Overexpression of APOBEC-1 Results in Mooring Sequence-dependent Promiscuous RNA Editing*  

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Apolipoprotein B (apoB) RNA editing involves site-specific deamination of a cytidine to a uridine. A mooring sequence, a spacer region, and a regulator region are components of the apoB RNA editing motif of which only the mooring sequence is both necessary and sufficient for editosome assembly and editing. The catalytic component of the editosome is APOBEC-1. In rat hepatoma, stable cell lines, overexpression of APOBEC-1 resulted in 3–6-fold stimulation of the editing efficiency on either rat endogenous apoB RNA or transiently expressed human apoB RNA. In these cell lines, cytidines in addition to the one at the wild type site were edited. The occurrence and efficiency of this “promiscuous” editing increased with increasing expression of APOBEC-1. Promiscuous editing was restricted to cytidines 5′ of the mooring sequence and only occurred on RNAs that had been edited at the wild type site. Moreover, RNAs with mutant editing motifs supported high efficiency but low fidelity editing in the presence of high levels of APOBEC-1. This study demonstrates that overexpression of APOBEC-1 can increase the efficiency of site-specific editing but can also result in promiscuous editing.

Apolipoprotein B (apoB)1 RNA editing (1, 2) is a post-transcriptional (3), site-specific deamination of a cytidine residue to a uridine. This nucleotide transition (at nt 6666, C6666) converts a glutamine codon (CAA) at amino acid position 2153 to an in-frame STOP codon (UAA), which results in the translation of a truncated apoB48 protein (reviewed in Ref. 4). An 11-nucleotide (UGAUCAGUAUA) mooring sequence (5–7) is both necessary and sufficient for site-specific editing in a variety of RNA backgrounds (6, 8–10). A macromolecular complex or “editosome” specifically assembles upon the mooring sequence and C6666 is a region of lax sequence specificity (12–14) but whose appropriate length is critical for efficient site-specific editing (12). Immediately 5′ of C6666 exists a regulator element (UGAUA). Enhancement of RNA editing efficiency by this element does not require a specific sequence, although TA immediately adjacent to C6666 is most effective (12).

The deamination reaction involved in apoB RNA editing is a zinc-dependent process mediated by APOBEC-1 (15–18). This protein has extensive sequence homology to other cytidine deaminases from Escherichia coli and mammals (4, 15, 19), especially within the zinc coordination domain wherein mutations abolish editing activity (16, 20, 21). The enzyme stimulates editing activity in vitro when complemented with suitable cell extracts (16), provides editing activity to human liver cell lines (HepG2) in vitro (22), and enhances apoB mRNA editing in mice when expressed by adenovirus-mediated gene transfer (23). The efficiency of apoB RNA editing is an important determinant in the proportion of full-length (apoB100) or truncated (apoB48) protein variants assembled as triglyceride-rich lipoprotein particles (24, 25).

APOBEC-1 has a weak and non-sequence-specific binding affinity for RNA (26, 27). The site-specific RNA editing activity of APOBEC-1 is absolutely dependent upon its assembly with one or more auxiliary proteins (15, 16, 18) as an editosome (11). Proteins with molecular masses of 66 and 44 kDa have been identified by ultraviolet light protein-RNA cross-linking as the presumptive factors for mooring sequence-specific RNA binding (9, 11, 28). Extracts from a variety of tissues, obtained from a number of species can complement APOBEC-1 in RNA editing activity and therefore must contain auxiliary factors (15, 16, 18, 20). Complementation of APOBEC-1 by these factors is not dependent upon whether the sources of the extract have the ability to either transcribe or edit apoB RNA. In fact, editing of transfected apoB RNAs can occur in cell lines that otherwise do not express apoB RNA (29). The data suggest that the capacity to edit RNA may be a more general phenomenon and that there may be RNAs other than apoB that could support C → U RNA editing.

