Two Efficiency Elements Flanking the Editing Site of Cytidine 6666 in the Apolipoprotein B mRNA Support Mooring-dependent Editing

Martin Hersberger‡ and Thomas L. Innerarity§

From the Gladstone Institute of Cardiovascular Disease, Cardiovascular Research Institute and the §Department of Pathology, University of California, San Francisco, California 94141-9100

Normally, apolipoprotein B (apoB) mRNA editing deaminates a single cytidine (C<sup>6666</sup>) in apoB mRNA. However, when the catalytic subunit of the editing enzyme complex, APOBEC-1, was overexpressed in transgenic mice and rabbits, numerous cytidines in the apoB mRNA and in a novel mRNA, NAT1, were aberrantly hyperedited, and the animals developed liver dysplasia and hepatocellular carcinomas. To identify the RNA motifs in the apoB mRNA that support physiological editing and those that support aberrant hyperediting, we constructed rabbit apoB RNA substrates and tested them in vitro for physiological editing and hyperediting. Three previously unrecognized RNA elements that are critical for efficient physiological editing at C<sup>6666</sup> were identified. In concert with the mooring sequence (6671–6681), the 5′ efficiency element (6609–6628), an A-rich region (6629–6640), and the 3′ efficiency element (6717–6747) increased editing at C<sup>6666</sup>. The 5′ efficiency element was the most potent, elevating physiological editing to wild-type levels in combination with the mooring sequence. The 3′ efficiency element was somewhat less important but increased physiological editing to levels approaching wild type. These elements encompass 139 nucleotides on the apoB RNA transcript and are sufficient for editing with the efficiency of full-length apoB mRNA. Furthermore, a distant downstream apoB region (6747–6824) may function as a recognition element in the apoB mRNA. Hyperediting at C<sup>6802</sup> in the rabbit apoB mRNA is mediated by RNA elements similar to those required for normal physiological editing at C<sup>6666</sup>. Similarly sized upstream and downstream flanking regions of C<sup>6802</sup> are necessary for hyperediting in combination with a degenerate mooring sequence.

RNA editing is the alteration of the genetic information present in nascent RNA transcripts. One form of RNA editing, apolipoprotein B (apoB)<sup>1</sup> mRNA editing, deaminates a single cytidine (C<sup>6666</sup>) in the apoB mRNA, which generates a uridine (1–4) changing codon 2153 from a genomically encoded CA(A-glutamine) to an in-frame stop codon (UAA) (5, 6).

‡ To whom correspondence should be addressed: Gladstone Inst. of Cardiovascular Disease, 4550 International Blvd., San Francisco, CA 94120-9990. Tel.: 415-285-7434; Fax: 415-285-7500; E-mail: martin_hersberger@gladstone.org

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1 The abbreviations used are: apoB, apolipoprotein B; nt, nucleotides; PCR, polymerase chain reaction.

The apoB mRNA editing complex comprises a catalytic subunit designated APOBEC-1 (16) and other, as yet unidentified, auxiliary proteins (17–19). Overexpression of APOBEC-1 in the livers of mice and rabbits resulted in liver dysplasia and hepatocellular carcinomas (20). Overexpression of APOBEC-1 also resulted in the aberrant editing of several other cytidines in the apoB mRNA (21–23). The highest degrees of editing occurred at C<sup>6738</sup>, C<sup>6743</sup>, C<sup>6762</sup>, C<sup>6782</sup>, and C<sup>6802</sup> (21). Numerous cytidines were also edited in a novel mRNA, NAT1 (22). This aberrant editing was termed hyperediting to distinguish it from the physiological editing at C<sup>6666</sup>. Sequence analysis of the RNA surrounding the hyperedited cytidines showed that no exact mooring sequence was present within the correct distance to support this hyperediting (21). In contrast, other mRNAs containing a mooring sequence were not hyperedited (20), indicating that elements in addition to the mooring sequence are necessary for hyperediting.

