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Three distinct RNA sequence elements are required for efficient apolipoprotein B (apoB) RNA editing *in vitro*

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ABSTRACT

Apolipoprotein B (apoB) mRNA is edited in rat liver and intestine to convert a CAA glutamine codon to a UAA translational stop codon by the direct conversion of cytidine to uridine at nucleotide 6666. We have proposed the 'mooring sequence' model for apoB RNA editing, in which editing complexes (editosomes) assemble on specific apo B mRNA flanking sequences to direct this site-specific editing event. One sequence element (approx. nts 6671 – 81, the presumed 'mooring sequence') has been previously identified as necessary for editing. We have identified two additional sequence elements which are necessary for efficient editing: (1) a 5′ 'Regulator' region which modulates editing efficiency and (2) a 'Spacer' region between the editing site and the 3′ mooring sequence, whose distance is critical for efficient editing. Utilizing this data, we have induced editing at a cryptic site and have defined a 22 nucleotide 'cassette' of specific apoB sequence which is sufficient to support wild-type levels of editing *in vitro* in a background of distal apoB RNA sequence.

INTRODUCTION

In rats, apolipoprotein B (apoB) exists as high molecular weight ($apoB_H$) and low molecular weight ($apoB_L$) isoforms. $ApoB_L$ is synthesized from the same primary transcript as $apoB_H$ through the direct editing of cytidine to uridine at nucleotide 6666, leading to the production of a UAA stop codon at codon 2153 (1,2). This reaction appears to be carried out by a site-specific cytidine deaminase (3), although direct replacement of the cytosine base has not been ruled out. Recently, it has been shown that mRNAs which code for glutamate receptor subunits in mouse and rat brain undergo an adenosine to guanosine conversion, possibly via a similar deamination mechanism (4). Diverse forms of RNA editing have also been described in the mitochondria of several organisms, including kinetoplastid protozoa (5–7), slime mold (8), and several plants (9).

Deletion mutagenesis has shown that 26 nucleotides of specific apoB sequence encompassing the editing site (6662–6687) are sufficient to support site-specific editing in an *in vitro* assay (10,11), although editing is more efficient with longer apoB RNA constructs (10,12). Mutant RNAs containing additional cytidines adjacent to the editing site (nucleotides 6665–6668) are also edited to a lesser extent at these residues (13). Deletion and point mutagenesis analyses of more distal sequences have shown that 3′ flanking sequence approximately encompassing nucleotides 6671–6681 are necessary (10,11) for site-specific editing, while 5′ flanking sequences and additional 3′ flanking sequences function to enhance editing efficiency (10).

These sequences have been proposed to function in part by binding proteins which are involved in the editing reaction. We have identified a 27S protein-containing macromolecular complex (the editosome) which specifically assembles on editing-competent apoB mRNA substrates *in vitro* (10,14). Assembly of this complex kinetically precedes the accumulation of edited RNA and inhibition of complex formation by vanadyl-ribonucleoside complexes (VRC) is accompanied by inhibition of editing. Translocation of 3′ sequences to an upstream CAA demonstrated that sequences 3′ of the editing site are necessary and sufficient for complex assembly and subsequent editing (10). Lau *et al.* (15), utilizing a different RNA gel mobility shift assay, have also described the sequence-specific binding of proteins to apoB mRNA substrates and have identified a 40 kDa protein which specifically UV crosslinks to apoB RNA sequences near the editing site (nucleotides 6661–6687). These results, along with the mutational analyses detailed above, support the 'mooring sequence' model for apoB mRNA editing, in which sequences flanking the editing site direct site-specific editing through interactions with specific proteins. We have proposed that cytidine deaminase activity within the editosome complex is positioned to scan for cytidines in the vicinity of nucleotide 6666. The term 'mooring' has been proposed for the apparent scanning mechanism which positions the deaminase over the site to be edited without actually docking it to a specific nucleotide. In this study we have constructed forward and revertant point mutations in apoB RNA sequence to further evaluate the roles of sequences 5′ of the editing site and of the position of the presumptive mooring sequence (approximately nts 6671–6681 in apoB mRNA sequence) relative to the nucleotide to be edited. The data presented here strongly support a 'mooring sequence' model in

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which proper positioning of the downstream mooring sequence relative to the editing site is required for efficient editing, while select sequences just 5′ of the editing site further regulate editing efficiency.

MATERIALS AND METHODS

Parental apoB plasmid constructs

Parental apoB construct pRSA13 contains 448 nts of specific apoB sequence (nts 6413−6860) and 50 nts of plasmid polylinker sequence (12,14). Translocation construct pRSA13Δ3′TL (TL) was prepared by digestion of pRSA13 with the restriction enzyme Bcl I (sites at nucleotides 6441 and 6672 in apoB sequence) and subsequent religation of the vector. In this construct, sequences 3′ of cytidine 6434 differ from those found at the wild-type editing site only by insertion of an A residue 4 nts downstream of the cytidine, while sequences 5′ of this cytidine are different than those found at the wild-type editing site (10). Parental clone 13-BD contains a single base mutation at nt 6445 (T to A) which destroyed the Bcl I site at nt 6441, enabling mutagenesis by the method of Vallette *et al.* (16). 13-BD supported a 1.6-fold higher level of editing than pRSA13, potentially due to mutation of a competing mooring sequence region (nts 6440−6450). ApoB construct US-6434Δ1 contains 351 nts of specific apoB sequence (nts 6262−6612) and 44 nts of plasmid polylinker sequence and was constructed by PCR cloning of a region of apoB construct pSX7 (10,12).