We have investigated the effect of APOBEC-1 overexpression on editing site specificity in rat liver cells. High levels of enzyme expression resulted in increased efficiency of apoB RNA editing at the wild type site, C6666. However, a mooring sequence-dependent phenomenon was observed in which multiple additional cytidines per RNA substrate were edited. Additional site editing was a direct consequence of overexpressing APOBEC-1. The data suggest that site-specific editing cannot be maintained if editing is up-regulated by only increasing the abundance of APOBEC-1. Moreover, mutant editing sites, which otherwise edited inefficiently, supported high efficiency but low fidelity RNA editing under high levels of APOBEC-1 expression. We propose that regulation of site-specific editing efficiency in tissues must involve coordinate expression of APOBEC-1 and auxiliary factors such that the relative abundance of APOBEC-1 is maintained as rate-limiting.

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¶1The abbreviations used are: apoB, apolipoprotein B; nt, nucleotide(s); PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; APOBEC-1, apoB mRNA editing enzyme, catalytic polypeptide 1.
**EXPERIMENTAL PROCEDURES**

Promiscuous RNA Editing through APOBEC-1 Overexpression

Plasmid Constructions—Wild type (wt) and mutant (TL, which contains a perfect mRNA sequence but imperfect spacer and regulatory elements) apob RNA expression vectors were generated by subcloning into the human apoB cassettes from pRSA13 and pRSA1333V (TL), respectively, as HindIII/KpnI fragments into HindIII/XbaI-linearized pRcCMV (Invitrogen). KpnI and XbaI restriction endonucleases were made blunt by the use of T4 DNA polymerase (Life Technologies, Inc.) and Klenow fragment of DNA Polymerase I (Promega) according to the manufacturer’s recommendations. apob-1 (18) cDNA was obtained from dil-got(dT)-primed rat small intestine, total cellular RNA using avian myoblastosis virus reverse transcriptase (Promega) and amplified with Pfu DNA polymerase (Stratagene) according to the manufacturer’s recommendations. PCR products were subcloned into pRcCMV and verified by dideoxy DNA sequencing (Sequenase, version 2.0, Amersham Corp.).

**Tissue Culture**—The rat liver hepatoma cell line McArdle RH7777 was obtained from ATCC (Rockville, MD) and maintained in DMEM containing 10% horse serum and 10% fetal bovine serum. Cells were transfected according to the method of Chen and Okayama (30). 48–72 h after DNA addition, cells were harvested for RNA isolation (transient transfections), or clonal stable transfected cell lines were obtained by limiting dilution under 500 μg/ml G418 (Life Technologies, Inc.), and harvested for RNA isolation (transient transfections), or clonal stable transfected cell lines were obtained by limiting dilution under 500 μg/ml G418 (Life Technologies, Inc.) selection.

RNA Isolations—Total cellular RNA was isolated from approximately 2 × 10^6 cells with Tri-Reagent (Molecular Research Center) according to the manufacturer’s recommendations. After isopropanol precipitation, RNAs were reprecipitated from 0.3 μM sodium acetate in ethanol. RNA preparations were digested with RQ-DNase I (Promega), according to the manufacturer's recommendations, for 30 min. Buffer conditions were modified appropriately, and an additional 30-min digestion was performed with a restriction enzyme having a recognition site between the PCR primer annealing sites of target substrates. These were EcoRI and RsaI for human and rat apoB RNA substrates, respectively. RNAs were phenol-extracted, precipitated, and quantified by spectrophotometry.

**Editing Assays**—The efficiency of editing of apoB RNAs was determined by an RT-PCR methodology. First strand cDNA was generated from 1 μg of dil-got(dT)-primed total RNA using avian myoblastosis virus reverse transcriptase (Promega) according to the manufacturer’s recommendations. Differential PCR amplification of the endogenous McArdle cell apoB sequences encompassing the editing site and those from the exogenously transfected human apoB cDNA was performed from the same first strand cDNA reaction using amplifiers ND1/ND2 (31) and SP6/T7 respectively. PCR was performed with Taq DNA polymerase (Promega) in the presence of 2.5 mM MgCl₂ according to the manufacturer’s recommendations. Thermal cycling conditions were as follows: one cycle at 94°C for 2 min; five cycles at 94°C for 45 s, 52°C for 1.5 min, 72°C for 1 min; and 30 cycles at 94°C for 45 s, 56°C for 1 min, 72°C for 1 min. The PCR product was subcloned into pRcCMV and verified by dideoxy DNA sequencing (Sequenase, version 2.0, Amersham Corp.).

