# Mutational analysis of apolipoprotein B mRNA editing enzyme (APOBEC1): structure–function relationships of RNA editing and dimerization

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**Abstract** **APOBEC1 is the catalytic subunit of an enzyme complex that mediates apolipoprotein (apo) B mRNA editing. It dimerizes in vitro and requires complementation factor(s) for its editing activity. We have performed a systematic analysis of the structure–functional relationship of APOBEC1 by targeted mutagenesis of various sequence motifs within the protein. Using in vitro RNA editing assay, we found that basic amino acid clusters at the amino-terminal region $R^{15}R^{16}R^{17}$ and $R^{33}K^{34}$, are essential for apoB mRNA editing. Mutation of $R^{15}R^{16}R^{17}$ to $K^{15}K^{16}K^{17}$ and mutation of $R^{33}K^{34}$ simultaneously to $A^{33}A^{34}$ almost completely abolished in vitro editing activity. The carboxy-terminal region of APOBEC1 contains a leucine-rich motif. Deletion analysis of this region indicates that residues 181 to 210 are important for in vitro apoB mRNA editing. Single amino acid substitutions demonstrate that $L^{182}$, $I^{185}$, and $L^{189}$ are important residues required for normal editing function. Furthermore, the double mutant P190A/P191A also lost >90% of editing activity which suggests that a β turn in this region of the molecule may be essential for proper functioning of APOBEC1. It was suggested that dimerization of APOBEC1 creates an active structure for deamination of apoB mRNA. When we examined the dimerization potential of truncated APOBEC1s using both amino and carboxy termini deletion mutants, we found that amino-terminal deletions up to residue $A^{117}$ did not impair dimerization activity whereas carboxy-terminal deletions showed diminished dimerization. The systematic and extensive mutagenesis experiments in this study provide information on the role of various sequence motifs identified in APOBEC1 in enzyme catalysis and dimerization.—**Teng, B-B., S. Ochsner, Q. Zhang, K. V. Soman, P. P. Lau, and L. Chan. **Mutational analysis of apolipoprotein B mRNA editing enzyme (APOBEC1): structure–function relationships of RNA editing and dimerization.** *J. Lipid Res.* **1999.** 40: **623–635.**

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RNA editing is a post-transcriptional process whereby a transcript is modified in its coding function. It encompasses a variety of modifications including insertion, deletion, substitution, or modification of nucleotides in mRNA transcripts (1, 2). The mechanism involved in each type of RNA editing is distinct, and it leads to a change in the information content of the mRNA. Apolipoprotein (apo) B mRNA editing consists of a C to U modification in the first base of a CAA codon. This modification in apoB-100 mRNA changes the glutamine codon at residue 2153 to a UAA stop codon. Translation of the edited mRNA produces a truncated form of apoB, called apoB-48, which is colinear with the N-terminal 48% of apoB-100 (3, 4). In humans, apoB-48 is synthesized in the small intestine and is an important component for the assembly and secretion of triglyceride-rich chylomicrons in response to a fatty meal. Translation of the unedited apoB mRNA in the liver produces apoB-100, which is an essential component of very low density, intermediate density, and low density lipoproteins (5, 6).

ApoB mRNA editing is mediated by an enzyme complex for which the catalytic component has been identified. This component has been named APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1) (7, 8). It is a cytidine deaminase-like enzyme (9, 10) that requires complementation factor(s) for catalytic activity (7, 11–18). Adenovirus-mediated gene transfer of APOBEC1 cDNA in mouse (19, 20) and rabbit liver (21–23) showed that APOBEC1 confers apoB mRNA editing activity to the liver in vivo. APOBEC1 is a 27 kDa protein that is widely distributed in various tissues in mice and rats

Abbreviations: apoB, apolipoprotein B; APOBEC1, apolipoprotein B mRNA editing enzyme, catalytic polypeptide #1; CAT, chloramphenicol acetyltransferase; HA-1, *Haemophilus influenza* hemagglutinin; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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(7, 24, 25), but is expressed only in the small intestine in humans (26, 27) and rabbits (16). APOBEC1 dimerizes in vitro (27), and binds to apoB mRNA nonspecifically in vitro (28–30). The complementation factors required for apoB mRNA editing appear to be present in tissue extracts from a wide variety of sources (7, 11, 15–18, 31). Presumably, complementation factors interact with both APOBEC1 and apoB mRNA to facilitate enzyme catalysis, as well as to confer additional sequence specificity to the editing process.

Using in vitro transcription and translation coupled system to synthesize APOBEC1, here we have performed a systematic analysis of APOBEC1 mutants to examine the major sequence motifs that may be important for the function of APOBEC1 as an apoB mRNA editing enzyme. We show that both the amino terminal domain, rich in basic amino acids, and the carboxy terminal region, containing the leucine-rich motif, are essential for apoB mRNA editing. We have also identified the region important for dimerization.

## METHODS

### Site-directed mutagenesis of rat small intestine apoB mRNA editing enzyme (APOBEC1)

Full-length rat APOBEC1 cDNA was cloned into pAlter-1 vector which contains multiple cloning sites flanked by SP6 and T7 RNA polymerase promoters (Promega, WI). Point mutations were inserted by using an Altered Sites *In Vitro* Mutagenesis System (Promega, WI). Briefly, a 20-base oligonucleotide with the mismatch located in the center and an ampicillin-repaired oligonucleotide were annealed to the template. In vivo host-directed mismatch repair mechanism was used to select the mutant. The efficiency of obtaining correct mutation was >90%. Each mutant was identified by sequencing. All mutant constructs were sequenced to confirm the mutated residue and to confirm the correct ATG site. Subsequently, we subcloned each mutant into pSP64 Poly(A) vector (Promega, WI) to obtain high-level protein expression by using TNT-coupled reticulocyte lysate system (Promega, WI).

### Deletion constructs and HA-tagged constructs of APOBEC1

APOBEC1 mutants of both amino and carboxy termini deletions were produced by polymerase chain reaction (PCR). Briefly, the carboxy-terminal deletion constructs were engineered as followed: pSP64 Poly(A) vector containing the wild-type rat APOBEC1 cDNA was amplified using forward primer BBTF and reverse primers, BBTP168, BBTE181, BBTL196, BBTL210, and BBTH221 at indicated residues, $P^{168}$, $E^{181}$, $L^{196}$, $L^{210}$, and $H^{221}$, respectively, followed by a translational stop codon (UGA). The PCR products were cloned into pSP64 Poly(A) vector designated pP168, pE181, pL196, pL210, and pH221. The amino-terminal deletion constructs were amplified using forward primers, BBTL14, BBTR33, BBTE35, BBTP78, and BBTA117 which started at indicated residues, $L^{14}$, $R^{33}$, $E^{35}$, $P^{78}$, and $A^{117}$, respectively, and were preceded by a methionine residue as the first residue and a reverse primer BBTR tagged with a 9-amino acid *Haemophilus influenza* hemagglutinin epitope (HA-1) preceded by a 3-alanine spacer (32). The PCR products were also cloned into pSP64 Poly(A) vector designated pHA-L14, pHA-R33, pHA-E35, pHA-P78, and pHA-A117. Wild-type rat APOBEC1 with HA tag was constructed by PCR using forward primer BBTF and reverse primer BBTR. The PCR product was cloned into pSP64 Poly(A) vector designed pHA-APOBEC1. Both strands of each construct were sequenced to confirm the sequence.

### Expression of recombinant APOBEC1

Wild-type, deletion, and mutant APOBEC1s were expressed by using TNT-coupled reticulocyte lysate system, a coupled transcription-translation system (Promega, WI) (33). Briefly, 1 μg of plasmid DNA was used to translate the protein as recommended by the manufacturer. [$^{35}$S]methionine was used to estimate the total amount of protein translated by the system. The product was separated on 12% SDS-PAGE, detected by fluorography, and quantitated using PhosphoImager SF (Molecular Dynamics, CA). Equal amounts of protein, deletion, and mutant APOBEC1s and wild-type APOBEC1 (~1 μl TNT-wild-type APOBEC1 = 55095 ± 1984 phosphoimage counts per h) were used for each assay. To be certain the assay was in the linear range, we usually used 3 to 4 different amounts of protein to carry out in vitro editing assay.

