 production may play a role in tumorigenesis and DNA damage-related pathogenesis.

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