**In Vitro RNA Transcription**—ApoB RNA substrates were synthesized using T7 RNA polymerase (Promega, in vitro transcription system) and 0.5 μg of PvuI linearized pRcCMV/WT(C) and pRcCMV/Wt(T) as templates according to the manufacturer’s recommendations. Deoxynucleotides—The following dNTPs were used in this study: dATP, dCTP, dGTP, and dTTP (Pharmacia). Two microliters of each dNTP were added to the reaction mixture for a final concentration of 200 μM. Incorporation of radioactive nucleotides into RNA was determined by PAGE. The specific activity of the [α-32P]dATP used was 6000 mCi/mmol (DuPont NEN).

**RESULTS**

McArdle RH7777, a rat hepatoma cell line, was selected for this study of apob-1 cDNA overexpression as these cells transcribe apoB RNA and edit it with relatively low efficiency (32). Clonal McArdle stable cell lines were selected under G418 in which apob-1 mRNA overexpression was directed from the human cytomegalovirus promoter. APOBEC-1 expression in the McArdle stable cell lines was confirmed by Western blotting, and the levels of APOBEC-1 protein in the cell line with the highest editing efficiency were determined to be at least 8-fold greater than that in the null vector transfected “control” McArdle cell line (Fig. 1).

The effect of APOBEC-1 overexpression on the editing efficiency of the endogenous rat apoB RNA was determined in triplicate, in two independently isolated total cellular RNA populations, from each of 12 independent cell lines (see “Experimental Procedures”). Poisoned primer extension analysis of RT-PCR products specific to endogenous rat apoB RNA demonstrated that the control cell line edited the wild-type site cytidine at nt position 6666 (C6666) with 12% efficiency (n = 8, S.E. = 0.03) (Fig. 2, lane 5). The editing efficiency of C6666 in three cell lines expressing low, medium, or high levels of apoB-1 was 20% (n = 8, S.E. = 1.0), 55% (n = 8, S.E. = 1.8), and 84% (n = 8, S.E. = 2.2), respectively (lanes 6–8). The correla-
tion between increased APOBEC-1 expression and an increase in RNA editing efficiency corroborates previous data from other systems (20, 22, 23, 33, 34).

The poisoned primer extension assay also suggested the presence of additional C → U editing events in the apob RNA substrate; endogenous refers to the rat apob RNA substrate. The positions of the primer and the extension products generated from unedited (CAA) and edited (UAU) RNAs are indicated. The length of these products is dependent upon the sequence 5′ of the first edited C in the RNA substrate and is therefore different between the rat and human substrates. Longer primer extension products indicative of additional editing site utilization are indicated at 2, 3, and 4.

Fig. 2. Additional editing site utilization in cells overexpressing APOBEC-1. Poisoned primer extension analyses were performed upon RT-PCR-amplified apob DNA templates as described under “Experimental Procedures.” Exogenous refers to the transfected human apoB RNA substrate; endogenous refers to the rat apoB RNA substrate. The positions of the primer and the extension products generated from unedited (CAA) and edited (UAU) RNAs are indicated. The length of these products is dependent upon the sequence 5′ of the first edited C in the RNA substrate and is therefore different between the rat and human substrates. Longer primer extension products indicative of additional editing site utilization are indicated at 2, 3, and 4.

To evaluate whether the additional C → U transitions were due to RNA editing or inaccuracies in the assay system, poisoned primer extension analysis was performed on RT-PCR products amplified from a mixture of in vitro transcribed, unedited and edited apoB RNA substrates. Primer extension beyond C6655 (to the additional sites) on RNA edited only at C6666 occurred with a frequency of less than 0.2% (n = 6). The proportion of read-through products was not altered at either extreme low or high concentrations of input RT-PCR product. This indicates a low but measurable error frequency due to a nucleotide incorporation error during RT-PCR or primer extension and may have produced the additional primer extension product (2) on the control McArdle RT-PCR products (Fig. 2, lanes 1 and 5).

Another limitation of the poisoned primer extension assay is that it would not be suitable for evaluating additional site editing on RNAs that were unedited at C6666 due to chain termination at this site. To confirm that the additional primer extension products were C → U editing events and not artifacts of the editing assay, the apob RNA RT-PCR products used in the primer extension analyses described above were cloned, and independent isolates were sequenced.