Here we report results from in vitro studies of physiological editing at C<sup>6666</sup> and hyperediting at C<sup>6802</sup> in the apoB RNA. Our results show that the essential elements for physiological editing at C<sup>6666</sup> of the rabbit apoB mRNA encompass 139 nt, consisting of defined upstream efficiency elements, the mooring sequence, and the 3′ efficiency element. Similar RNA features are necessary for the hyperediting of cytidines when APOBEC-1 is overexpressed.

EXPERIMENTAL PROCEDURES

Generation of ApoB RNAs—ApoB RNAs were produced by in vitro transcription from polymerase chain reaction (PCR) constructs, that included a T7 promoter. The apoB cDNA constructs were generated by PCR amplification from the plasmid pRabSK, a derivative of pHab-1 (24), encoding a 354-base pair rabbit apoB cDNA segment. The PCR products were purified by agarose gel electrophoresis and eluted by Qiagen II gel extraction (Qiagen, Santa Clarita, CA). The primers designed on the computer program Oligo 4.0 were 18–25-nt-long and were purchased from Life Technologies, Inc. The T7 promoter sequence

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with GGA (5′-GGATCTTATACGACTCATATAGGA-3′) added to achieve efficient expression was incorporated into the PCR constructs by a second PCR amplification. RNA was produced by the T7-MEGA shortscript in vitro transcription kit (Ambion, Austin, TX). The RNA was analyzed by agarose gels, treated with DNAse I, and purified by phenol-chloroform extraction and ethanol precipitation. The names of the RNAs refer to the first nucleotide of the RNA according to the apoB cDNA position (e.g. B6629 starts at nt 6629), and the length of the RNAs is given in nucleotides.

The DNA constructs for the P1 (GenBank accession number X62154) and the N-myc RNA (GenBank accession number M12731) were produced by in vitro transcription-PCR from normal mouse liver RNA. The primers for the N-myc amplification were M1L6879/5′-GCCGCTGTATATTTGTTTATGAT-3′ and M1L7053/5′-AACAAAATACGATACCAAAAGGAA-3′. For the P1 amplification, the primers were P1U1279/5′-GCCCCGGCCTGACAGGTGGTTTTGGTGA-3′ and P1L1454/5′-CCCTGTATTGGTGCATCCTAA-3′. The N-myc and the P1 PCR fragments were TA subcloned (Invitrogen, San Diego, CA) and sequenced. Chimeric RNAs were produced from PCR-derived RNA constructs by recombinant PCR (25). The PCR products were purified, and the RNAs were produced as described for the apoB RNAs. Substitution mutagenesis was accomplished by incorporating the alterations in the primers used to produce the PCR constructs.

The chimeric RNA MS-P1 (196 nt) consists of 58 nt of apoB RNA (6629–6686) and 138 nt of P1 RNA (1337–1474). The N-myc chimeric RNA (566 nt of apoB RNA 6662–6666) and 129 nt of N-myc RNA (6936–7074). The second set of N-myc chimeric RNAs used the 138-nt N-myc RNA MY (6859–6996) as a control. M-MOOR-M (138 nt) has 51 nt of N-myc RNA (6859–6909), 26 nt of apoB RNA (6662–6687), and 61 nt of N-myc RNA (6936–6996). EFF-MOOR-EFF (138 nt) has 20 nt of apoB RNA (6669–6689), 31 nt of N-myc RNA (6859–6909), 26 nt of apoB RNA (6662–6687), 30 nt of N-myc RNA (6936–6965), and 31 nt of apoB RNA (6717–6747). The third set consists of the F-M-P RNA (139 nt), which has 53 nt of apoB RNA (6669–6666), 36 nt of N-myc RNA (6910–6955), and 60 nt of apoB RNA (6868–6747). M-F RNA (137 nt) has 77 nt of N-myc RNA (6859–6935) and 60 nt of apoB RNA (6868–6747). F-M RNA (140 nt) has 53 nt of apoB RNA (6609–6661), 86 nt of N-myc RNA (6859–6909), and 51 nt of apoB RNA (6717–6747). The fourth set of apoB/N-myc chimeric RNAs used the 196-nt N-myc RNA M (6859–7074) as a control. M-B6747 has 118 nt of apoB RNA (6879–6969) and 78 nt of apoB RNA (6747–6824). The PCR templates for all the apoB/N-myc chimeric RNAs were sequenced.