Site-directed mutagenesis

PCR mutagenesis was carried out either by the incorporation of a restriction site into the mutagenic oligonucleotide (16) utilizing the Bcl I restriction site at nt 6672. Mutant PCR products were subsequently double-digested with restriction enzymes, gel-isolated and cloned into double-digested gel-isolated parental vector. Clones of interest were characterized by double-stranded dideoxy sequencing of the mutagenized region utilizing Sequenase® 2.0 (United States Biochemical). Purified plasmid was isolated using Qiagen columns (Qiagen, Inc.).

Extract preparation

Rat enterocyte cytosolic S100 extracts were prepared as previously described (10). These extracts were stored at −20°C and remained stable for at least 6 months.

In vitro editing reactions and primer extension analysis

Plasmid DNAs were linearized with Kpn I (site in 3′ polylinker), transcribed with T7 RNA polymerase in the presence of ^{3}H-labeled ATP and capped *in vitro*. Mutant RNAs were then purified, quantitated and assayed for their ability to support editing *in vitro*. In the standard editing assay, 20 fmoles of RNA was incubated for 3 h at 30°C in a 50 μl reaction containing 10 mM Hepes pH 7.9, 10% glycerol (v/v), 50 mM KCl, 50 mM EDTA, 0.25 mM DTT, 40 units of RNasin® (Promega) and 60 μg of protein as rat enterocyte S100 extract. No edit controls (NEC's) were performed as described above except that incubation was at 0°C. Reactions were stopped as previously described (12), phenol-chloroform extracted, and ethanol precipitated. The purified RNA was then annealed to 30 fmoles of the appropriate ^{32}P-end-labeled DNA primer which was subsequently primer extended in the presence of an excess of ddGTP as described (10). Primer extension of unedited substrates terminates at the editing site (first stop, CAA), while primer extension of edited substrates terminates at the next cytidine 5′ of the editing site (second stop, TAA). Primer extension products were resolved on 10% denaturing acrylamide gels and subsequently autoradiographed. Editing efficiency was determined by direct scintillation counting of excised first and second stop gel bands.

Primer extension oligonucleotides

The following primer extension oligonucleotides were synthesized on a Milligen Cyclone™ Plus DNA synthesizer and gel isolated:

DD3: AATCATGTAAATCATAACTATCTTTAATATACTGA, 35-mer with 3′ end at 6674
JB-1: CTCTGTATTTTCTTACAAATTGATC, 25-mer with 3′ end at 6441
JB-3: TGTAAATCATAACTATCTTTAACGCCCTG, 29-mer with 3′ end at 6675
JBΔ93: CTGCTCTGTATTTTCTTACAAACTGATC, 28-mer with 3′ end at 6441
JBΔ94: CTGCTCTGTATTTTCTTACATATTGATC, 28-mer with 3′ end at 6441
JBΔ95: CTGCTCTGTATTTTCTTATAAATTGATC, 28-mer with 3′ end at 6441
JBΔ96: CTGCTCTGTATTTTCTTATATACTGATC, 28-mer with 3′ end at 6441

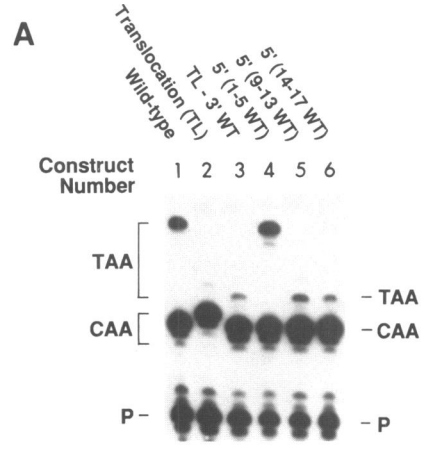
Oligonucleotides DD3 and JB-1 are complementary to wild-type human apoB sequence. JB-3, JBΔ93, JBΔ94, JBΔ95, and JBΔ96 are complementary to apoB mutants 13Δ6678−81GGCG, US-6434Δ3, US-6434Δ4, US-6434Δ5 and US-6434Δ6, respectively. Oligonucleotides were labeled with (γ-^{32}P)ATP and T4 polynucleotide kinase (United States Biochemical) by the vendor's protocol.

RESULTS

Sequence requirements 5′ of the editing site

We have previously reported that translocation of sequences 3′ of the apoB RNA editing site to an upstream cytidine induces editing of this cytidine (cytidine 6434, ref. 10). This construct, pRSA13Δ3′TL (TL, Figure 1, Lane 2), supported approximately 8% the level of *in vitro* editing observed at the wild-type editing site in parental construct pRSA13 (Lane 1). The TL construct has an A residue inserted 4 nt 3′ of the editing site and contains 5′ sequences different than those found at the wild-type editing site. Removal of the additional A residue (between the editing site and the mooring sequence, construct TL-3′WT, Lane 3) tripled the level of editing to 25% of wild-type. We attempted to further restore editing by reversion of sequences 5′ of the TL editing site to those of the wild-type editing site. Reversion of sequences 9−13 nucleotides and 14−17 nucleotides 5′ of the editing site (Lanes 5 and 6, respectively) had little effect on editing activity; however, reversion of sequences 1−5 nucleotides 5′ of the editing site (Lane 4) increased editing efficiency 6-fold, to 160% of wild-type.