Fig. 3 shows the C and T sequence between nt 6573 and nt 6719 of representative clones of the transiently transfected exogenous human apoB RNA substrates isolated from the highest stable APOBEC-1-expressing (A) and control McArdle (B) cell lines. C → U editing at C6666 was observed in 18 of the 21 exogenous human apoB clones from the highest APOBEC-1-expressing cell line. This was equivalent to the 85% C6666 specific editing efficiency determined by poisoned primer extension assay (Fig. 2, lane 4). Of the 18 exogenous RNAs that were edited at C6666 in the highest expressing APOBEC-1 line, 8 were edited at additional sites 5′ of nt 6666 involving a total of 15 C → U transitions. Representative clones highlighting the nature and extent of additional editing site utilization are shown (Fig. 3A, lanes 3-8). Lanes 3–6 each demonstrated a single and different additional editing event at C6655, C6645, and C6643, respectively. Lanes 7 and 8 are representative of five isolates, which demonstrated that a single RNA could be additionally edited at multiple sites involving a wide array of cytidines. Additional cytidines that were edited included C6639, C6626, C6597, and C6583. Depending on the clone, one or more cytidines between C6666 and the additional edited base(s) appeared to remain unedited, e.g., C6604, C6617, C6616, and C6623.

No cytidine to uridine editing was observed 3′ of C6666, and no clones were isolated in which additional site C → U editing had occurred upon an RNA that was not edited at C6666. These data suggest that additional site editing was mooring sequence-dependent and, consistent with this model (7, 10), only cytidines 5′ of the mooring sequence were edited.

Only 4 of 23 exogenous clones isolated from the control McArdle cell line demonstrated C → U editing at C6666 by direct DNA sequencing (Fig. 3B) This was equivalent to an editing efficiency of 17%, comparable with that determined by the poisoned primer extension assay (Fig. 2, lane 1). None of the 23 clones showed additional site editing, supporting the possibility that the additional primer extension product seen on apob RNA from control McArdle cells was due to an assay system read-through error. Importantly, however, these control data underscore the importance of APOBEC-1 overexpression in additional site editing.

Fig. 4 shows the C and T sequence between nt 6572 and nt 6683 of representative clones of endogenous rat apob RNA substrates isolated from the highest APOBEC-1-expressing (lanes 1-3) and control McArdle (lanes 4 and 5) cell lines. C → U editing at C6666 was observed in 18 of 22 endogenous rat apob clones isolated from the highest APOBEC-1-expressing cell line. This was equivalent to the 82% C6666 specific editing efficiency determined by poisoned primer extension assay (Fig. 2, lane 8). Two of the 18 clones edited at C6666 were additionally edited at C6648, C6617 and C6597 for a total of four C → U
transitions (Fig. 4, lanes 2 and 3). Lane 1 shows the sequence of an isolate only edited at C6666. Similar to the exogenous substrates isolated from this cell line, additional editing site utilization was only observed upon RNAs that were edited at C6666, and no C → U transitions were observed 3' of the mooring sequence. Fig. 4, lanes 4 and 5, show representative endogenous sequences from 24 clones isolated from the control McArdle cell line. Lane 4 is an unedited isolate, and lane 5 is representative of the four C6666 edited clones. None of these four clones showed editing at additional sites, consistent with the role of APOBEC-1 overexpression in this process.

The disparate spacing of additional editing events relative to the mooring sequence suggests that the constraints on the spacer (and perhaps regulator) elements of the tripartite editing motif had become lax in cells expressing high levels of APOBEC-1. Previous studies have shown that a mutant human apoB RNA substrate (TL), which contains a perfect mooring sequence but a five-base pair spacer element (compared with four in the wt) and no 5' regulator element (6, 12), supported 3% editing in transfected McArdle cells and less than 7% of wild type levels in vitro (6, 12). We therefore chose to examine the potential of this mutant RNA substrate to support additional site editing in the highest APOBEC-1-expressing cell line. ApoB RNAs from duplicate transient transfections of the wt (positive control) and TL expression constructs were analyzed by poisoned primer extension assay as described above. Remarkably, greater than 60% editing efficiency (n = 6, S.E. = 2.2) was observed at the primary editing site (C6434 (6, 12)) compared with 67% (n = 3, S.E. = 1.4) at C6666 observed on the wt substrate (Fig. 5, lanes 2 and 1, respectively). Additional primer extension products were also observed on the wt and TL RNA substrates at a combined efficiency of 23% (n = 4, S.E. = 4.0) and 26% (n = 4, S.E. = 1.2) relative to that at C6666 and C6434, respectively. These products corresponded to editing of additional 5'-located cytidines. These results corroborate those