### In vitro apoB mRNA editing assay

Synthetic apoB RNA was prepared from pRBF-CAA, a rat apoB cDNA fragment of 470 base-pairs (nucleotides 6512–6982) spanning the RNA editing region (15). The in vitro editing assay was carried out as described by Teng and Davidson (15), using 2 ng of synthetic RNA substrate in the presence of indicated amount of TNT-expressed protein and 5 μg of chicken enterocytes S-100 extract. The products after primer extension were fractionated on an 8% polyacrylamide-urea gel (National Diagnostics, GA) and radiolabeled bands of UAA (edited) and CAA (unedited) were quantitated by PhosphoImager SF.

### Dimerization of epitope-tagged APOBEC1 and non-tagged APOBEC1 in vitro

Dimerization of HA-tagged APOBEC1 and non-tagged APOBEC1 was carried out using two different methods described as follows. A: HA-tagged wild-type and mutant APOBEC1 plasmid vectors were transcribed and translated individually in vitro in the presence of [$^{35}$S]methionine using TNT-coupled reticulocyte system. The synthetic HA-tagged wild-type protein was then mixed with 1- to 5-fold excess of the mutant APOBEC1 protein. The mixtures were incubated at 37°C for 30 min to allow dimerization to occur (34). At the end of reaction, the mixture was immunoprecipitated against HA-1 specific antibody as described (27). B: In consideration that nascent protein chain may play a critical role in dimerization, co-translation of two proteins was carried out. In general, 0.1 μg of HA-tagged wild-type and 1 μg of mutant APOBEC1 plasmid vectors were co-translated in the presence of [$^{35}$S]methionine using TNT-coupled reticulocyte lysate system. The reaction was carried out at 30°C for 2 h. At the end of the reaction, 1 μl of reaction mixture was used for SDS-PAGE analysis to measure the amount of proteins synthesized and 10–20 μl of reaction mixture was used for immunoprecipitation. Immunoprecipitation was carried out as described (27). Pre-immunoprecipitated sample and sample control without addition of antibody were included in SDS-PAGE analysis. The gel was processed by fluorography and quantitated by PhosphoImager SF. In each assay, controls of untagged wild-type or untagged mutant proteins were carried out in the same manner. These controls were always included with each assay to make sure there were no aggregated proteins that bound nonspecifically and to make sure all the washing steps were complete.

### Protein–protein interaction; mammalian matchmaker two-hybrid system

Mammalian matchmaker two-hybrid system (Clontech Laboratories, Inc., CA) was used to examine protein–protein interac-

tion. Briefly, full-length rat APOBEC1 cDNA was cloned into pM vector, which generated a fusion protein of the GAL4-binding domain and APOBEC1. Full-length APOBEC1 with and without the HA tag, amino terminal deletion mutants, HA-R33, HA-E35, HA-P78, HA-A117, and carboxy terminal deletion mutants, P168, E181, L196, L210, and H221, were cloned into pVP16 vector, which generated a fusion protein of VP16 activation domain and the protein of interest. Each vector was sequenced and confirmed by using automated sequencing system (ABI 337, Perkin-Elmer, Norwalk, CT). Transfection experiment was performed using FuGene 6 (Boehringer Mannheim) with three plasmid vectors into COS cells; pM-APOBEC1 (0.2 μg), pVP16-APOBEC1 wild-type or pVP16-APOBEC1-deletion mutants (2 μg), and pG5CAT (0.4 μg). pG5CAT is a reporter vector which contains the CAT gene downstream of five consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene. At the end of experiment (~72 h), the interaction between two proteins was assayed by measuring CAT gene expression. CAT assay was carried out with a commercial kit, CAT ELISA (Boehringer Mannheim), a colorimetric enzyme immunoassay for the quantitative determination of chloramphenicol acetyltransferase (CAT).

### Oligonucleotides

The oligonucleotides used in this study are listed in **Table 1**. Oligonucleotides H61A, V62A, V64L, F66L, L180A, L182A, I185A, L187A, L189A, P190A, P191A, P190A/P191A, C192S, L193A, L196A, L196S, L196E, P201A, P201N, L203A, L210A, P219A, P220A, P219A/P220A, R15A, R15K/R16K/R17K, R33A, K34A, and R33A/K34A were used for point mutation. Oligonucleotides BBTF and BBTR were used to construct the HA-tagged APOBEC1. Oligonucleotides BBTP168, BBTE181, BBTL196, BBTL210, and BBTH221 were used to engineer 3 carboxyl terminal deletion constructs. Oligonucleotides BBTL14, BBTR33, BBTE35, BBTP78, and BBTA117 were used to engineer amino terminal deletion constructs.

### Secondary structure predictions

Two different methods, those of Chou and Fasman (35, 36) and of Garnier, Osguthorpe, and Robson (37) and Garnier, Gibrat, and Robson (38), referred to as the Chou-Fasman, and GOR methods, respectively, were used to predict secondary structures from amino acid sequences. Both methods make use of conformational parameters for amino acid residues derived from a data base of protein crystal structures and the final outputs are predictions for each residue of the chain. The conformational parameters (denoted in the Chou-Fasman method as $P_\alpha$, $P_\beta$, and $P_t$) are essentially the frequencies of occurrence of a given residue in helices, sheets, and turns, respectively. They are a measure of residue preferences to occur in α helices, β sheets, and turns.

The widely used Chou-Fasman algorithm uses a set of empirically derived rules to predict secondary structures. The procedures for helix and sheet prediction are similar, and the principle is briefly explained below. The protein sequence is scanned starting from the amino-terminus in overlapping, multi-residue, and reading frame until a helix/sheet nucleation site is found. This is then extended in both directions until tetrapeptide breakers are encountered. The criteria for nucleation and continuation are defined empirically in terms of average $P_\alpha$ or $P_\beta$ values of the residues in the reading frame. Also, a helix should not contain any proline residues except at the first three positions. Turn predictions are handled in a somewhat different way from the above nucleation–extension approach, in order to take into account the positional preference of residues. Turn probability at a residue is calculated as the product of frequencies of occurrence (in the data base) of that residue at the four different positions of a turn ($f_i$, $f_{i+1}$, $f_{i+2}$, and $f_{i+3}$); predictions are then made based on these probabilities and the values of $P_t$. While these are the basic principles, there are a number of additional detailed rules to resolve overlaps of predicted regions according to Nishikawa (39) and Matsuo and Nishikawa (40) which improve and automate the predictions.

The GOR algorithm takes a more sophisticated approach to prediction in that it is based on information theory. However, it has the advantage of being programmed easily and objectively. The implementation of the secondary structure predictions we used is the program "Peptide Structure" (41) of GCG Sequence Analysis Package from Genetics Computer Group, version 8 from University of Wisconsin, Madison, WI. The calculations were run on the MBCR (Molecular Biology Computing Resources) computer at Baylor College of Medicine.

## RESULTS

### Strategies for examining the structure–function relationship of rat APOBEC1

To systematically examine the structure–function relationship of rat APOBEC1, we used TNT-coupled transcription-translation reticulocyte lysate system to produce a biologically active recombinant APOBEC1 (16, 33). With this method, we would be able to test readily the activity of wild-type APOBEC1 and large numbers of mutant APOBEC1s using an in vitro RNA editing assay, and we would also be able to study the in vitro physical interaction (dimerization) of wild-type and mutant APOBEC1s easily. Protein–protein interaction of APOBEC1 was also evaluated quantitatively by mammalian two-hybrid system in COS cells.

As shown in **Fig. 1**, in vitro RNA editing assay was carried out with increased amount of [$^{35}$S]methionine-labeled wild-type APOBEC1 (1 μl = 55,095 phosphoimage counts/h) in the presence of 5 μg chicken enterocyte S-100 extract as complementation factors and synthetic rat apoB mRNA as template. It is evident that the in vitro synthesized APOBEC1 edited apoB RNA efficiently. Under our reaction condition, the assay was linear up to 55,095 phosphoimage counts/h of TNT wild-type-APOBEC1 which edited 47.5 ± 2.3% ($n = 4$, $r^2 = 0.995$) of apoB RNA; we performed all our in vitro editing experiments within the linear range of the assay. Additional amounts of APOBEC1 led to a gradual nonlinear increase of editing activity. This may be due to a required stoichiometry between complementation factors and APOBEC1 for maximal editing efficiency or the presence of nonspecific inhibitors in the reticulocyte lysate system that interfered with the reaction.