In Vitro ApoB mRNA Editing Assay—For the in vitro apoB mRNA editing assay (26), 100 μg of synthetic RNA prepared as described above, 100 μg of rabbit liver S100 extract, 5 μg of recombinant APOBEC-1 (MBP-APOBEC-1) (21), and 1 μg of Escherichia coli RNA, and 40 units of RNasin (Promega, Madison, WI) in buffer containing 1 mm dithiothreitol in a reaction volume of 100 μl. dATP, dGTP, and dTTP and 5 mm dCTP were used for the transcription-PCR, and the resulting single-band PCR products were purified over a microspin S-300 HR column (Amersham Pharmacia Biotech).

Primer Extension Analysis—Primer extension was performed essentially as described (1). Except in the experiments with primers PE6762, PE6782/3, and PE6802, the coding strand instead of the noncoding strand was analyzed for hyperediting. For these experiments, 1 μm each dATP, dGTP, and dTTP and 5 μm dCTP were used for the primer extension PCR. The extent of editing was determined with a radiolabeled imaging system (AMBIS, San Diego, CA). The primers used for primer extension in apoB apoB were M51 at C6666 (5′-ATCACATATTATATCCG-3′), PE673R at C737B (5′-ATTITTTATTATTTTCTGCA-3′), PE674R at C737D (5′-GTTACTACAAATGTTTTATTAT-3′), PE6762 at C737C (5′-TACATCATATTACATTGAA-3′), PE6782/3 at C6782 and C6783 (5′-AATCTCTTATGAGTCATC-3′), and PE6802 at C6609 (5′-TACATCATATTACATTGAAA-3′). The primer extension products were analyzed by sequencing the apoB mRNA editing cassette (designated MOOR). The primer used to detect editing in N-myc was MPE6919R at C6919 (5′-TACGAAAAATAATAGATGAA-3′). The percentage of editing is given as the mean plus and minus the deviation of n experiments.

RESULTS
To investigate the influence of distal flanking sequences on editing of C6666 and hyperediting of C6609 in the apoB mRNA, we used an in vitro editing assay of small apoB RNAs. The RNAs were produced by in vitro transcription and incubated in the presence of rabbit liver extract (as a source of auxiliary proteins) and an excess of recombinant APOBEC-1. The percentage of editing of a specific cytidine was then determined by primer extension analysis.

Editing at C6666—To determine the impact of downstream elements on physiological editing of C6666, we first generated RNAs with 3′ sequences of different lengths (Fig. 1). Editing at C6666 was determined in an in vitro editing assay after 1, 2, 4, and 16 h. The parental RNA B6507 (354 nt) was highly edited (82%) after 16 h (Fig. 1). Editing of smaller RNAs was similar; even B6629A-77 (119 nt) was edited to 77% after 16 h of incubation. However, removal of an additional 31 nt at the 3′ end (6717–6747) decreased editing by almost half. We termed this 31-nt spanning region located 51–81 nt downstream of C6666 the “3′ efficiency element.” That the loss of editing efficiency was not simply due to the length of the RNA was shown by using the N-myc chimeric RNA MS-M (197 nt) and the P1 chimeric RNA MS-P1 (196 nt) in which sequences 3′ of the mooring sequence were replaced with N-myc or P1 sequences. The AU-rich apoB/N-myc RNA and B6629A-118 were edited to a similar extent. The GC-rich apoB/P1 RNA was marginally edited (MS-P1, Fig. 1).

