Commitment of apolipoprotein B RNA to the splicing pathway regulates cytidine-to-uridine editing-site utilization

Mark P. SOWDEN*† and Harold C. SMITH*†‡§[1]

*Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, U.S.A., †Department of Pathology, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, U.S.A., ‡Environmental Health Sciences Center, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, U.S.A., and §The James P. Wilmot Cancer Center, University of Rochester School of Medicine and Dentistry, 601 Elmwood Avenue, Rochester, NY 14642, U.S.A.

A tripartite motif located in the centre of the 7.5 kb exon 26 of apolipoprotein B (apoB) mRNA directs editosome assembly and site-specific cytidine-to-uridine editing at nucleotide 6666. apoB mRNA editing is a post-transcriptional event, occurring primarily at the time exon 26 is spliced or at a time after splicing, but before nuclear export. We show, through reporter RNA constructs, that RNA splice sites suppress editing of precursor RNAs when placed proximal or distal to the editing site. Processed RNAs were edited more efficiently than precursor RNAs. Mutation of both the splice donor and acceptor sites was necessary for RNAs to be edited efficiently. The results suggested that commitment of pre-mRNA to the splicing and/or nuclear-export pathways may play a role in regulating editing-site utilization. The HIV-1 Rev–Rev response element ('RRE') interaction was utilized to uncouple the commitment of precursor RNAs to the spliceosome assembly pathway and associated nuclear-export pathway. Under these conditions, unspliced reporter RNAs were edited efficiently. We propose that pre-mRNA passage through the temporal or spatial restriction point where they become committed to spliceosome assembly contributes regulatory information for subsequent editosome activity.

Key words: editsome, nucleus, pre-mRNA, transcription.

INTRODUCTION

RNA editing, the co- or post-transcriptional alteration of RNA sequence other than that arising from splicing and poly-adenylation, is becoming recognized as a widespread phenomenon amongst eukaryotes as a means of increasing the diversity of protein isoforms from that which is encoded in the genome. The most thoroughly characterized mammalian RNA editing activities are those involved in the deamination of cytidine to form uridine in apolipoprotein B (apoB) mRNA, and of adenosine to form inosine in neuronal glutamate receptor (GluR) and 5-hydrotryptamine receptor subunits [1–4].

Adenosine-to-inosine editing of pre-mRNA substrates requires adenosine deaminases active on RNA (ADARs) and the presence of a complementary intron sequence to form a partially double-stranded RNA structure that is recognized by the appropriate ADAR1 or ADAR2 deaminase [3,5,6]. The critical role of *cis*-acting intronic sequences indicates that deamination is a nuclear event and must occur prior to pre-mRNA splicing. Editing of the ADAR2 transcript by its own translation product to create a new alternative 5′ splice acceptor sequence further underscores the temporal and functional relationship between RNA splicing and adenosine-to-inosine editing [7].

Under physiological conditions, apoB RNA editing is a nuclear event [8,9]. It was first evident at the time pre-mRNAs were polyadenylated and spliced and was completed by the time the pre-mRNAs had been fully processed [8,10]. The results suggested that editing occurred maximally at the time exon 26 containing the editing site was spliced or a point thereafter, but that pre-mRNA may not be the preferred substrate. An explanation for this is that, despite the fact that the apoB editing site resides in the centre of the 7.5 kb exon 26, editosome assembly and/or function may be inhibited by spliceosome assembly and/or function. In this regard, 500–2000 nt of apoB RNA expressed from cDNA edited with a 2-fold or greater efficiency in McArdle rat hepatoma cells than the natively expressed apoB mRNA [10,11]. Cytidine-to-uridine editing required only a 21 nt tripartite motif surrounding the edited cytidine residue. This motif includes an 11-nucleotide mooring sequence 3′ of C^{6666}, a 4 nt spacer element between the edited cytidine and the mooring sequence and an enhancer element immediately upstream of C^{6666} [12–14]. Though less efficient, transcripts as short as 55 nt supported editing [11,15,16], and the tripartite motif alone supported editing when placed in a foreign RNA context [12,17]. Subsequently, additional sequences 5′ and 3′ of the tripartite motif were shown to contain efficiency elements [15,16,18,19] and secondary structure [13,14,20,21] that promoted editosome assembly and editing activity. These findings suggest that the editing site should be efficiently utilized with a sufficient amount of flanking sequence, and therefore raise the question of why exon 26 in pre-mRNA does not edit efficiently before splicing is completed.