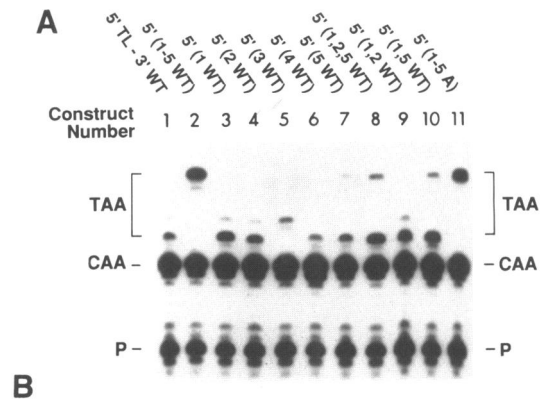
We further investigated the 5′ sequences required for this enhancement by creating single point-revertants in the region 1−5 nucleotides 5′ of the editing site and assaying the level of editing supported by these mutant constructs (Figure 2). Reversion of the nucleotide adjacent to the editing site (Lane 3) accounted for approximately 50% of the increase in editing efficiency, while reversion of sequences 2 and 5 nucleotides 5′ of the editing site (Lanes 4 and 7, respectively) had more modest (1.5−2-fold) effects. Double point-reversion mutants of 1 and 2 nucleotides (Lane 9) and 1 and 5 nucleotides (Lane 10) 5′ of the editing site



B

CONSTRUCT	SEQUENCE	RELATIVE EDITING
(1) Wild-type	5'-TGCAGACATATATGATACAATTTGATC-3'	100%
(2) TL	5'-aGaAacCtgAagcacatCAATaTTGATC-3'	7%
(3) TL - 3'WT	5'-aGaAacCtgAagcacatCAATTTGATC-3'	25%
(4) 5' (1-5 WT)	5'-aGaAacCtgAagTGATACAATTTGATC-3'	160%
(5) 5' (9-13 WT)	5'-aGaAGACATAagcacatCAATTTGATC-3'	30%
(6) 5' (14-17 WT)	5'-TGCAacCtgAagcacatCAATTTGATC-3'	25%

Figure 1. Primer extension analysis of editing supported by 5′ clustered point reversion mutants. **A)** ApoB RNAs were assayed for the ability to support editing in 60 μg rat enterocyte S100 extract as described in Materials and Methods. The products of the editing reactions were analyzed by primer extension in the presence of an excess of dideoxy-GTP. The positions of the primer (P) and the extension products generated from unedited (CAA) and edited (TAA) RNAs are indicated. The positions of the unedited (CAA) and edited (TAA) primer extension products are dependent on the position of the editing site relative to the primer and the position of the next cytidine in the RNA and varies between constructs. **B)** The sequence in the immediate vicinity of the editing site is shown for each construct. The cytidine which is being assayed for editing is outlined. Sequences different than those surrounding the wild-type editing site are in lower case and mutated bases are underlined. Editing efficiencies were determined as described in Materials and Methods and were normalized relative to parental construct pRSA13.



B

CONSTRUCT	SEQUENCE	RELATIVE EDITING
Wild-type	5'-TGATACAATTTGATC-3'	100%
(1) TL - 3'WT	5'-cacatCAATTTGATC-3'	25%
(2) 5' (1-5 WT)	5'-TGATACAATTTGATC-3'	160%
(3) 5' (1 WT)	5'-cacaACAATTTGATC-3'	85%
(4) 5' (2 WT)	5'-cacTtCAATTTGATC-3'	55%
(5) 5' (3 WT)	5'-caAatCAATTTGATC-3'	25%
(6) 5' (4 WT)	5'-cGcatCAATTTGATC-3'	20%
(7) 5' (5 WT)	5'-TacatCAATTTGATC-3'	40%
(8) 5' (1,2,5 WT)	5'-TacTACAATTTGATC-3'	100%
(9) 5' (1,2 WT)	5'-cacTACAATTTGATC-3'	75%
(10) 5' (1,5 WT)	5'-TacaACAATTTGATC-3'	80%
(11) 5' (1-5 A)	5'-aaAaACAATTTGATC-3'	125%

Figure 2. Primer extension analysis of editing supported by 5′ single point reversion mutants. **(A)** ApoB RNAs were assayed for the ability to support editing in 60 μg rat enterocyte S100 extract as described in Materials and Methods. The products of the editing reactions were analyzed by primer extension. The positions of the primer (P) and the extension products generated from unedited (CAA) and edited (TAA) RNAs are indicated. The length of the edited (TAA) primer extension product is dependent on the position of the next cytidine in the RNA and varies between constructs. **B)** The sequence in the immediate vicinity of the editing site is shown for each construct. The cytidine which is being assayed for editing is outlined. Sequences different than those surrounding the wild-type editing site are in lower case and mutated bases are underlined. Editing efficiencies were determined as described in Materials and Methods and were normalized relative to parental construct pRSA13.

had no additional effect beyond mutation of the nucleotide adjacent to the editing site. Simultaneous reversion of all three nucleotides (Lane 8) increased editing efficiency 4-fold, but did not match the stimulatory effect observed when all five bases were reverted. Interestingly, mutation of this region to all A's (Lane 11) had a similar stimulatory effect on editing as reversion of all five nucleotides to the sequences found 5′ of the wild-type editing site.

If sequences 1−5 nucleotides 5′ of the editing site are truly important in the regulation of editing efficiency, then mutations in this region which change wild-type sequences to those found in the translocation construct should impair editing. As predicted, mutation of the nucleotide adjacent to the wild-type editing site to the nucleotide present in this position of the translocation construct (Figure 3, Lane 2) reduced editing efficiency (almost 2-fold). Mutation of this entire region to translocation sequence (Lane 3) reduced editing efficiency at the wild-type editing site 6−7-fold, to approximately the same level observed with the translocation construct TL-3′WT. These results together provide strong evidence that sequences 1−5 nucleotides 5′ of the editing site regulate editing efficiency.

Sequence requirements between the editing site and the mooring sequence

The observation that the presence of an additional nucleotide between the editing site and the mooring sequence reduced editing efficiency more than 3-fold (Figure 1, TL vs TL-3′WT) suggests either that the sequence in this region is important or that the length of sequence between the editing site and the mooring sequence is important. To assess the sequence requirements in this region, we mutated nucleotides 6667−6670 (wild-type sequence AATT) to all A's, T's, or G's (Figure 4). Mutation of this region to all T's or A's (Lanes 3 and 4, respectively) reduced editing by only 30−40%, while mutation of this region to all G's (Lane 2) dropped editing efficiency to 40% of wild-type. G point mutations in this region (Lanes 5−8) showed that the inhibitory effect of G's in this region was maximized if the G residue was placed directly adjacent to the editing site (Lane 5). The data taken together suggest a lax sequence requirement in the region between the editing site and the mooring sequence.