To investigate the influence of upstream flanking regions on editing of C6666, we transcribed small RNAs lacking the 3′ efficiency element. RNAs starting at nt 6609 (e.g. B6609L (78 nt) and B6609L (98 nt)) were edited with the same efficiency as the parental construct B6507, resulting in 80–90% editing within 4 h of incubation (Fig. 2). However, deletion of 20 nt in the 5′ end resulted in a 50% decrease in editing after 16 h of incubation (Fig. 2, B6629A-108 (88 nt) and B6629A-118 (78 nt)). The RNAs were not responsible for the difference in editing, because B6629A-118 and B6609 are the same length (78 nt). Thus, the difference in editing efficiency was due to the RNA element 6609–6628, located 38–57 nt upstream of C6666, which we termed the “5′ efficiency element.” Because RNAs lacking the 5′ efficiency element were still edited to about 50% of the wild-type level after 16 h, we deleted more 5′ sequences to define closer regions that promote editing at C6666. Additional deletion of 12 nt from the 5′ end resulted in an RNA, B6641 (46 nt), that was edited only to 7% (n = 3) after 16 h of incubation (data not shown), indicating the importance of a second region 26–37 nt upstream of C6666 for efficient editing at C6666. This second region is A-rich (AAAUAGAAA), and mutations in this A-rich region (6629–6640) revealed that three A → U and three A → G substitutions decreased editing at C6666 (data not shown). However, introduction of a mooring sequence (UGAUCAUGAUA) returned editing efficiency to high levels (65% after 16 h [data not shown]).

To distinguish further whether the 5′ and 3′ efficiency elements are part of the minimal flanking requirement for efficient physiological editing at C6666 or whether they are specific enhancer elements for physiological editing, we made apoB/N-myc RNAs (Fig. 3). Previous deletion and mutagenesis experiments indicated that the 26-nt apoB editing cassette, including C6666 and the 78 nt 3′ sequence, is sufficient for low level editing in certain heterologous RNA contexts (10, 12). We therefore exchanged sequences flanking this apoB-editing cassette with N-myc sequences, resulting in 20 ± 14% (M-MOOR-M) editing at C6666 after 16 h. This represents about 25% of the editing activity detected in the parental apoB RNA (B6609C). However, chimeric RNAs containing the apoB 5′ efficiency element were edited to wild-type levels (EFF-MOOR-M and EFF-MOOR-EFF), emphasizing the importance of the 5′ efficiency element (6609–6628) for efficient mooring-dependent editing. Adding the 3′ efficiency element (6717–
did not further increase editing efficiency when the 5' efficiency element was present (EFF-MOOR-EFF), but in the absence of the 5' efficiency element, the 3' efficiency element increased editing 2-fold (M-MOOR-EFF). These data indicate that in combination with the apoB editing cassette, the 5' efficiency element and to a lesser extend the 3' efficiency element restored editing at C6666 to wild-type levels.

Our data indicate that efficient physiological editing at C6666 is enhanced by both the 5' efficiency element (38–57 nt upstream) and by the 3' efficiency element (51–81 nt downstream). If these apoB elements enhance editing at C6666, they may promote hyperediting in N-myc RNA containing a mooring-like motif. To test this possibility, we exchanged the 26-nt apoB editing cassette for the equivalent N-myc sequences (Fig. 4). The N-myc RNA (MY) was not hyperedited, although it contains mooring-like motifs and its AU content is equivalent...
FIG. 3. The apoB 5' and 3' efficiency elements increase mooring-dependent editing at C6909. RNAs were incubated for 16 h in an in vitro editing assay, and the editing of C6909 was determined by primer extension analysis (n = 3). A, B6609C is the parental apoB RNA. M-MOOR-M consists of the 26-nt apoB editing cassette including the mooring sequence (black bar), flanked by N-myc RNA (line). EFF-MOOR-M consists of the apoB 5' efficiency element and the apoB editing cassette flanked by N-myc RNA. EFF-MOOR-EFF has the apoB 5' efficiency element, the apoB editing cassette, and the 3' efficiency element linked by N-myc RNA. M-MOOR-EFF has the apoB editing cassette and the apoB 3' efficiency element flanked by N-myc RNA. The numbers in the top part of the figure refer to the apoB cDNA position. ApoB RNA is drawn as a bar. B, primer extension analysis of C6909 in the apoB RNA is shown. The top one or two bands represent the deamination of C6909 to a uridine (U at 6909). The second band from the bottom represents the unmodified cytidine (C at 6909). The extent of editing was determined with a radioanalytic imaging system.