Cytidine deamination *in vitro* and *in vivo* required a multi-protein complex or 'editosome' [22–25]. Editosome assembly in cells [22,26] or *in vitro* [11,15,22,27] required a complex protein composition with an aggregate size of 27 S. Minimally, *in vitro* editing activity required a homodimer of APOBEC-1 (catalytic subunit for cytidine-to-uridine editing of apolipoprotein B mRNA-1), the zinc-dependent catalytic subunit of cytidine deaminase [28,29] and a 65 kDa RNA binding protein, ACF/ASP (<u>A</u>POBEC-1 <u>c</u>omplementation <u>f</u>actor/<u>A</u>POBEC-1 <u>s</u>timulatory <u>p</u>rotein) [30,31]. APOBEC-1 demonstrated a weak and non-specific RNA binding activity to AU-rich apoB

---

sequences [32] and alone could not edit apoB mRNA. Editing-site specificity and RNA editing activity are imparted upon APOBEC-1 through its interactions with ACF/ASP [30,31] and potentially other auxiliary proteins which bind to APOBEC-1 and/or apoB mRNA and modulate the efficiency of the editing reaction [23,24,33–35]. In terms of the potential effect of pre-mRNA splicing on apoB mRNA editing, some of the auxiliary proteins implicated in apoB mRNA editing, including hetero-geneous ribonucleoprotein (hnRNP) C, hnRNP D, APOBEC-1-binding protein and KSRP (KH type splicing regulatory protein) have a role in pre-mRNA splicing and/or hnRNP structure [31,36–38]. Tissue-specific heterogeneity in auxiliary protein expression and function [16,27], and the widespread expression of the auxiliary proteins, even in cells that lack apobec-1 or apoB mRNA [23,24,26,30,33], has suggested an additional role(s) for these auxiliary proteins.

The 'gating hypothesis' was proposed to account for the observation that the proportion of apoB mRNAs edited within an intracellular population was a function of the number of editosomes in the cell and not, paradoxically, dependent upon the number of transcribed apoB mRNA substrates. The 'gate' at which a decision to edit or not edit an RNA is made was envisioned as a nuclear restriction point temporally or spatially related to pre-mRNA splicing through which all apoB mRNAs must pass during processing [10]. The present study demonstrates that, in wild-type McArdle rat hepatoma cells, the ability of pre-mRNAs to commit to the spliceosome assembly pathway de-termined the editing efficiency of these RNAs. Moreover, RNAs that would otherwise not be edited efficiently due to splice-site interference, could be efficiently edited when the 5′ and 3′ splice sites were mutated or when interactions with the splicing ma-chinery were by-passed utilizing the Rev–Rev-response element (RRE) interaction. The results suggested that the regulation of editing-site utilization is consequential to the commitment of pre-mRNAs to the splicing and associated nuclear-export pathways.

EXPERIMENTAL

All plasmids were constructed by standard recombinant DNA methods and verified by DNA sequencing. The intervening sequence (IVS)–apoB construct has been described previously [10]. Mutation of 6 bp at the 5′ splice donor sequence, including the intronic GU dinucleotide (IVS–Δ5′apoB) and deletion of 20 bp encompassing the 3′ splice acceptor and polypyrimidine tract sequences (IVS–Δ3′apoB), was accomplished by 'run-around' PCR using primers that included an XhoI site to facilitate subsequent re-ligation of the PCR product [39]. IVS–Δ3′5′apoB was created by ligation of the appropriate halves of the above molecules.

McArdle RH7777 cells were maintained as previously de-scribed [40] and transfected in six-well clusters with 2 μg of DNA using LIPOFECTAMINE® (Gibco BRL) according to the manufacturer's recommendations. RNAs were harvested 48 h post-transfection in TriReagent (Molecular Research Center, Cincinnati, OH, U.S.A.) and subjected to reverse-transcriptase (RT)-PCR for amplification of intron-containing or exonic apoB specific transcripts using appropriate PCR primers as previously described [10] and outlined in the Figure legends. Editing efficiencies were determined by poisoned-primer-extension assay on purified PCR products [10] and quantified by analysis on a PhosphorImager (model 425E; Molecular Dynamics).