2 M. Sowden and H. C. Smith, unpublished findings.
DISCUSSION

This study has revealed C → U editing at sites other than C\(^{6666}\) whose efficiency of utilization was dependent upon APOBEC-1 abundance. These sites were not utilized in normal tissues or cells but were unique to cells that overexpress APOBEC-1 following transfection with apobec-1 cDNA. We therefore consider this activity as "promiscuous editing." The data also demonstrated that promiscuous editing was sequence-dependent, as only RNAs that were edited at C\(^{6666}\) were further edited at the promiscuous sites. Furthermore, consistent with the orientation specificity of the mooring sequence (10), only cytidines 5' of the mooring sequence were promiscuously edited.

The occurrence of the promiscuous editing events has been documented by primer extension and dideoxy sequencing analyses. Both assays were dependent on RT-PCR and could therefore have errors due to nucleotide misincorporation. We have determined that the cumulative error frequency in the primer extension assay was ≤0.2%. Only the additional primer extension product seen on apoB RNA from control McArdle cells fell within this range. The efficiency of promiscuous site utilization by APOBEC-1-overexpressing cells was much higher and ranged from 0.9 to 42%. More importantly, the efficiency of both C\(^{6666}\) editing and promiscuous site utilization increased with the degree to which APOBEC-1 was overexpressed in stable transfected cell lines.

The C → U transitions proposed as promiscuous editing sites also occurred with significantly greater efficiency than the assay error rate involved in the DNA sequencing analyses. 9.1% of the total cytidine residues 5' of C\(^{6666}\) in the exogenous substrates and 1.4% of the total cytidine residues 5' of C\(^{6666}\) in the endogenous substrates were converted to uridine in the clones isolated from the highest APOBEC-1-expressing cell line. A total of 24 base errors (not including editing site conversions) were observed following the sequencing of all four nucleotides of 90 independent exogenous and endogenous RNA isolates for a total of over 11,500 bases read. This was equivalent to 0.21%, or 1 base error in every 476 bases, resulting from reverse transcriptase, PCR, or dideoxy sequencing nucleotide misincorporation. These errors were assorted, e.g. A→G, T→C, G→A, were randomly located throughout the different substrates, and were not specific to edited substrates. Notably, there were no C→T errors observed either at C\(^{6666}\) or beyond 100 bases 5' of C\(^{6666}\). No cytidines were observed that had been converted to nucleotides other than uridine.

DNA sequence analyses clearly demonstrated the extent and variety of sites promiscuously edited. These data confirmed those from primer extension analyses and extended the information by demonstrating that promiscuous site utilization only occurred on RNAs that had been edited at C\(^{6666}\) and that all of these sites resided 5' of the mooring sequence. The analysis
The unique occurrence of promiscuous editing sites 5’ of the mooring sequence suggests that cis-acting elements must also be mechanistically important. The predominance of a four-nucleotide UGAU motif has been noted as a curious feature of RNA flanking the apoB RNA editing site (5, 10, 28). This motif, which comprises the 5’ head of the 11 nt mooring sequence, would be expected to only occur randomly every 256 nucleotides (once in 4th nt). In human apoB RNA, the motif occurs 12 times in a 400-nucleotide region (nt 6440–6839), an average of once every 33 nucleotides. More surprisingly, this motif occurs eight times in the 193-nucleotide region analyzed in these sequencing studies. It has been recently demonstrated that APOBEC-1 fusion proteins can interact with a similar RNA motif with low affinity in the absence of auxiliary factors (26, 27). Overexpression of APOBEC-1 in our study may have resulted in C → U editing due to inappropriate binding of APOBEC-1 homodimers (35) at one or more of these motifs.