FIG. 4. Independent 5’- and 3’-flanking regions of the apoB RNA promote hyperediting of a previously unedited cytidine in chimeric RNAs. RNAs were incubated for 16 h in an in vitro editing assay, and the hyperediting of C6914 in the N-myc RNA was determined by primer extension analysis (n = 3). MY consists of 138 nt of N-myc RNA (line), including C6914 and the mooring-like motif (UUAUACUCUUAAU, small box). EFF-M-EFF consists of the apoB 5’ efficiency element fused to N-myc RNA and to the apoB 3’ efficiency element. M-F has N-myc sequences fused to the apoB 3’-flanking region. F-M-F consists of the apoB 5’-flanking region fused to the 26-nt N-myc hyperediting cassette and the apoB 3’-flanking region. F-M has the apoB 5’-flanking region fused to N-myc RNA. M has the 3’ last 78 nt of N-myc RNA replaced with the 78-nt apoB sequence, B6747, that includes the apoB mooring-like motif (striped bar). The numbers in the top part of the figure refer to the apoB cDNA position.

to that of apoB RNA. When the apoB 5’ and 3’ efficiency elements were added to the N-myc RNA, no hyperediting was detected (Fig. 4, EFF-M-EFF). Thus, the apoB 5’ and 3’ efficiency elements promote editing in combination with the apoB editing cassette (Fig. 3). However, providing the entire apoB upstream flanking region (6609–6661), including the 5’ efficiency element (F-M) or the entire apoB downstream flanking region (6688–6747), including the 3’ efficiency element (M-F), resulted in hyperediting of one (C6914) or two cytidines (C6914 and C6909) in the N-myc RNA. The flanking regions of the editing cassette in the apoB RNA therefore act as recognition elements for hyperediting of a cytidine in the N-myc RNA context. Because we hypothesized that several elements in the apoB mRNA could function as recognition elements and could support mooring-dependent editing (22), we examined the influence of the more downstream apoB RNA element, B6747, on hyperediting of the N-myc RNA (Fig. 4). The apoB sequence applied in these chimeric RNAs does not cover sequences relevant for efficient editing of C6909, but B6747 starts directly downstream of the 3’ efficiency element mapped above. Again, this longer N-myc RNA (M) was not hyperedited (Fig. 4). In M-B6747, however, the substitution of 78 nt of apoB RNA (B6747) in the N-myc RNA caused editing of two cytidines 5’ of the N-myc mooring-like motif (Fig. 4). These results indicate that an element in the apoB RNA more than 81 nt downstream of C6909 influences editing.

**Hyperediting—**Our data indicate that both upstream and downstream flanking regions contribute to editing of C6909. To compare these findings to hyperediting, we first mapped in vitro the minimal upstream sequence required for hyperediting of C6802 (Fig. 5). B6629 includes C6909, the mooring sequence downstream, and the region with the clustered cytidines that are hyperedited in the apoB mRNA from transgenic mice overexpressing APOBEC1. All cytidines investigated in B6629 (196 nt) were hyperedited in vitro in a pattern similar to that observed in vivo (21). At C6802, the level of hyperediting was 4.9 ± 1.7% (n = 3). Deletion of the mooring sequence (Fig. 5, B6687, 138 nt) abolished hyperediting at C6743 and increased hyperediting 2-fold at C6802 (11.8 ± 4%, n = 3). Deletion of an additional 60 nt of 5’ sequences (Fig. 5, B6766, 78 nt) resulted in the loss of combined hyperediting of C6782 and C6783 (C6782/3), but C6802 was still hyperedited (4.2 ± 1.5%, n = 3). However, deletion of an additional 13 nt at the 5’ end abolished hyperediting at C6802 (Fig. 5, B6760, 65 nt), indicating that sequences 42–55 nt upstream are essential for hyperediting at this site.