### Identification of regions in APOBEC1 required for apoB mRNA editing activity

*Deletion and point mutation analysis of amino-terminal region of APOBEC1*. The amino-terminal region of APOBEC1 contains a basic amino acid domain (residues $Arg^{15}Arg^{16}Arg^{17}$-$(X)_{15}$-$Arg^{33}Lys^{34}$) and the catalytic center (residues $His^{61}$, $Cys^{93}$, and $Cys^{96}$) which require Zn ion for catalytic activity. The putative catalytic active site of APOBEC1 has been studied by different laboratories using site-specific

TABLE 1. Oligonucleotides used

| Oligonucleotides | Sequence |
|---|---|
| Point Mutation | |
| H61A | AACACCAACAAA**GCC**GTTGAAGTCAAT |
| V62A | ACCAACAAACAC**GCC**GAAGTCAATTTC |
| V64L | AAACACGTTGAA**CTC**AATTTCATAG |
| F66L | GTTGAAGTCAAT**CTC**ATAGAAAAAT |
| L180A | AGGCTGTACGTA**GCG**GAACTCTACTGC |
| L182A | TACGTACTGGAA**GCT**TACTGCATCATT |
| I185A | GAACTCTACTGC**GCC**ATTTTAGGACTT |
| L187A | TACTGCATCATT**GCA**GGACTTCCACCA |
| L189A | ATCATTTTAGGA**GCT**CCACCCTGTTTA |
| P190A | ATTTTAGGACTT**GCA**CCCTGTTTAA |
| P191A | TTAGGACTTCCA**GCC**TGTTTAAATA |
| P190A/P191A | ATTTTAGGACTT**GCAGCC**TGTTTAAATATT |
| C192S | GGACTTCCACCC**TCT**TTAAATATTTTA |
| L193A | CTTCCACCCTGT**GCA**AATATTTTAAGA |
| L196A | TGTTTAAATATT**GCA**AGAAGAAAACAA |
| L196S | TGTTTAAATATT**TCA**AGAAGAAAACAA |
| L196E | TGTTTAAATATT**GAA**AGAAGAAAACAA |
| P201A | AGAAGAAAACAA**GCT**CAACTCACGTTT |
| P201N | AGAAGAAAACAA**AAT**CAACTCACGTTT |
| L203A | AAACAACCTCAA**GCC**ACGTTTTTCACG |
| L210A | TTCACGATTGCT**GCT**CAAAGCTGCCAT |
| P219A | TACCAAAGGCTA**GCA**CCCCACATCC |
| P220A | CAAAGGCTACCA**GCC**CACATCCTGT |
| P219A/P229A | TACCAAAGGCTA**GCTGC**ACACATCCTGTGG |
| R15A | GATCCCACTCTG**GCT**AGAAGAATTGAT |
| R15K/R16K/R17K | GTAGCTGTTGATCCCACTCTG**AAAAAGAAA**ATTGAG |
| | CCCCACGAGTTTGAA |
| R33A | CCCCGGGAACTT**GCT**AAAGAGACCTGT |
| K34A | CGGGAACTTCGG**GCA**GAGACCTGTCTG |
| R33A/K34A | CCCCGGGAACTT**GCTGCA**GAGACCTGTCTG |
| HA-tagged | |
| BBTF | CGC**AAGCTT**TCCGAGGAAGGAGTCCAGAGACA |
| BBTR | CC**GGATCC**TCAAGCGTAATCTGGAACATCGTATGGG |
| | TAGGCTGCGGCTTTCAACCCTGTGGCCCCACAGGAT |
| Deletion | |
| BBTP168 | CC**GGATCC**TCATGGCCAATGAGCT |
| BBTE181 | CC**GGATCC**TCATTCCAGTACGTAC |
| BBTL196 | CC**GGATCC**TCATAAAATATTTAAACAGGG |
| BBTL210 | CC**GGATCC**TCAAAGAGCAATCGTGAAAAA |
| BBTH221 | CC**GGATCC**TCAGTGGGGTGGTAGCCTTTG |
| BBTL14 | CGC**AAGCTT**ATGCTGAGGAGAAGAATTGAGCCC |
| BBTR33 | CGC**AAGCTT**ATGCGGAAAGAGACCTGTCTGCTG |
| BBTE35 | CGC**AAGCTT**ATGGAGACCTGTCTGCTGTATGAG |
| BBTP78 | CGC**AAGCTT**ATGCCAAACACCAGATGCTCCATT |
| BBTA117 | CGC**AAGCTT**ATGGCACGGCTTTATCACCACGCA |

Nucleotide positions refer to the published sequence for rat APOBEC1. All the oligonucleotides are shown 5′ to 3′ with restriction enzyme sites or mutated codons in bold and ATG and stop codon underlined.

mutagenesis (11, 16, 29, 30, 42). To explore whether other sequences besides the catalytic residues are required for editing, we constructed a series of amino terminal deletion mutants where residues 2–13, 2–32, 2–34, 2–77, and 2–116 were deleted. The constructs were designated pL14, pR33, pE35, pP78, and pA117, respectively; the first residue (after methionine) in each construct is identified by both the amino acid and its position in wild-type APOBEC1 (**Fig. 2A**). pR33 excluded part of the basic amino acid domain $R^{15}/R^{16}/R^{17}$, whereas pE35 excluded the entire basic amino acid domain. pP78 removed residues upstream of proline 78, including part of the catalytic site. pA117 removed all residues from 2 to 116, and the truncated protein was only half the size of wild-type APOBEC1. The deletion mutant proteins were synthesized from their respective DNA constructs by using the TNT transcription-translation system, a representative result of the proteins expressed is shown in Fig. 2B. A representative result of RNA editing assay using increased amount of specific radioactivity of amino-deletion mutant APOBEC1 is shown in Fig. 2C, and the results of the mean from three experiments are shown in **Table 2**. Deletion mutant L14 had essentially the same editing activity (95 ± 0.9%, n = 3) as that of wild-type APOBEC1. In contrast, deletion mutants R33 and E35 had completely lost their editing activity and, as expected, P78 and A117, which lack the catalytic domain partly or totally, also had no detectable editing activity. Therefore, the data indicate that the region encompassing residues 14 to 35 of APOBEC1 is essential for apoB RNA editing activity. As noted, when excess amount of inactive TNT-expressed protein was used for the assay, we observed a decrease amount of substrate

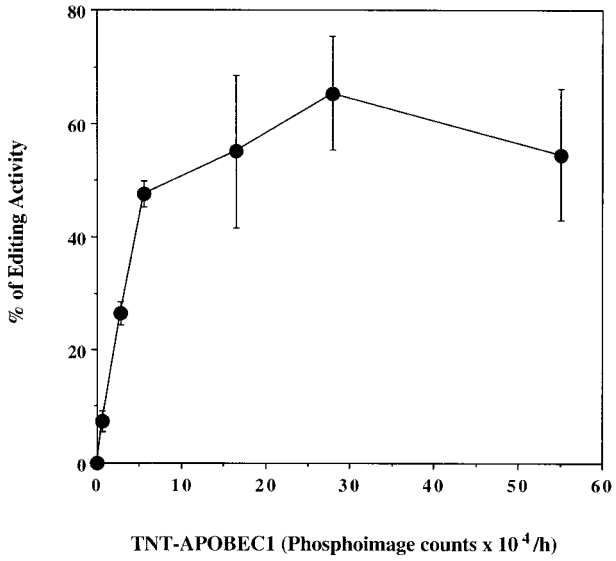


**Fig. 1.** In vitro RNA editing activity of TNT-expressed wild-type APOBEC1. Recombinant wild-type APOBEC1 was expressed using a TNT-coupled transcription–translation reticulocyte lysate system in the presence of [$^{35}$S]methionine. The translation product (1 μl) was separated on 12% SDS-PAGE, followed by fluorography and quantitated using PhosphoImager SF. The specific radioactivity is expressed as phosphoimage counts × $10^4$/h. The indicated amount (phosphoimage counts × $10^4$/h) of TNT expressed wild-type apobec1 was incubated with 5 μg of chicken enterocyte S-100 extract and 2 ng of synthetic rat apoB RNA for apoB RNA editing activity in vitro. The data of % editing activity were the mean of three or more assays, and the data are presented as mean ± standard deviation.