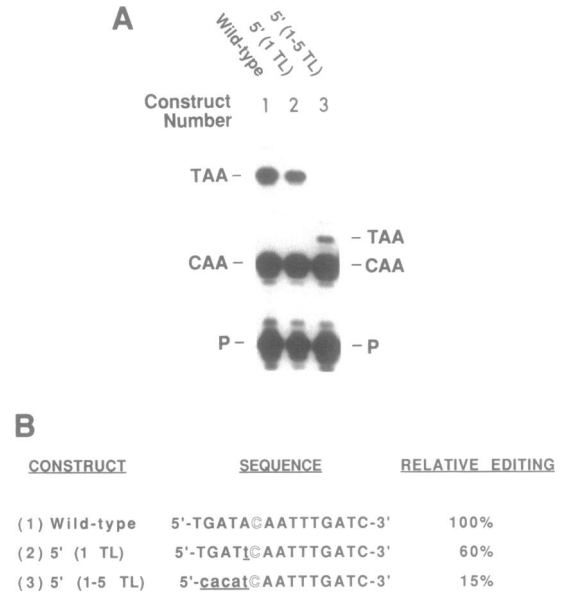


Figure 3. Primer extension analysis of editing supported by 5′ single and clustered point mutants. (**A**) ApoB RNAs were assayed for the ability to support editing in 60 μg rat enterocyte S100 extract as described in Materials and Methods. The products of the editing reactions were analyzed by primer extension. The positions of the primer (P) and the extension products generated from unedited (CAA) and edited (TAA) RNAs are indicated. The length of the edited (TAA) primer extension product is dependent on the position of the next cytidine in the RNA and varies between constructs. **B**) The sequence in the immediate vicinity of the editing site is shown for each construct. The cytidine which is being assayed for editing is outlined. Sequences different than those surrounding the wild-type editing site are in lower case and mutated bases are underlined. Editing efficiencies were determined as described in Materials and Methods and were normalized relative to parental construct 13-BD.

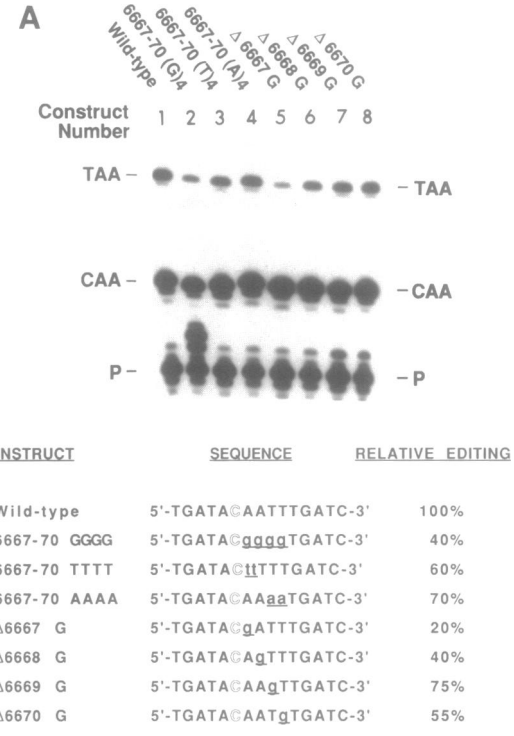


Figure 4. Primer extension analysis of editing supported by 'spacer region' mutants. (**A**) ApoB RNAs were assayed for the ability to support editing in 60 μg rat enterocyte S100 extract as described in Materials and Methods. The products of the editing reactions were analyzed by primer extension. The positions of the primer (P) and the extension products generated from unedited (CAA) and edited (TAA) RNAs are indicated. **B**) The sequence in the immediate vicinity of the editing site is shown for each construct. The cytidine which is being assayed for editing is outlined. Sequences different than those surrounding the wild-type editing site are in lower case and mutated bases are underlined. Editing efficiencies were determined as described in Materials and Methods and were normalized relative to parental construct 13-BD.

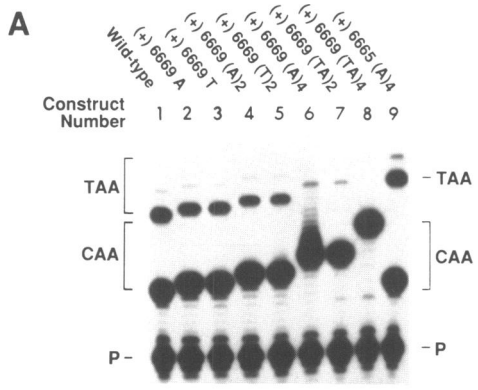
Addition and deletion of sequence between the editing site and the mooring sequence

The previous analyses suggest that any sequence added in the region between the editing site and the mooring sequence to evaluate the importance of distance in this region should consist of A and/or T residues. Therefore, we added varying amounts of A and/or T sequence between nucleotides 6668−6669 (Figure 5). Addition of a single nucleotide in this region (Lanes 2 and 3) reduced editing efficiency approximately 2-fold, while addition of 2, 4, and 8 nucleotides (Lanes 4 and 5, Lanes 6 and 7 and Lane 8, respectively) in this region reduced editing efficiency 4-fold, 12−20-fold, and 65-fold, respectively. These results suggest that small (1−2 nucleotides) insertions in this region are tolerated to some extent, while larger insertions of sequence severely impair editing. To rule out the possibility that the effects observed with sequence insertion were due to a change in the distance between the mooring sequence and specific sequences 5′ of the editing site, we inserted 4 A residues immediately 5′ of the editing site. The ability of this construct (Lane 9) to support editing at near wild-type levels demonstrates that the inhibitory effect of addition of sequence in this region is truly due to a change in the distance between the editing site and the downstream mooring sequence.