Intriguingly, the sequence 42–55 nt upstream of C6802 is AU-rich (AAAAUUUUAAA), similar to the A-rich region (AAAAUUUUAAA) that contributes to physiological editing of C6909. Furthermore, the AU-rich sequence contains a cryptic polyadenylation signal (AAUAAA). We tested the possibility that the cryptic polyadenylation motif (6752–6757) influences
Control of ApoB mRNA Editing

Previous studies indicated that several sequence elements are needed for efficient editing of apoB mRNA. For example, small apoB RNAs and chimeric RNAs with only 26–63 nt of specific apoB sequences were edited inefficiently (11, 14, 15, 28). Specifically, in the GC-rich apoE RNA, 63 nt of specific apoB sequence were not edited (15). In contrast, when 354 nt of specific apoB sequence were translocated into the same locus of the apoE mRNA, wild-type levels of editing were detected, indicating that all sequence requirements for efficient editing at C6662 are present within this segment (15). In this in vitro study, we have defined essential RNA sequence elements in the apoB mRNA necessary for its physiological editing and for its aberrant hyperediting.

Fig. 5. In vitro hyperediting of short apoB RNAs. RNAs were incubated for 16 h in an in vitro editing assay, and the editing of each cytidine was determined by primer extension analysis. B6629 (196 nt) is the parental RNA containing the mooring sequence (dark bar) and several mooring-like motifs downstream. One of these mooring-like motifs (6809–6819) is marked by a striped bar. B6687 (138 nt), B6747 (78 nt), B6760 (65 nt), and B6779 (46 nt) lack the mooring sequence and have 5’ sequences progressively deleted. The name of the RNA refers to the first nucleotide of the RNA according to the apoB cDNA position (e.g. 6629 starts at 6629). The numbers in the top part of the figure refer to the apoB cDNA. The cytidines investigated for editing and hyperediting are indicated at the bottom of the figure.

Fig. 6. Scrambling the cryptic polyadenylation site (AUUAAA) in the 78-nt apoB RNA B6747 did not decrease hyperediting of C6802. RNAs were incubated for 16 h in an in vitro editing assay, and the editing of C6802 was determined by primer extension analysis. B6747 (n = 3) is a 78-nt parental apoB RNA used for the mutagenesis studies including the mooring-like motif at 6809–6819 (striped bar). UB6747 has U → A substitutions in the region of the cryptic polyadenylation site (U). MB6747 has the exact 11-nt mooring sequence introduced at the same site (dark bar), and GB6747 has G substituted for A (G). Underlined letters indicate substituted nucleotides. The numbers in the top part of the figure refer to the apoB cDNA position. The percentage of editing is given as the mean plus or minus the deviation.

hyperediting of C6802, because we and others have shown a link between editing and activation of this cryptic polyadenylation signal in the apoB mRNA (5, 6, 15, 27). The 78-nt apoB RNA, B6747, contains the cryptic polyadenylation signal (Fig. 6). Mutation of the cryptic polyadenylation signal to AUUUA by the substitution of a U for A did not diminish hyperediting of C6802 in UB6747 (Fig. 6). Moreover, in the MB6747 RNA, the replacement of the cryptic polyadenylation signal with a mooring sequence (UGAUCAGUAUA) had no effect on hyperediting of C6802. Therefore, the cryptic polyadenylation signal (AUUAAA) itself is not necessary for hyperediting at C6802. However, three A → G substitutions in this region abolished hyperediting at C6802 (Fig. 6, GB6747). Thus, there appears to be a preference, if not a necessity, for an AU-rich sequence in the 5′-flanking region 42–55 nt upstream of C6802.

To investigate the importance of the mooring-like motif (6809–6819) downstream of C6802, we used the constructs shown in Fig. 7. This mooring-like motif is located five nucleotides from the 3′ end of all short apoB RNAs investigated and is the only mooring-like motif downstream of C6802. In rabbit apoB RNA, this mooring-like motif matches only six out of the 11 nucleotides in the mooring sequence and has a 6-nt instead of a 4-nt spacer region (Fig. 7). In the 138-nt B6687 RNA template, all of the cytidines investigated were hyperedited except for C6743 (Fig. 7). Scrambling the mooring-like motif at 6809–6819 influenced hyperediting of cytidines up to 27 nt upstream, but hyperediting of C6762, located 47 nt upstream, was unaffected by this mooring-like motif.