The poisoned-primer-extension assay relies on the annealing of a ^{32}P-end-labelled primer 3′ of the editing site to the heat-denatured single-stranded PCR product. Extension of this primer

using RT in the presence of dATP, dCTP, dTTP and dideoxy (dd)-GTP produces an extension product eight nucleotides longer if the cytidine has not been edited (CAA in the Figures); that is, incorporation of ddGTP causes chain termination. If editing has created a uridine, then primer extension continues a further 11 nucleotides to the next 5′ cytidine, where chain termination then occurs (UAA in the Figures). Quantification of the level of editing is accurately determined using laser scanning densitometry. The linear exposure range of the PhosphorImager screen is sufficiently great to permit precise determination of low counts in the UAA bands whilst the high levels of counts in the CAA band remain in the linear range. Editing percentages were calculated as the counts in the UAA band divided by the total counts in the CAA plus UAA bands times 100. This assay has a lower level of detection of 0.1% editing and remains linear up to 99.5% and is independent, between 1 ng and 500 ng, of the total amount of template PCR product used (M. P. Sowden, unpublished work).

Rev complementation/editing assays [41] were performed in duplicate in McArdle cells seeded in six-well clusters. Briefly, a total of 2 μg of DNA, comprising 1 μg of reporter DNA, 0.75 μg of transactivator DNA (pRc/CMV vector or a nucleocyto-plasmic shuttling competent Rev–Rex fusion; a gift of Dr Thomas J. Hope, Infectious Disease Laboratory, Salk Institute for Biological Studies, La Jolla, CA, U.S.A.) and 0.25 μg of pRSV-β-galactosidase [internal control for chloramphenicol acetyl-transferase (CAT) assays] were introduced into McArdle cells using LIPOFECTAMINE® as described above. Cells were harvested at 48 h post-transfection, protein extracts prepared by freeze–thawing, and β-gal [42] and CAT [43] assays performed as previously described. All extracts were normalized for β-gal activity. Parallel transfections were harvested for RNA prepa-ration and RT-PCR amplification of the apoB RNA. Editing efficiencies were quantified as described above.

RESULTS

Introns interfere with editing

Our previous studies demonstrated that the editing efficiency of apoB RNA was dramatically reduced when an intron was placed ⩽ 350 nt 5′ or 3′ of the target cytidine [10]. To provide proof that it was specifically RNA splicing and/or spliceosome assembly that had affected editing efficiency, splicing-competent and splicing-defective RNA transcripts were evaluated for their ability to support RNA editing in transfected McArdle rat hepatoma cells. The apoB pre-mRNA reporter construct contained an abbreviated splicing cassette from the adenovirus late leader sequence fused to 450 nt of wild-type apoB mRNA (Figure 1A). Unspliced pre-mRNA and spliced mRNA were amplified from total cellular McArdle cell mRNA using the MS1/MS2 and SP6/T7 amplier pairs respectively (Figure 1A). Consistent with previous results, the splicing cassette impaired the ability of the IVS–apoB RNA transcript to be edited, either before (pre-mRNA) or after (mRNA) it was spliced relative to a control transcript (pRc-apoB) that contained only apoB sequence (Figure 1B). These results corroborate previous findings suggesting that there is a window of opportunity for editing apoB mRNA in the nucleus and that no further editing occurs in the cytoplasm of wild-type hepatic cells. Specifically, recently published sub-cellular-fractionation studies have shown that the low level of editing measured on this transcript as mRNA (1%) occurred while the RNA was still in the nucleus [9].