In support of this possibility, the promiscuous editing sites on the human exogenous RNA at C6583 and C6597 are 6 and 13 nt (respectively) 5’ of UGAU motifs. Edited cytidines C6539, C6643, C6645, C6648, C6651, and C6659 are located between 5 and 21 nt 5’ of a third UGAU motif. Also consistent with this possibility, from the point of view of distance constraints, was the finding that C6517, C6616, C6623, or C6626, which lie more than 30 nt 5’ of a UGAU motif, were not edited. Only three UGAU motifs exist within the 207-base pair region of the endogenous rat apoB RNA that was analyzed. One of these motifs is located within the mooring sequence, and it could have been responsible for the promiscuous editing at C6661 and C6659. Consistent with the potential quantitative relationship between UGAU motifs and sites of promiscuous editing, less extensive promiscuous editing occurred on rat apoB RNA compared with human apoB RNA.

Contrary to the data supporting multiple foci for editing complex assembly was the finding that C6604 on the exogenous human transcript was not edited, although it only resides six nt 5’ of a UGAU motif. Moreover, five UGAU motifs exist within 140 nt 3’ of the mooring sequence, yet no editing occurred within this region. In the endogenous rat apoB RNA, C6583 and C6572 were not edited, although they were proximal to a UGAU motif at nt 6590. Interestingly, there was no UGAU motif suitably located to support the observed editing at C6617 and C6597 on the rat endogenous apoB RNA transcript. Moreover, none of the promiscuous editing sites on the human or rat apoB RNAs are flanked by optimal spacer or regulatory elements. The data suggest therefore that although cis-acting elements must play an important role, promiscuous editing cannot be solely accounted for by the occurrence of multiple UGAU motifs, individually capable of binding to editing complexes. We cannot currently distinguish between the two alternate hypotheses for promiscuous editing, although it is clear that APOBEC-1 overexpression is of central importance.

Promiscuous editing upon the endogenous rat apoB RNA can be predicted to induce a number of silent amino acid changes, some conservative amino acid changes, several alterations anticipated to effect protein structure, and even the introduction of a translation STOP codon seven amino acids N-terminal to that which would be created by utilization of the normal editing site. A similar array of amino acid alterations were predicted as a result of promiscuous editing upon the human RNA including the introduction of translation STOP codons at amino acid residues 2145 and 2148. A number of different apoB mutations associated with hypobetalipoproteinemia have been mapped that include the introduction of numerous STOP codons throughout the apoB gene (reviewed in Ref. 36). Under normal circumstances, it is unlikely that promiscuous editing would...
occur in human liver, as it does not express sufficient APOBEC-1 to edit its own apoB RNA (22). The emphasis that is currently being placed on regulating serum low density lipoprotein levels as a means of reducing the risk of atherogenic diseases raises the possibility of using apoB-1 cDNA in gene therapy (23, 34). The current studies suggest that such transfections in humans would be inappropriate due to the potential of inducing promiscuous editing.

To this extent, we believe that promiscuous editing may not be limited to apoB RNA. Induction of site-specific editing within heterologous RNA contexts both in vitro and in cells has been described through the insertion of the 11-nucleotide motoring sequence (6, 8, 9, 37). The high efficiency with which C→U transition in the TL mutant RNA was edited and the occurrence of promiscuous editing suggest that RNAs containing regions with low homology to the apoB RNA editing motif might become editing substrates if APOBEC-1 was overexpressed without the concordant increase in auxiliary factor expression. The potential that RNAs other than apoB are edited has also been suggested by the finding that cell lines of diverse origin that did not express apoB RNA had the ability to edit a transfected apoB cDNA (29).

A search of GenBank™ using the Pearson and Lipman type FASTA algorithm (38) for human and rodent RNA transcripts that contain homology to the 11-nucleotide motoring sequence demonstrated that only apoB contains a perfect match for a complete 21-nucleotide editing motif. Six human and rodent RNAs were identified that contained a perfect 11-nucleotide sequence-dependent C→U editing of alternative RNA substrates and genotoxicity resulting in hepatic dysplasia.

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