Single base deletions in the region between the editing site and the mooring sequence (Figure 6, Lanes 2 and 3) had effects similar to those seen with the addition of a single nucleotide (2−3-fold impairment of editing). Deletion of any 2 nucleotides in this region (Lanes 4−6), however, reduced editing efficiency 65−100-fold. Further deletion of sequence in this region (Constructs 7−9) reduced editing efficiency below the detection limit of scintillation quantitation (<0.5% of wild-type). Once again, the possibility that these effects were due to a change in the distance between the mooring sequence and sequences 5′ of the editing site was evaluated by the addition of 2 nucleotides immediately 5′ of the editing site in constructs which previously had 2 nucleotides deleted between the editing site and the mooring sequence (Constructs 10 and 11). The inability of these 5′ sequence additions to restore efficient editing demonstrates that the observed decrease in editing efficiency resulted from a reduction in the distance between the editing site and the mooring sequence.

Correction of 5′ sequences and 3′ spacer distance induces editing of a cryptic site in apoB sequence

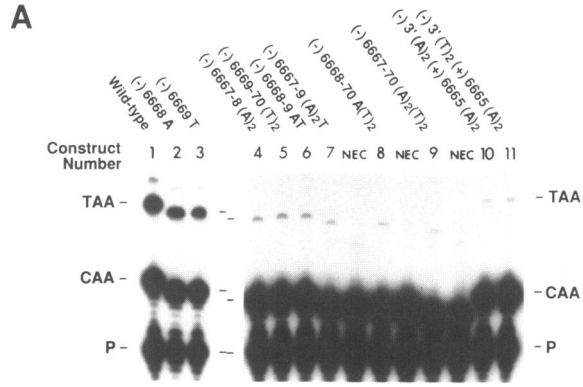
Our analyses have shown that at least three distinct sequence elements are required for efficient editing. This suggests that there may be cryptic mooring sequences which are not utilized for editing because they do not have a cytidine positioned at the correct distance and/or they have unsuitable sequences 5′ of this



B

CONSTRUCT	SEQUENCE	RELATIVE EDITING
(1) Wild-type	5'-TGATA©AATTTGATC-3'	100%
(2) (+) 6669 A	5'-TGATA©AAaTTTGATC-3'	50%
(3) (+) 6669 T	5'-TGATA©AAtTTTGATC-3'	45%
(4) (+) 6669 AA	5'-TGATA©AAaaTTTGATC-3'	25%
(5) (+) 6669 TT	5'-TGATA©AAttTTTGATC-3'	25%
(6) (+) 6669 A_4	5'-TGATA©AAaaaaTTTGATC-3'	8%
(7) (+) 6669 TATA	5'-TGATA©AAtatatTTTGATC-3'	5%
(8) (+) 6669 $(TA)_4$	5'-TGATA©AAtatatatatTTTGATC-3'	1.5%
(9) (+) 6665 AAAA	5'-TGATAaaaa©AATTTGATC-3'	80%

Figure 5. Primer extension analysis of editing supported by spacer region addition mutants. (**A**) ApoB RNAs were assayed for the ability to support editing in 60 μg rat enterocyte S100 extract as described in Materials and Methods. The products of the editing reactions were analyzed by primer extension. The positions of the primer (P) and the extension products generated from unedited (CAA) and edited (TAA) RNAs are indicated. The positions of the unedited (CAA) and edited (TAA) primer extension products are dependent on the position of the editing site relative to the primer and the position of the next cytidine in the RNA and varies between constructs. **B**) The sequence in the immediate vicinity of the editing site is shown for each construct. The cytidine which is being assayed for editing is outlined. Inserted sequences are in lower case and underlined. Editing efficiencies were determined as described in Materials and Methods and were normalized relative to parental construct 13-BD.



B

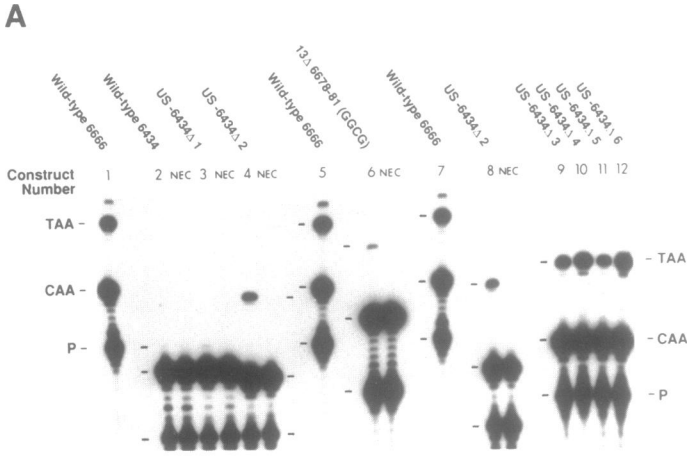
CONSTRUCT	SEQUENCE	RELATIVE EDITING
(1) Wild-type	5'-TGATA©AATTTGATC-3'	100%
(2) (-)6668 A	5'-TGATA©A*TTTGATC-3'	45%
(3) (-)6669 T	5'-TGATA©AA*TTGATC-3'	35%
(4) (-)6667-8 AA	5'-TGATA©**TTTGATC-3'	1%
(5) (-)6669-70 TT	5'-TGATA©AA**TGATC-3'	1.5%
(6) (-)6668-9 AT	5'-TGATA©A**TTGATC-3'	1.25%
(7) (-)6667-9 AAT	5'-TGATA©***TTGATC-3'	B.D.
(8) (-)6668-70 ATT	5'-TGATA©A***TGATC-3'	B.D.
(9) (-)6667-70 AATT	5'-TGATA©****TGATC-3'	B.D.
(10) (-)3'AA(+)6665AA	5'-TGATAAA©**TTTGATC-3'	1%
(11) (-)3'TT(+)6665AA	5'-TGATAAA©AA**TGATC-3'	0.6%