To investigate this mooring-like motif at 6809–6819 without the influence of distal elements, we concentrated on the smallest apoB RNA template, B6747, that was still hyperedited at C6802. In contrast to the 138-nt B6687 RNA (Fig. 7), where scrambling of the mooring-like motif decreased hyperediting of C6802, in the smaller 78-nt RNA B6747, the same mutations abolished hyperediting at C6802 (Fig. 8). Changing the mooring-like motif to an exact 11-nt mooring sequence (B6747M) motif enhanced editing of C6802 2-fold. Furthermore, restoring the pattern of the mooring-like motif with an extra A in the spacer 9

DISCUSSION

FIG. 5.

FIG. 6.
Control of ApoB mRNA Editing

Physiological Editing of ApoB mRNA—We found that for normal physiological editing, an apoB RNA fragment of 139 nt consisting of the upstream 5′ efficiency element (6609–6628), an upstream 5′ A-rich element (6629–6640), the proximal efficiency sequence defined by Driscoll et al. (6648–6661) (11), the mooring sequence (6671–6681) (10–12), and the 3′ efficiency element (6717–6747) were sufficient for editing with an efficiency equal to that of full-length apoB mRNA. We also found evidence that sequences even further downstream (6747–6824) contributed to editing at C6666 (Fig. 10). The importance of the 5′ efficiency element for physiological editing was demonstrated by using short apoB RNA constructs in which the 5′ efficiency element was present or absent (Fig. 2) and by testing chimeric apoB/N-myc RNAs (Fig. 3). In these chimeric RNAs, the presence of the apoB 5′ efficiency element with the apoB editing cassette (6662–6687) was sufficient to give wild-type levels of editing at C6666. How the two elements increased editing of C6666 to wild-type levels is unknown. One possibility is the formation of Watson-Crick base pairing between the two elements. However, according to RNA folding data obtained with the Mfold program (29, 30), no secondary structures are favored between the 5′ efficiency element and the editing cassette (data not shown).

Our data also indicate that a second apoB RNA element supported physiological editing of C6666. This A-rich element is located at position 6629–6640, directly downstream of the 5′ efficiency element. Deletion and mutagenesis studies in this A-rich element influenced editing at C6666 severalfold (data not shown). Furthermore, a third proximal efficiency sequence at positions 6648–6661 was reported to promote editing in combination with the mooring sequence (11).

Independent of other sequences, the 3′ efficiency element of the apoB mRNA increased editing at C6666. A previous study indicated that 3′-flanking sequences of the apoB mRNA increase editing at C6666 (14), but no efficiency element was defined. Our results indicate that the 3′ efficiency element located within a 31-nt fragment (6717–6747) was necessary for efficient physiological editing in the apoB RNA (Fig. 1). It also increased editing in combination with the apoB editing cassette (Fig. 3), although to a lesser extent than did the 5′ efficiency element. Furthermore, in the presence of the 5′ efficiency element, the 3′ efficiency element did not further increase editing (Fig. 3), indicating that the 5′ efficiency element had a stronger impact on editing of C6666 in small apoB RNA at least in vitro.