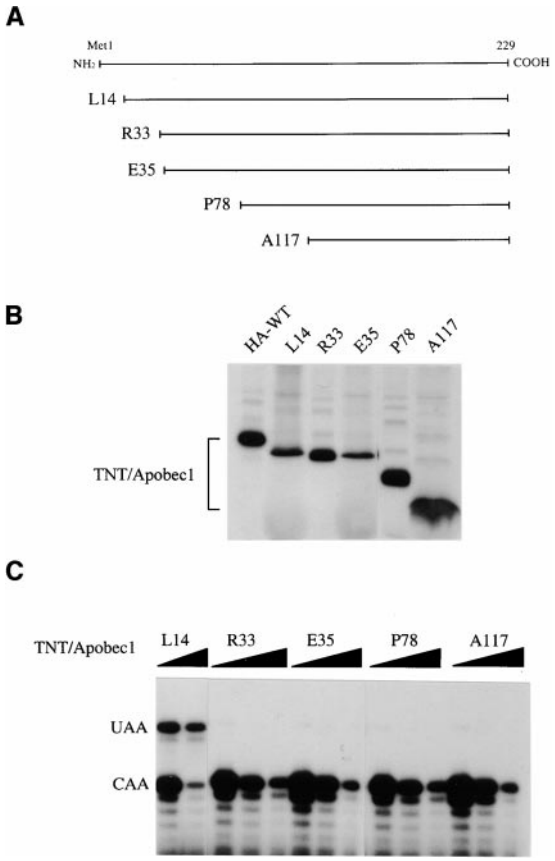


**Fig. 2.** In vitro editing activity of amino-terminal deletion mutants. A: A schematic diagram of amino terminal deletion constructs. Wild-type APOBEC1 containing 229 amino acids. Deletion mutant pL14 lacked residues 2–13. Deletion mutants pR33, pE35, pP78, and pA117 lacked residues 2–32, 2–34, 2–77, and 2–116, respectively. B: Wild-type and deletion mutant APOBEC1s were expressed using TNT-reticulocyte lysate system in the presence of [$^{35}$S]methionine. Five μl of translation product was separated on 12% SDS-PAGE, followed by fluorography and autoradiogram. A representative of the result is shown and TNT/APOBEC1 is marked. C: In vitro apoB RNA editing assay. A representative of RNA editing result is shown. In vitro editing assay was performed using increased phosphoimage counts per h (5510, 27550, and 165285 pch, respectively) of TNT-expressed deletion mutant proteins L14, R33, E35, P78, and A117 in the presence of 5 μg of chicken enterocyte S-100 extract and 2 ng of synthetic rat apoB RNA. The products were analyzed on 8% polyacrylamide urea gel. Radiolabeled bands of edited (UAA) and unedited (CAA) products are indicated.

RNA recovered (Fig. 2C). The reason for this observation is unclear.

Residues 14–35 contain the basic amino acid domain ($Arg^{15}Arg^{16}Arg^{17}$-$(X)_{15}$-$Arg^{33}Lys^{34}$). To examine whether this sequence motif is important for apoB RNA editing, we engineered point mutations in the basic amino acid domain, R15A, R33A, and K34A. We also examined the editing activity of a double mutant, R33A/K34A, and two triple mutants, R15K/R16K/R17K and R15A/R16A/R17A. The editing activities of wild-type and mutant APOBEC1s were assayed in vitro (Table 2). Single substitution mutants, R15A, R33A, and K34A had similar editing activity as wild-type APOBEC1 (98 ± 0.5%, n = 4; 97 ± 3.0%, n = 4; and 96 ± 1.4%, n = 4; respectively, compared to wild-type). In contrast to the single substitution mutant R15A which had the same editing activity as wild-type, the triple mutant APOBEC1 with all three arginine residues in the basic amino acid cluster substituted by lysine residues (R15K/R16K/R17K) lost over 90% of its editing activity (9.3 ± 1.2%, n = 4). Unexpectedly, we were not able to produce sufficient protein to perform editing assay for the triple mutant where all three arginine residues substituted to alanine (R15A/R16A/R17A). We speculate that the basic amino acid cluster substitution of Arg to Ala had significant impact on protein conformation and greatly impaired its translation efficiency. Interestingly, mutants R33A and K34A had essentially normal editing activity, but the double substitution mutant R33A/K34A, which had both positively charged residues replaced by a nonpolar residue alanine, had almost completely lost its editing activity (2.9 ± 0.1%, n = 4). Therefore, these results indicate that many of the conserved residues in the region of residues 14–35 are needed for efficient apoB mRNA editing. The two basic amino acid clusters ($R^{15}$, $R^{16}$, $R^{17}$) and ($R^{33}$, $K^{34}$) seem to play especially important structural roles in maintaining the necessary conformation for apoB mRNA editing.

We also generated point mutants (H61A, V62A, V64L, and F66L) around the catalytic domain and examined the editing activity of these mutants. These mutants were con-

TABLE 2. Summary of assay results

| | Editing Activity | Dimerization | CAT Activity |
|---|---|---|---|
| Wild-type APOBEC1 | 100% | 100% | 100% |
| 5′ Deletion | | | |
| L14 | 95 | 95 | – |
| R33 | 0 | 70 | 88 |
| E35 | 0 | 85 | 93 |
| P78 | 0 | 86 | 88 |
| A117 | 0 | 77 | 67 |
| 5′ Point mutants | | | |
| R15A | 98 | – | – |
| R15K/R16K/R17K | 9.3 | – | – |
| R33A | 97 | – | – |
| K34A | 96 | – | – |
| R33A/K34A | 2.9 | – | – |
| Catalytic domain | | | |
| H61A | 0 | – | – |
| V62A | 5.4 | – | – |
| V64L | 92 | – | – |
| F66L | 4.6 | – | – |
| 3′ Deletion | | | |
| P168 | 0 | 17 | 0 |
| E181 | 0 | 20 | 8 |
| L196 | 21 | 28 | 29 |
| L210 | 91 | 74 | 39 |
| H221 | 98 | 51 | 17 |
| 3′ Point mutants | | | |
| L180A | 82 | – | – |
| L182A | 47 | – | – |
| I185A | 6.0 | – | – |
| L187A | 101 | – | – |
| L189A | 2.0 | – | – |
| P190A | 98 | – | – |
| P191A | 96 | – | – |
| P190A/P191A | 7.0 | – | – |
| C192S | 128 | – | – |
| L193A | 98 | – | – |
| L196A | 98 | – | – |
| P201A | 105 | – | – |
| L203A | 102 | – | – |
| L210A | 96 | – | – |
| P219A | 117 | – | – |
| P220A | 115 | – | – |
| P219A/P220A | 94 | – | – |

The results of in vitro apoB RNA editing activity, dimerization assay by immunoprecipitation, and CAT activity by the mammalian two-hybrid system are shown for wild-type, deletion, and mutant APOBEC1 proteins. In vitro RNA editing activity is presented quantitatively as % of wild-type APOBEC1, and equal amounts of mutant and wild-type APOBEC1 were used (protein used = 27,550 phosphoimage counts per h). Dimerization is presented quantitatively as % of wild-type APOBEC1 dimerization. CAT activity is shown quantitatively as % of chloramphenicol acetyltransferase (CAT) activity for wild-type APOBEC1 dimerization. The data of editing activity, dimerization, and CAT activity were the mean of three or more assays; (–) represents sample not analyzed.

structed to be used as control to confirm the fidelity of in vitro TNT-generated APOBEC1 proteins. As shown in Table 2, mutant H61A was found to be completely inactive (0%, n = 3). $Val^{62}$ was conserved among APOBEC1s of all species reported (25), but this position was an alanine residue among the other members of the cytidine deaminase family (43). The mutant V62A was almost totally inactive (Table 2, 5.4 ± 0.1% of wild-type, n = 3). However, mutant V62A in rabbit APOBEC1 retained most of the editing activity (89% of wild-type) (16). $Val^{64}$, like $Val^{62}$, was also a conserved residue among all the species (25) but was not conserved between deaminases (43); mutant V64L had the same editing activity as wild-type APOBEC1 (92 ± 3.1%, n = 4). Single substitution of $Phe^{66}$ by Leu resulted in markedly decreased editing activity (to 4.6 ± 0.7%, n = 4). Therefore, except for mutant V62A, editing activity of TNT mutant proteins (H61A, V64L, and F66L) was comparable to that reported by others (11, 16, 29, 30).