Figure 6. Primer extension analysis of editing supported by spacer region deletion mutants. (**A**) ApoB RNAs were assayed for the ability to support editing in 60 μg rat enterocyte S100 extract as described in Materials and Methods. The products of the editing reactions were analyzed by primer extension. The positions of the primer (P) and the extension products generated from unedited (CAA) and edited (TAA) RNAs are indicated. The positions of the unedited (CAA) and edited (TAA) primer extension products are dependent on the position of the editing site relative to the primer and the position of the next cytidine in the RNA and varies between constructs. NEC, no edit control reaction. **B**) The sequence in the immediate vicinity of the editing site is shown for each construct. The cytidine which is being assayed for editing is outlined. Nucleotides which have been deleted are denoted by a '*'. Editing efficiencies were determined as described in Materials and Methods and were normalized relative to parental construct 13-BD. B.D., below detection limit.

cytidine to support efficient editing. This possibility is supported by the dramatic enhancement (20-fold) of editing activity observed when the translocation construct pRSA13Δ3'TL (TL) has both the distance between its mooring sequence and editing site corrected and the sequences 1−5 nucleotides 5′ of the editing site reverted to those found in the wild-type construct (Figure 1, Lanes 2 and 4). We directly tested the ability to activate a cryptic editing site utilizing a construct which contains cytidine 6434 and its surrounding sequence, but which lacks the wild-type editing site (US-6434Δ1). As detailed in Figure 7, this potential editing site contains an imperfect mooring sequence (3 nucleotides out of 11 different than the wild-type mooring sequence), an extra A residue in the region between the editing site and the mooring sequence and sequences 5′ of the editing site which are different than those of the wild-type editing site. Cytidine 6434 is not edited in the parental construct pRSA13 (Figure 7, Construct 2 and ref. 10). Removal of the wild-type editing site (US-6434Δ1, Construct 3) induced a detectable, but not quantitatible (<0.5% of wild-type) level of editing at cytidine 6434. Correction of the distance between this cytidine and the imperfect downstream mooring sequence, concomitant with mutation of the region 1−5 nt 5′ of cytidine 6434 to the sequence found at the wild-type editing site (US-6434Δ2, Construct 4), induced editing of cytidine 6434 at 20% of the level supported by the wild-type construct pRSA13.

The data of Shah *et al.* (11) suggest that the entire mooring sequence present 5−15 nucleotides 3′ of the wild-type editing site (nts 6671−81 in apoB sequence) is necessary for efficient editing, with the possible exception of nucleotide 6677. The construct which we are testing (US-6434Δ2) differs from the wild-type editing site at 3 out of 11 nucleotides in this region, although one of these is at nucleotide 6677 and therefore may be tolerated. The two potentially deleterious differences in the mooring sequence region of US-6434Δ2 may account for the diminished level of editing supported by this construct. This possibility is supported by the low level of editing (1% of wild-type) observed with a construct which contains 4 consecutive mutations in this region, all of which are predicted from the data

A



B

	CONSTRUCT	SEQUENCE	RELATIVE EDITING
(1,5,7)	Wild-type - nt 6666	5'-TGATA©AATTTGATCAGTATA-3'	100%
(2)	Wild-type - nt 6434	5'-cacat©AATaTTGATCAaTtTg-3'	N.D.
(3)	US-6434Δ1	5'-cacat©AATaTTGATCAaTtTg-3'	B.D.
(4,8)	US-6434Δ2	5'-TGATA©AATTTGATCAaTtTg-3'	20%
(6)	13Δ6678-81 GGCG	5'-TGATA©AATTTGATCAGggcg-3'	1%
(9)	US-6434Δ3	5'-TGATA©AATTTGATCAGTtTg-3'	30%
(10)	US-6434Δ4	5'-TGATA©AATTTGATCAaTATg-3'	100%
(11)	US-6434Δ5	5'-TGATA©AATTTGATCAaTtTA-3'	25%
(12)	US-6434Δ6	5'-TGATA©AATTTGATCAGTATA-3'	95%

Figure 7. Primer extension analysis of editing supported by mutants which correct sequences surrounding a cryptic editing site. (**A**) ApoB RNAs were assayed for the ability to support editing in 60 μg rat enterocyte S100 extract as described in Materials and Methods. The products of the editing reactions were analyzed by primer extension. The positions of the primer (P) and the extension products generated from unedited (CAA) and edited (TAA) RNAs are indicated. The positions of the unedited (CAA) and edited (TAA) primer extension products are dependent on the position of the editing site relative to the primer, the position of the next cytidine in the RNA and varies between constructs and the length of the primer extension primer. NEC, no edit control reaction. **B**) The sequence in the immediate vicinity of the editing site is shown for each construct. The cytidine which is being assayed for editing is outlined. Sequences different than those surrounding the wild-type editing site are in lower case and mutated bases are underlined. Editing efficiencies were determined as described in Materials and Methods and were normalized relative to parental constructs pRSA13 and 13-BD. N.D., not detectable. B.D., below detection limit.

of Shah *et al.* to be deleterious to the ability to support editing (mutant 13Δ6678−81 GGCG, Construct 6).