Physiological editing at C6666 may also be enhanced by elements more than 81 nt from the editing site. Although these sequences were not required for efficient editing, the apoB RNA region encompassing C6662 may function as a recognition element for the editing complex (Figs. 4 and 10). According to our two-step model for apoB mRNA editing, such recognition could bring the editing complex into closer proximity to the editing site at C6666 (22). In a second step, the enzyme complex may then bind with a much higher affinity to the editing site at C6666 than to the sequence elements that compose the binding site for the C6662 (Fig. 5).
Hyperediting of ApoB mRNA—When APOBEC-1 is overexpressed, multiple cytidines are hyperedited (21–23). Detailed examination of the editing of one of these cytidines, C^6666, in small apoB RNAs showed that sequences similar to those important in physiological apoB mRNA editing are also involved in the hyperediting of C^6666. Hyperediting at C^6666 was supported by three RNA regions in the apoB mRNA: a proximal 5'-flanking region (6747–6759), a mooring-like motif (6809–6819), and a 3'-flanking region (6853–6882) (Fig. 10). The 5'-flanking region has a greater influence than the 3'-flanking region on the hyperediting of C^6666. A proximal 5'-flanking region (6747–6759) and 5' sequences upstream from that element plus the mooring-like motif were all that was necessary for high level (11.8%) hyperediting of C^6666 (Fig. 5). Like the 5'-rich element that enhanced physiological physiological apoB mRNA editing, the proximal 5'-flanking region (6747–6759) that enhanced the hyperediting of C^6666 is AU-rich (Fig. 6). Three A → G substitutions in this element abolished hyperediting (Fig. 6). The importance of the 3'-flanking region for the hyperediting of C^6666 was demonstrated in the experiment shown in Fig. 9. An apoB RNA substrate lacking the proximal 5'-flanking region was not hyperedited at C^6666 until the 3'-flanking region (6853–6882) was added. Intriguingly, the minimal size of flanking regions required for hyperediting at C^6666 was comparable to that required for efficient physiological editing at C^6666. Hyperediting and physiological editing required 55–57 nt of upstream flanking sequences and approximately 80 nt of downstream flanking sequences (Fig. 10). The similar size of the required flanking sequences and the importance of the mooring-like motif for hyperediting at C^6666 (Fig. 8) suggest that hyperediting depends on loose recognition of RNA features that define the physiological editing site at C^6666.

Besides the flanking sequences, a mooring-like motif supports hyperediting at C^6666. Several mutagenesis studies demonstrated that physiological apoB mRNA editing depends on the highly conserved mooring sequence and the spacer region (10–13). Single substitution mutations in the mooring sequence either drastically decreased or abolished editing (10, 12), and alterations in the 4-nt spacer decreased editing severalfold (10, 13). However, the mooring-like motif downstream of C^6666 has only 6 of the 11 nt in the mooring sequence and has a spacer region of 6 rather than 4 nt. Furthermore, it consists of seven Us and four As and lacks the TGAT motif previously suggested to support hyperediting (23). Nevertheless, this mooring-like motif supported hyperediting of C^6666, and scrambling the mooring-like pattern abolished hyperediting of C^6666 in short apoB RNAs and reduced hyperediting in longer apoB RNAs (Figs. 7 and 8). Furthermore, the introduction of an exact mooring motif (6747M) increased hyperediting at this site 2-fold (Fig. 8). These results emphasize that the mooring-like pattern is essential for hyperediting at C^6666 and that not every AU-rich sequence supports hyperediting at C^6666, although APOBEC-1 has been shown to bind to AU-rich RNA (31, 32).

The mooring-like motif at C^6666 supports hyperediting of C6782 located up to 27 nt upstream. When the mooring-like motif downstream of C^6666 was mutated, hyperediting of C^6666 was diminished or abolished (Figs. 7 and 8). Furthermore, both mutations abolished hyperediting at C^6666 (Fig. 7), indicating that hyperediting of C^6666 depends also on the mooring-like motif located 26–27 nt farther downstream. Hence, hyperediting has a relaxed spacer constraint compared with physiological editing, which was abolished by increasing the spacer to 12 nt (10, 11, 33).

Our in vitro study shows that the essential elements for physiological editing at C^6666 of the apoB mRNA encompass 139 nt, consisting of defined upstream efficiency elements, the mooring sequence, and the 3’ efficiency element. Further investigations on the secondary structure of the apoB mRNA editing locus are needed to define the interaction of these elements. Our study also shows that similar RNA features are necessary for the hyperediting of cytidines when APOBEC-1 is overexpressed.

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