*Deletion and point mutation analysis of the carboxy-terminal region of APOBEC1.* It was demonstrated that a truncated form of APOBEC1 lacking residues downstream from $P^{168}$ had completely lost its mRNA editing activity (7). This region of APOBEC1 contains two leucine-rich motifs ($L^{182}$ to $L^{203}$ and $L^{189}$ to $L^{210}$) and has been postulated to be involved in protein–protein interaction (7, 44). Here, we have constructed a series of carboxy-terminal deletion constructs in order to systematically further define the role of this region in apoB mRNA editing. Carboxy-terminal deletion constructs ending at residues $P^{168}$, $E^{181}$, $L^{196}$, $L^{210}$, and $H^{221}$ were constructed as shown in **Fig. 3A**; a representative result of the TNT expressed protein is shown in Fig. 3B. The editing activities of the truncated proteins were assayed in vitro (Fig. 3C and Table 2). As shown in Fig. 3C and Table 2, truncated APOBEC1s terminating at residues $P^{168}$ and $E^{181}$ were completely inactive. Extending the carboxy terminus to residue $L^{196}$ restored approximately 20% of the wild-type APOBEC1 activity (21 ± 3.2%, n = 4). Further increasing the length of the deletion construct APOBEC1 to residues $L^{210}$ and $H^{221}$ completely restored editing activity (91 ± 4.7%, n = 4 and 98 ± 3.1%, n = 4, respectively). Thus the minimal carboxy-terminal region required for apoB RNA editing resides between residues 181 and 210.

To examine specific amino acids within the carboxy-terminal region that may be important in apoB mRNA editing, we engineered the following point mutants: L180A, L182A, I185A, L187A, L189A, P190A, P191A, C192S, L193A, L196A, L196S, L196E, P201A, P201N, L203A, L210A, P219A, P220A, and two double mutants, P190A/P191A and P219A/P220A. Of these residues, $L^{180}$, $L^{182}$, $I^{185}$, $L^{187}$, $L^{189}$, $P^{190}$, $C^{192}$, and $L^{193}$ are consensus residues for a class of leucine-rich motif (16, 44). It is evident that single amino acid substitutions involving amino acids beyond $C^{192}$ did not reduce the editing activity of APOBEC1 (Table 2). In contrast, a number of mutations within the consensus leucine-rich motif spanning residues $L^{180}$ to $P^{191}$ resulted in significant loss of activity. In particular, point mutants I185A and L189A were essentially inactive (6.0 ± 1.7%, n = 4 and 2.0 ± 1.0%, n = 4, respectively). Two other point mutants, L182A and L180A, retained partial editing activity (47 ± 2.4%, n = 4 and 82 ± 1.1%, n = 4, respectively). When the double prolines, residues 190 and 191, were mutated to alanine individually, there was no loss of activity. However, when they were both mutated simultaneously, the mutant APOBEC1 lost over 90% of its activity (down to 7.0 ± 1.3%, n = 4). This was not the case with the other double prolines further downstream, residues 219 and 220. Each retained its fully editing activity either as single substitution mutants, P219A and P220A, or as the double mutant P219A/P220A.

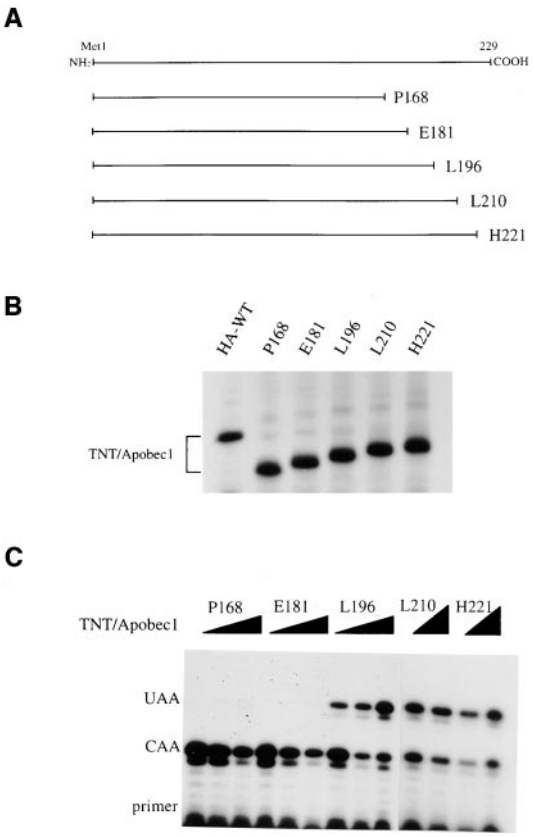


**Fig. 3.** In vitro editing activity of carboxy-terminal truncated mutants. A: A schematic diagram of carboxy-terminal truncated mutants. Wild-type APOBEC1 contains 229 amino acids. Truncated mutants pP168, pE181, pL196, pL210, and pH221 terminated at residues P168, E181, L196, L210, and H221, respectively. B: HA-tagged wild-type APOBEC1 and deletion mutant APOBEC1s were expressed using TNT-reticulocyte lysate system in the presence of [$^{35}$S]methionine. Five μl of the translation product was separated on 12% SDS-PAGE, followed by fluorography and autoradiogram. A representative result is shown and TNT/APOBEC1 is marked. C: In vitro apoB RNA editing assay of carboxy-terminal truncated mutants. Increasing phosphoimage counts per h (5510, 27550, and 165285 pch, respectively) of TNT-expressed carboxy-terminal truncated proteins, P168, E181, L196, L210, and H221, were incubated with 5 μg of chicken intestinal S-100 extract and 2 ng of synthetic rat apoB RNA. A representative result is shown. The products after primer extension were separated on 8% polyacrylamide urea gel electrophoresis. Edited (UAA), unedited (CAA), and primer radiolabeled bands are indicated.

### APOBEC1 dimerization in vitro

*Amino terminus of APOBEC1.* As it was demonstrated that APOBEC1 forms a spontaneous homodimer in vitro (27), we set out to define the regions that are necessary for dimerization. The method we used was to tag APOBEC1 with an HA-epitope sequence and study the co-immunoprecipitation of untagged and HA-tagged APOBEC1 by an HA-1 specific antibody, an approach similar to our previous study (27). Co-immunoprecipitation of two proteins as described here is a well-accepted method that has been used by many investigators to study protein–protein interaction and dimerization (34, 45–50). To define whether only nascent APOBEC1 chains were competent in dimer formation or whether completed APOBEC1 proteins were also able to dimerize efficiently, we did the analysis with both tagged and untagged APOBEC1 co-translated in vitro, or by mixing the tagged and untagged proteins after they were translated separately. We found identical results using the two methods, which indicated that dimerization can occur on full length APOBEC1 and folding of the nascent chain is not required for such interactions. Furthermore, the protein–protein interaction was evaluated quantitatively by the mammalian two-hybrid system in COS cells.