Individual reversion of these bases (11 and 15 nucleotides 3′ of the editing site) in construct US-6434Δ2 (Constructs 9 and 11, respectively) had little stimulatory effect on editing efficiency, while mutation of the base 13 nucleotides downstream (Construct 10) to the sequence found in the wild-type mooring sequence increased editing efficiency to wild-type levels. Reversion of all three nucleotides simultaneously (Construct 12) also induced wild-type levels of editing. These results support previous evidence which has shown that the sequences present in the mooring sequence region are critical for efficient editing (11,10). The data presented here taken together suggest that the distance between the editing site and the 3′ mooring sequence, the sequences 1−5

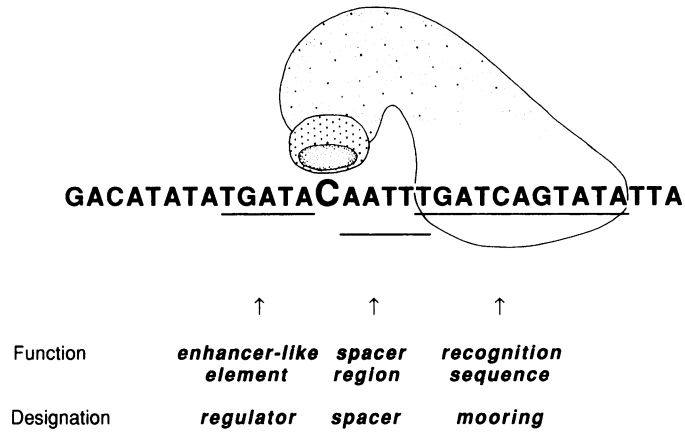


Figure 8. The 'mooring sequence' model for apoB mRNA editing. The mooring sequence model originally proposed by Smith *et al.* (14) has been modified to reflect the presence of a single mooring sequence, as well as to indicate the positions of the 'regulator' and 'spacer' sequence elements described in the Discussion.

nucleotides 5′ of the editing site and the sequences present in the mooring sequence region together determine the level of *in vitro* editing which can be supported at any given cytidine residue.

DISCUSSION

What constitutes a C→U RNA editing site?

The purpose of this study was to define the sequence elements which are required for efficient apoB RNA editing *in vitro*. We have evaluated the role of sequences flanking the editing site in the regulation of apoB mRNA editing. Our data suggest that apoB mRNA editing has at least three distinct sequence requirements beyond the presence of a cytidine to support editing (see Figure 8):

(1) The 'mooring sequence' (wild-type site at nts 6671−81 in apoB mRNA sequence) is required for editing and is proposed to be the initial site for protein recognition and binding.

(2) The length of the 3′ 'spacer' sequence element (nts 6667−70) is proposed to be necessary for correct positioning of the editing activity relative to the editing site.

(3) The upstream 'regulator' sequence element (nts 6661−5) is proposed to modulate the level of editing through secondary structure interactions and/or enhancement of editing site utilization by editing factors.

One of these sequence elements, the mooring sequence, has been previously characterized and defined (11,10). This sequence itself is clearly not sufficient to direct efficient editing of a cytidine in all contexts. For example, the entire mooring sequence is contained in translocation construct 13Δ3′TL (TL), yet it edits at 8% of the level supported by wild-type construct pRSA13. All of the constructs which have had sequences added and deleted in the 'spacer' sequence element also contain an intact mooring sequence, yet some edit at levels below the detection limit of the primer extension assay. Beyond the presence of the mooring sequence, a cytidine residue at the proper distance 5′ of this sequence is also necessary. Sequences just 5′ of this cytidine (the 'regulator' sequence element), especially the nucleotide adjacent to the editing site, also have major effects on editing efficiency.

The level of editing supported by these specific sequences is also dependent on the context into which they are placed. For example, these sequences placed in the context of a small construct containing 55 nucleotides of specific apoB RNA sequence edits 80-fold more efficiently than a construct with 26 nucleotides of specific sequence (10). A smaller amount (20−22 nucleotides) of specific sequence, however, supports wild-type levels of editing when placed in the context of large (approximately 400 nucleotides) apoB constructs (US-6434Δ4 and US-6434Δ6).

The 'spacer' region data suggest that there may be structural limitations in the ability of the cytidine deaminase to interact with RNA and/or scan for cytidines which favors (≥25% of wild-type editing levels) cytidines which are 4−7 nucleotides 5′ of the mooring sequence, with an optimal distance of 5 nucleotides. It is not known whether the editing process described above could be carried out by one multifunctional enzyme or by a macromolecular complex containing specific protein subunits for recognition and catalysis. Data from our lab (10,14, Harris *et al.*, submitted) supports recognition and catalysis by a macromolecular complex (the editosome).

The 'regulator' sequence element

We have defined and mapped a specific region 5′ of the editing site which regulates the efficiency of apoB RNA editing. We have shown by clustered-point mutagenesis that sequences 1−5 nucleotides 5′ of the editing site dramatically regulate the level of editing at this site. Mutation of sequences in the regulator region of the translocation construct TL-3′WT to those found at the wild-type editing site increases editing efficiency 6−7-fold; correspondingly, forward mutation of wild-type sequences in this region to the sequences present in the translocation mutant depresses editing to a similar extent (to 15% of wild-type levels). The nucleotide immediately 5′ of the editing site accounts for approximately 50% of this effect. Mutation of this nucleotide in the translocation construct to that found at the wild-type editing site stimulates editing at least 3-fold, while mutation of this nucleotide in the wild-type construct decreases editing efficiency almost 2-fold. We have demonstrated that mutation of this region to all A residues, either in the context of the wild-type construct or the translocation construct, accomplishes near wild-type levels of editing. This is the same sequence found immediately 5′ of the additional editing site discovered at nucleotide 6802 in apoB mRNA sequence (17). This site is edited at a low level both *in vitro* and *in vivo* even though it contains a compromised mooring sequence.