Amino-terminal deletion constructs of APOBEC1, pHA-R33, pHA-E35, pHA-P78, and pHA-A117, tagged with HA epitope at their carboxy terminus, were individually co-translated with untagged wild-type APOBEC1 in vitro, and dimer formation was analyzed by immunoprecipitation and SDS/PAGE. A representative result is shown in **Fig. 4** and the data are summarized in Table 2. In all cases, the amino-terminal deletion proteins HA-R33, HA-E35, HA-P78, and HA-A117 dimerized with untagged wild-type APOBEC1, the dimerization activities varied from 70% to 86% compared to wild-type APOBEC1 dimerization. Deletion construct L14 without tagged epitope was also translated together with HA-tagged wild-type APOBEC1, and L14 was found to dimerize efficiently with tagged wild-type APOBEC1 (Table 2, 95 ± 6.7%, n = 4). Control assay of untagged wild-type APOBEC1 by itself did not produce any immunoprecipitate (data not shown). Therefore, the data suggest that the amino terminal sequence of APOBEC1 up to A117 was not required for efficient dimerization in vitro.

To evaluate the protein–protein interaction in vivo, we chose the mammalian two-hybrid system using CAT gene as the reporter (51, 52). Plasmid vectors of wild-type, mutant APOBEC1, and CAT reporter were co-transfected into COS cells. Control experiments of background expression of wild-type with CAT reporter, mutant APOBEC1 with CAT reporter, or vector only, were performed simultaneously to subtract for basal expression level of CAT protein. CAT activity was measured by ELISA. The results are expressed as % of CAT activity for wild-type APOBEC1 dimerization, and are summarized in Table 2. The amino-terminal deletion mutants, R33, E35, and P78, had similar CAT activity compared to wild-type (Table 2, 88–93%, n = 4), but the deletion mutant of A117 had decreased CAT activity (Table 2, 67%, n = 4). In general, the results of in vivo mammalian two-hybrid system support our conclusion using in vitro immunoprecipitation that the amino-terminal region of APOBEC1 plays a minor role in dimerization.

*Carboxy terminus of APOBEC1.* We next studied the dimerization potential of untagged carboxy-terminal deletion constructs, pP168, pE181, pL196, pL210, and pH221 against HA-tagged wild-type APOBEC1 using the co-immunoprecipitation method. A representative result is shown in **Fig. 5** and the data are summarized in Table 2. Carboxy-terminal deletion mutant proteins, P168, E181, and L196, lost the ability to dimerize with wild-type APOBEC1 protein (17 ± 0.08%, n = 6; 20 ± 0.05%, n = 6; 28 ± 0.09%, n = 6, compared to 100% wild-type APOBEC1 dimeriza-

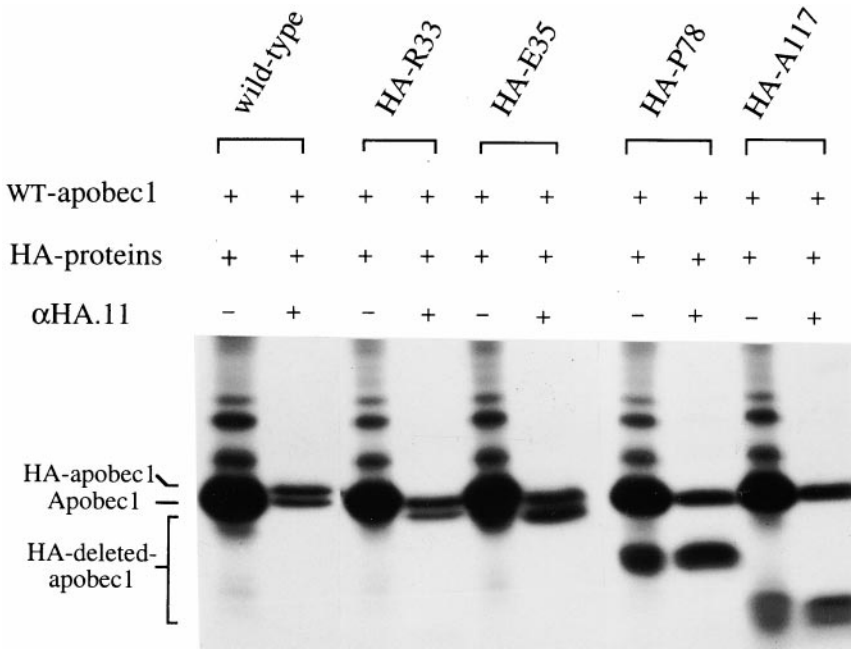


**Fig. 4.** Co-immunoprecipitation of wild-type (WT) APOBEC1 and amino-terminal deletion mutants. One μg of untagged wild-type (+) and 0.1 μg of HA-tagged wild-type APOBEC1 (+) or HA-tagged deletion mutants (+), HA-R33, HA-E35, HA-P78, and HA-A117 DNAs were co-translated using TNT-reticulocyte lysate system in the presence of [$^{35}$S]methionine. Translation products before immunoprecipitation (−) and after immunoprecipitation with antibody αHA.11 (+) were analyzed on 12% SDS-PAGE. Wild-type APOBEC1 (APOBEC1), HA-tagged wild-type APOBEC1 (HA-APOBEC1), and HA-tagged deletion APOBEC1s (HA-deleted-APOBEC1) are indicated.

tion, respectively). In contrast, efficient dimer formation was observed with L210 (74 ± 1.3%, n = 4), but efficiency decreased slightly with the deletion mutant H221 (51 ± 5.6%, n = 6). Therefore, the data suggest that carboxy-terminal residues from Leu$^{196}$ to Leu$^{210}$, and possibly His$^{221}$ to Lys$^{229}$, are important for dimer formation in vitro.

The potential importance of protein–protein interaction of these carboxy-terminal deletion mutants was evaluated by the mammalian two-hybrid system in COS cells. The data of reporter gene CAT activity are shown in Table 2. Deletion mutant proteins, P168 and E181, lost the ability to interact with wild-type APOBEC1 (0% and 8 ± 0.03%, n = 5, respectively). Deletion mutant protein L196 regained some of the ability to interact with wild-type APOBEC1 (29 ± 0.2%, n = 4). Inclusion of residues from L196 to L210 increased the protein–protein interaction ability slightly to 39 ± 2.2% (n = 4); however, the activity is ~2-fold less than that of amino-terminal deletion mu-

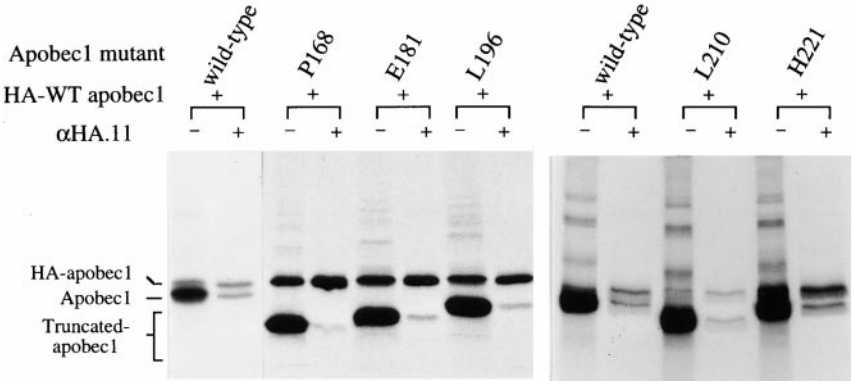


**Fig. 5.** Co-immunoprecipitation of wild-type APOBEC1 and carboxy-terminal truncated mutant proteins. HA-tagged wild-type (WT) APOBEC1 DNA (+) were co-translated with untagged wild-type APOBEC1, or with carboxyl-terminal truncated mutants P168, E181, L196, L210, and H221 DNAs using TNT-reticulocyte lysate system in the presence of [$^{35}$S]methionine. Translation products before immunoprecipitation (−) and after immunoprecipitation with antibody αHA.11 (+) were analyzed on 12% SDS-PAGE. HA-tagged wild-type APOBEC1 (HA-APOBEC1), wild-type APOBEC1 (APOBEC1), and truncated APOBEC1s (Truncated-APOBEC1) are indicated.

tants (Table 2). Further extension of APOBEC1 to residues H221 resulted in losing its ability to interact with wild-type APOBEC1 again (17 ± 0.34%, n = 4). Therefore, the results of the mammalian two-hybrid system, in general, correlate with the observation from in vitro co-immunoprecipitation assay. They both suggest that the carboxy-terminal region is important for the protein–protein interaction of APOBEC1 in dimer formation.