Previously, we have shown that removal of the region 5−17 nucleotides 5′ of the editing site from a small construct (pBS55) reduces editing efficiency >10-fold (10). This removal cannot be compensated for by addition of 5′ polylinker distal to the apoB sequence. Reversion of sequences in this region of the translocation mutant 13Δ3′WT, however, had little effect on editing efficiency. These conflicting results support the concept that the context in which a mutation is made is important in determining the effect of that mutation on editing efficiency. For example, the 5′ polylinker present in the small constructs described above is GC-rich (68%); in contrast, the 100 nucleotides 5′ of cytidine 6666 in apoB sequence contains 49 A residues and 26 T residues for a combined AT content of 75%. In fact, the entire apoB insert present in the wild-type construct pRSA13 (nts 6413−6860) is 73% AT (195 A's and 130 T's out of 448 nts). For these reasons, we carried out our mutational analyses in the context of pRSA13, a large wild-type apoB construct.

The 'spacer' sequence element

The observation that correction of the distance between the mooring sequence and the editing site in the translocation construct 13Δ3′TL (TL) induces a 3-fold increase in editing activity suggested to us that this distance may be a critical factor regulating editing of cytidines upstream of potential mooring sequences. This possibility is supported by the data of Shah *et al.* (11), who observed that deletion of a single nucleotide from this region of the construct pBS55 (17E37) depresses editing efficiency to approximately 40% of wild-type. We observed that small additions of sequence in this region are somewhat tolerated, with 2-fold and 4-fold decreases in editing efficiency observed with the addition of 1 and 2 nucleotides of A and/or T sequence between nucleotides 6668 and 6669. Similar effects are observed with the insertion of 1 and 2 nucleotides of random sequence between nucleotides 6670 and 6671 (data not shown). Larger additions of sequence in this region are much more deleterious to editing; additions of 4 and 8 nucleotides of sequence in this region reduced editing efficiency 12−20-fold and 65-fold, respectively. Deletions in this region are even more detrimental to editing. Removal of a single base in this region reduces editing efficiency only 2.5-fold, but removal of any 2 nucleotides in this region results in a 65−100-fold reduction in editing efficiency. Removal of additional nucleotides drops editing levels below the quantitation limit of the assay (<0.5% of wild-type).

In the case of additions or deletions of sequence, it could be argued that the reductions in editing efficiency could result from changing the distance between the mooring sequence and sequences 5′ of the editing site. This possibility was ruled out in the case of deletions of sequence by adding back sequence 5′ of the editing site in an attempt to recover editing efficiency. No increases in editing efficiency are induced by these additions of sequence. Similarly, addition of 4 nucleotides 5′ of the editing site has little effect on editing efficiency, again showing that the decrease in editing efficiency observed when sequence is added 3′ of the editing site results from a change in the distance between the mooring sequence and the editing site.

Induction of editing at a cryptic site

A strong proof of the importance of sequences 5′ of the editing site and of the distance between an editing site and a mooring sequence is to be able to induce editing of a non-edited site by correction of these sequences to those found at the wild-type site. We carried out this test in the context of cytidine 6434, which contains a 3′ sequence with 8 out of 11 nucleotides identical to those in the wild-type mooring sequence region shown to be required for editing. This site has the same sequences 5′ of this impaired mooring sequence as those found in the translocation construct 13Δ3′TL. Removal of the additional A residue between cytidine 6434 and the impaired 3′ mooring sequence, concomitant with mutation of nucleotides 6429−6433 to the sequence present 1−5 nucleotides 5′ of cytidine 6666, induces editing of cytidine 6434 at 20% of the level supported by the wild-type construct pRSA13. This result is very strong evidence that these two regions are essential for efficient editing.

We have further shown that wild-type levels of editing may be imparted upon this enhanced cryptic editing site (in

US-6434Δ2) by reconstruction of a wild-type mooring sequence. Mutation of the base 13 nucleotides 3′ of cytidine 6434 to the base found in the wild-type mooring sequence and collective reversion of all three nucleotides both induce wild-type levels of editing. It is surprising that the single base revertant edits at wild-type levels, given that it contains two differences in the mooring sequence region, one of which has been shown by Shah *et al.* (11) to inhibit editing by approximately 75% in the context of the small wild-type apoB construct pBS55. Several possibilities could account for this discrepancy, including interactions in secondary structure between sequences 5′ and 3′ of the editing site and/or general context differences {AT-rich apoB sequence (data presented here) versus GC-rich polylinker sequence (11)}.

Our results suggest that 20−22 nucleotides of specific apoB RNA sequence (5 nucleotides upstream, a cytidine, and 14−16 nucleotide downstream) are all that are necessary to support wild-type levels of editing in the context of AT-rich apoB RNA sequence. We have found, however, that a similar amount of specific apoB RNA sequence is not sufficient to support wild-type levels of editing when placed in the context of a human albumin construct (data not shown). This result is consistent with the conflicting *in vivo* data of Davies *et al.* (18) and Boström *et al.* (3). Davies *et al.* found that 26 nucleotides of specific apoB sequence, when placed in the context of approximately 900 nucleotides of distal apoB sequence, is sufficient to support high levels of editing in a rat hepatoma cell line (McArdle 7777); in contrast, Boström *et al.* found that 63 nucleotides of specific apoB sequence is insufficient to support editing in human colon adenocarcinoma (CaCo-2) and normal mouse liver (BNL CL.2) cell lines when placed in the context of an apoE construct which is approximately 70% GC.

CONCLUSIONS

We have presented direct evidence for the role of three sequences in the apoB RNA editing reaction: (1) the previously described 'mooring sequence', (2) the 'spacer' sequence element between the mooring sequence the the edited cytidine, and (3) the 'regulator' sequence element, present 1−5 nucleotides 5′ of the edited cytidine. Our data show that all three of these sequence elements are essential in order to direct efficient editing *in vitro*. We have further shown that correction of these sequences surrounding a cryptic editing site to those found at the wild-type apoB RNA editing site is able to impart wild-type levels of editing to the cryptic site.

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