## DISCUSSION

In this study, based on the postulated functional importance of APOBEC1, we set out systematically to make a series of deletion and point mutations on APOBEC1 to examine the structure–function relationship of this enzyme. We have identified a number of amino acids in APOBEC1 that are important for apoB mRNA editing. These residues are scattered over several structural motifs (**Fig. 6**). The basic amino acids in the amino terminal region, $R^{15}$, $R^{16}$, $R^{17}$, $R^{33}$, and $K^{34}$, have been postulated to be part of a bipartite nuclear localization signal (16, 53). Here we show that they are also important for the editing reaction itself. The leucine-rich region in the carboxy-terminal of APOBEC1 are also important in apoB mRNA editing in vitro. Amino acid substitutions involving $L^{180}$, $L^{182}$, $I^{185}$, and $L^{189}$ all led to partial to almost complete loss of editing. When the proline doublet, $P^{190}$ and $P^{191}$, was substituted individually with alanine, the mutant APOBEC1 retained full activity. However, a double P190A/P191A mutant was essentially inactive. Finally, we evaluated the potential dimerization domain using co-immunoprecipitation method in vitro, and confirmed by a mammalian two-hybrid system in COS cells. The data implicate the carboxy terminus of APOBEC1 around residues 196–210 and 221–229 to be important in dimer formation.

Analysis of the predicted secondary structures of APOBEC1 suggests that the basic amino acid region in the amino terminus of APOBEC1 (residues 1 to 50) forms α-helix (H) and β-sheet structure (B) which continues with a turn (T) (**Table 3**). Arginine and lysine are both positively charged residues, although their side chains have different pKas (12.4 for Arg and 10.0 for Lys); substitution of the $R^{15,16,17}$ cluster to Lys residues markedly inhibited apoB mRNA editing activity, and substitution to Ala residues resulted in the inhibition of recombinant protein synthesis. As shown in Table 3, substitution of Arg cluster to Lys or to Ala altered the local secondary structure by interrupting an α-helix structure with a β-sheet in Chou-Fasman prediction, and by disrupting the β-sheet to strong helix structure (H) as suggested using GOR prediction. Therefore, the side chain structure of the Arg residues in this region is important in stabilizing the active conformation of APOBEC1. Another basic amino acid dipeptide further into the APOBEC1 sequence ($R^{33}K^{34}$) also probably plays a role in editing because the double mutant R33A/K34A is essentially inactive. As shown by secondary structure predictions, the mutation of R33A/K34A disrupted the α-helix structure (h) by generating a turn (T) in this region (Table 3). The other possible function of this cluster of basic amino acids may be in protein–protein interaction. The recent study of Mehta, Banerjee, and Driscoll (14) indicates that the amino terminal region of APOBEC1 may be involved in binding to complementation protein(s). We note that the tetrapeptide RRRI (residues 15–18) is 14 residues away from a hydrophobic $L^{32}$, an arrangement reminiscent of a variety of calmodulin-binding domain peptides (such as myosin light chain kinase) (54, 55), which also contain a tetrapeptide RRKL separated from a Leu or a Phe by 14 amino acid residues. Whether the recognition of APOBEC1 by a complementation protein occurs via analogous secondary structures as calmodulin–peptide complexes (56) must await the structural analysis of the interaction of APOBEC1 and its cognate complementation factor(s).

We have previously shown that the leucine-rich motif in the carboxy-terminal region of APOBEC1 was important for apoB mRNA editing (7, 42). A study by MacGinnitie, Anant, and Davidson (30) showed that mutation of leucine to isoleucine involving residues 182, 189, 196, and 203 simultaneously reduced the editing activity of the mutant proteins. Our systematic analysis of the leucine and proline residues corroborates and extends the previous observations. The point mutations involving residues in this region are especially revealing. It is particularly noteworthy that mutants L182A, I185A, L189A, and the double mutant, P190A/P191A, almost completely lost their apoB RNA editing activity, whereas a large number of other leucine to alanine mutations were apparently innocuous (Table 2). To examine whether the functional consequences of the leucine-rich region mutations could be correlated with structural changes, we analyzed the pre-

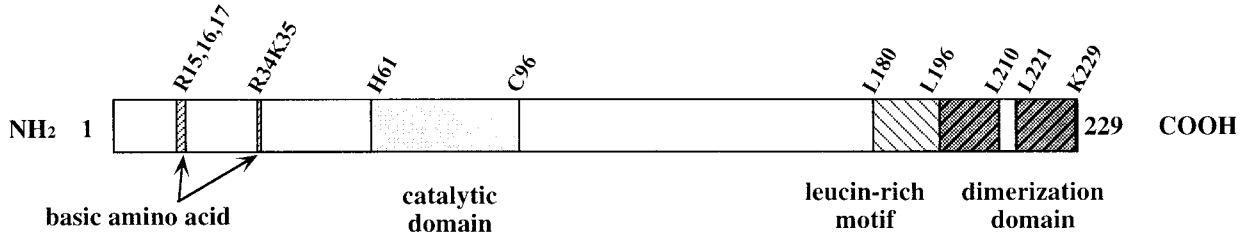


**Fig. 6.** Schematic diagram of the important functional domains of APOBEC1. Basic amino acid clusters, ($R^{15}R^{16}R^{17}$) and ($R^{33}K^{34}$), catalytic domain region, $H^{61}$–$C^{96}$, leucine-rich region; leucine-rich motif, $L^{180}$–$L^{196}$, and dimerization domains, $L^{196}$–$L^{210}$ and $L^{221}$–$K^{229}$, of APOBEC1 are indicated.

TABLE 3. Secondary structure predictions of residues 1–50 from APOBEC1 wild-type, triple mutants, R15K/R16K/R17K, R15A/R16A/R17A, and double mutant, R33A/R34A

| | | |
|---|---|---|
| | | 1-MSSETGPVAVDPTLRRRIEPHEFEVFFDPRELRKETCLLYEINWGGRHSI-50 |
| Wild Type | Ch-F | ..tttt.....TT....hhhhhhhhhhhhhhhhhhBBBBBBBBtttt.. |
| | GOR | .......BBBBBB..TT....HHHHHHHHHHHHHHHHHBBBBTTT...... |
| R15K/R16K/R17K | Ch-F | ..tttt.....TT.HHHHHH...BBBBBhhhhhhhBBBBBBBBtttt.. |
| | GOR | ........HHHHHHHHH.HHHHHHHHHHHHHHHHHHHBBBBTTT...... |
| R15A/R16A/R17A | Ch-F | ..tttt.....TT.HHHHHH...BBBBBhhhhhhhBBBBBBBBtttt.. |
| | GOR | ........HHHHHHHHH.HHHHHHHHHHHHHHHHHHHBBBBTTT...... |
| R33A/K34A | Ch-F | ..tttt.....TT....hhhhhhhhhhTTHHHHHHHBBBBBBBtttt.. |
| | GOR | .......BBBBBB..TT....HHHHHHHHHHHHHHHHHHHHTTTT...... |

Primary protein sequence of residues 1–50 is listed. The mutated amino acids used for secondary predictions are underlined. Prediction of Chou-Fasman (Ch-F) is listed in line 1 and that of Garnier, Osguthorpe, and Robson (GOR) in line 2. In Ch-F prediction, h, b, and t denote α-helix, β-strand, and turn, respectively, and H, B, and T denote corresponding "strong" predictions. In GOR prediction, H, B, and T denote α-helix, β-strand, and turn, respectively. The dot (.) denotes no prediction at that position.

dicted secondary structure for residues 173 to 198 (**Table 4**). Wild-type APOBEC1 and all the mutants studied, except the double mutant P190A/P191A, have the same predicted local structure in the form of two β-sheet strands connected by a turn at the two proline residues 190 and 191. However, when both prolines were mutated to alanine, an α-helix was predicted for the leucine-rich region. This change in conformation would drastically affect the backbone structure at the carboxy-terminal half of the peptide. Interestingly, mutation of either one of the two prolines (to Ala) is insufficient to trigger the predicted conformational change (Table 4), and is compatible with normal editing activity (Table 2).

Unlike the double Pro to Ala mutations, mutations of Leu or Ile to Ala at positions 182, 185, and 189 are conservative substitutions not associated with any significant change in the predicted secondary structure. However, the substantial loss of enzyme activity among mutants involving residues 182, 185, and 189 suggests that the side chains of these residues may be involved in protein–protein interaction. We note that the side chain volume decreases significantly from Leu or Ile to Ala. Therefore, if these residues are involved in a hydrophobic protein–protein interaction, the void created could lead to a different local packing, destabilizing the enzymatically active form of the complex (57). A recent study by Yang, Yang, and Smith (58) has put the significance of the amino-terminal and carboxy-terminal regions of APOBEC1 in perspective. They demonstrated that the amino-terminal 56 amino acids (residues 1–56) were necessary for the nuclear distribution of APOBEC1 and the carboxy-terminal leucine-rich domain (residues 173–196) had the presumptive cytoplasmic localization signal or nuclear export signal. They suggested that complementation factors may contribute to the editosome complex by interacting with either the amino-terminal or carboxy-terminal region of APOBEC1 to determine the localization of APOBEC1 (nucleus vs. cytoplasmic), which in turn plays a role in regulating the efficiency of editing.

TABLE 4. Secondary structure predictions of residues 173–198 from APOBEC1 wild-type, single point mutants, L189A, P190A, and P191A, and double mutants, P190A/P191A, L182A/L187A, and L182R/L187R

| | | |
|---|---|---|
| | | 173-LWVRLYVLELYCIILGLPPCLNILRR-198 |
| Wild Type | Ch-F | BBBBBBBBBBBBBB..tTTBBBBBB |
| | GOR | BBBBBBBBBBBBBB..TTBBBB |
| L189A | Ch-F | BBBBBBBBBBBBBB..ttBBBBBB |
| | GOR | BBBBBBBBBBBBB...TTBBBB |
| P190A | Ch-F | BBBBBBBBBBBBBB...ttBBBBB |
| | GOR | BBBBBBBBBBBBBB..TTBBBB |
| P191A | Ch-F | BBBBBBBBBBBBBB...bbbbbbb |
| | GOR | BBBBBBBBBBBBBB...BBBBB |
| P190A/P191A | Ch-F | BBBBBBBBBBBBBB.hhhhhhhhhhh |
| | GOR | BBBBBBBBBBBBHHHHHHHHHH |

Primary protein sequence of residues 173–198 is listed. The mutated amino acids used for secondary predictions are underlined. Prediction of Chou-Fasman (Ch-F) is listed in line 1 and that of Garnier, Osguthorpe, and Robson (GOR) in line 2. In Ch-F prediction, h, b, and t denote α-helix, β-strand, and turn, respectively, and H, B, and T denote corresponding "strong" predictions. In GOR prediction, H, B, and T denote α-helix, β-strand, and turn, respectively. The dot (.) denotes no prediction at that position.

The crystal structure of *E. coli* cytidine deaminase (ECCDA) (59) indicates that ECCDA is a homodimer of two identical 31.5 kDa subunits. Like ECDDA, APOBEC1 dimerizes in vitro (27, 42). The ECDDA and APOBEC1 monomers are of similar molecular size, and APOBEC1 contains the homologous sequences of the catalytic active site as ECCDA (60). The active site of both enzymes binds Zn ion to catalyze the deamination. The distinctive features of APOBEC1 that differentiate it from ECCDA are the bipartite basic amino acid domain near the amino terminus and the leucine-rich domain in the carboxy-terminal region. Recently, Navaratnam et al. (61) constructed a molecular model for APOBEC1 based on the homology between ECCDA and APOBEC1. The model is supported by extensive mutagenesis experiments on RNA editing activity, RNA binding, and dimerization. The model organizes APOBEC1 with two independent domains consisting of an amino-terminal (residues 31–116) and a carboxy-terminal (residues 154–229) region, linked by a stretch of amino acids (residues 120–153). They further postulated that the two monomers of APOBEC1 are orientated in a head-to-tail fashion with sufficient space to accommodate an RNA substrate. Direct proof of this model awaits experimental verification.

Navaratnam et al. (61) showed that deletion of ten or more amino acid residues from the amino terminus of APOBEC1 completely abolished dimerization, RNA binding, and RNA editing. In contrast, our data showed that amino terminal deletion to residue 14 had no effect on either RNA editing or dimerization; deletion mutant L14 had similar editing activity and dimerization capacity as wild-type APOBEC1 (Table 2). Further deletion from the amino terminal (R33, E35, P78, and A117) resulted in completed lost of editing activity but had no significant effect on dimerization as demonstrated by immunoprecipitation in vitro and two-hybrid system in vivo (Table 2). The experimental approaches of these two studies vary significantly. Navaratnam et al. (61) used glutathione S-transferase (GST) fusion deleted form of APOBEC1 to determine RNA editing activity, and they used GST-fusion deletion protein and HA-tagged APOBEC1 to do the protein–protein physical interaction. GST-APOBEC1 contains a GST domain of a molecular mass of 27 kDa, the same molecular weight as the full-length APOBEC1. Therefore, GST-APOBEC1 fusion protein is twice the size of the actual form of APOBEC1. We used the APOBEC1 representing its native form for RNA editing assay, and the immunoprecipitation was processed with HA-tagged-APOBEC1 deletion mutant and untagged-APOBEC1. Furthermore, we found that TNT-expressed APOBEC1 had much higher editing activity than GST-APOBEC1 fusion protein. As shown in MacGinnitie et al. (30), they used μg amounts of GST-fusion APOBEC1 to produce only 12% editing activity. Similarly, deletion mutant from carboxy terminus to residue 224 of Navaratnam et al. (61) also completely abolished both editing activity and dimerization whereas our data showed that deletion from carboxy terminal to residue 221 retained the editing activity but had very weak dimerization capacity (Table 2). Therefore, it is possible that the difference in experimental system and the use of a fusion protein in one series of experiments versus the use of the APOBEC1 without the GST protein in the other may contribute to the contradictory experimental outcomes.

The model by Navaratnam et al. (61) suggests that residues H61 and C93 in the active site, residues L135, and F156 in the linker region, and residues A165, P168, and P171 in the carboxy terminal domain are all involved in dimer formation. Their model and experimental data revealed that homodimerization is crucial for RNA editing activity. Previously, Oka et al. (42) also suggested that dimerization of APOBEC1 is critical for RNA editing; they demonstrated that carboxy terminal deletion mutant (residues 1–172) had no editing activity and had lost its dimerization ability; it failed to inhibit wild-type APOBEC1 editing activity when it was co-expressed with wild-type in vitro. Our amino-terminal deletion mutants (R33, E35, P78, and A117) dimerized sufficiently with wild-type APOBEC1 but had no editing activity. The carboxy-terminal deletion mutants of residues 1–196 abolished both editing activity and dimerization capacity, and further extension of residues to L210 and H221 recovered the editing activity to the same level as wild-type but not the dimerization capacity. Therefore, the results of our deletion mutants suggest that the carboxy terminal is important for dimer formation, and our observations do not completely support a linear relationship between RNA editing and dimerization.

It has been demonstrated that APOBEC1 has only a weak nonspecific RNA binding activity (28–30). However, the model of Navaratnam et al. (61) proposes that one active site of APOBEC1 binds to substrate RNA and the other active site binds to the product RNA, and RNA binding also showed correlation with editing. Recent work by Mehta and Driscoll (18) suggests that a 65 kDa complementing factor binds to both apoB mRNA and interacts with APOBEC1. Data from Lau et al. (13) suggest that additional complementation proteins interact with both APOBEC1 and apoB mRNA may exist, and data from Greeve et al. (62) demonstrated that heterogeneous nuclear ribonucleoprotein C1 also interacts with apoB mRNA and APOBEC1. Therefore, our understanding of the structure–function relationship of APOBEC1 is quite primitive. As the contributions of these complementation proteins in the structure and conformation of APOBEC1 and its interactions with apoB mRNA have not been rigorously defined in any of these studies, a complete understanding of the structure–function aspects of APOBEC1 in the context of an active editosome complex must await the elucidation of the complete structure of this macromolecular complex.

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