Deletion of the polypyrimidine tract/branch point sequences and the 3′ splice acceptor site in the IVS–Δ3′apoB transcript (Figure 1A) ablated the ability of this pre-mRNA to be spliced, as the SP6/T7 amplier pair yielded only PCR products
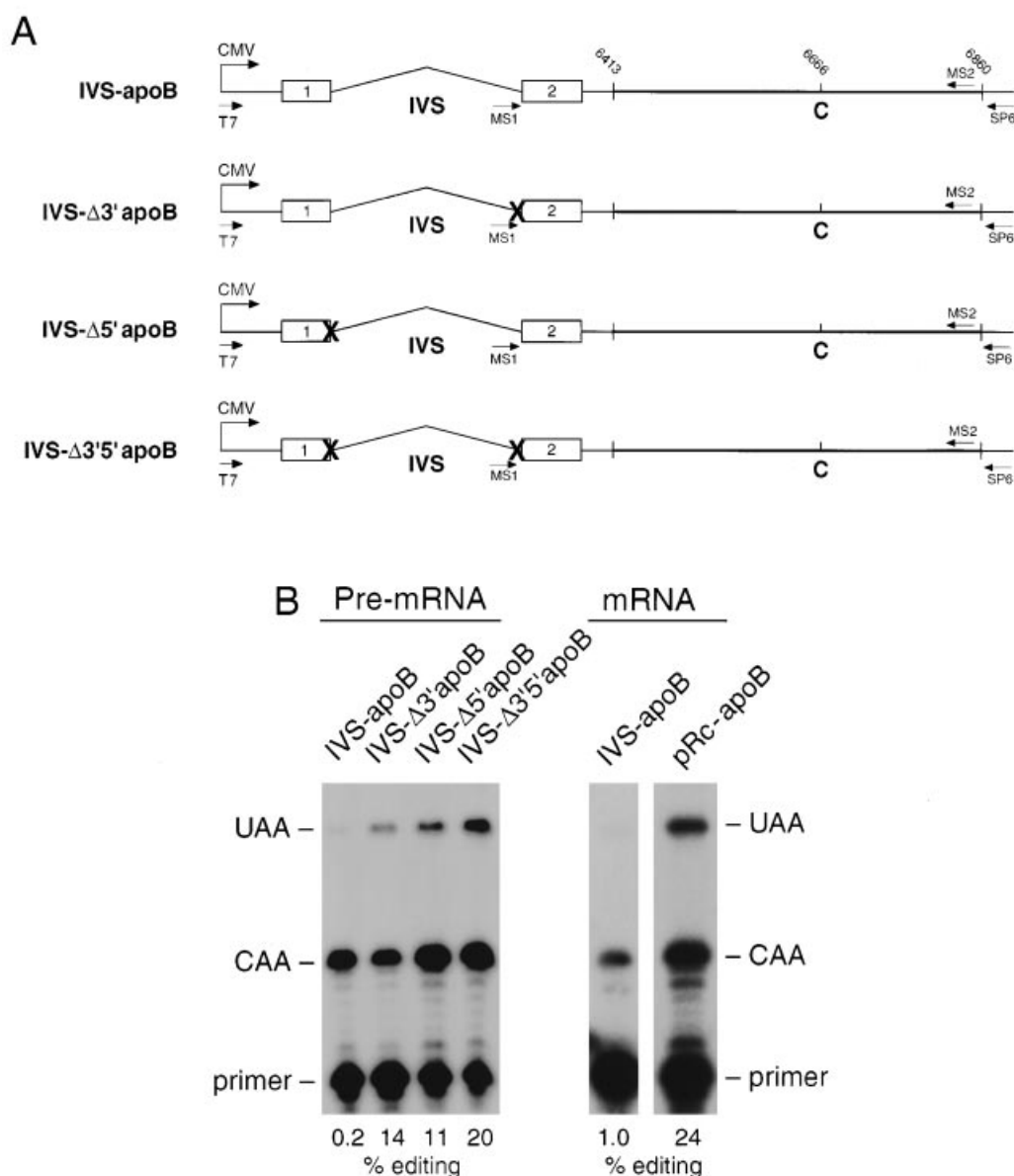
Figure 1   Effect of introns on editing efficiency

(**A**) Diagram of the chimaeric apoB expression constructs. The intron sequence (IVS) is derived from the adenovirus late leader sequence. Co-ordinates of the human apoB sequence are shown and the location of PCR amplimers indicated. **X** indicates the deleted 5′ splice donor or 3′ splice acceptor sequences. CMV, cytomegalovirus. (**B**) Poisoned-primer-extension assays of amplified apoB RNAs. Pre-mRNA and mRNA were amplified with the MS1/MS2 or SP6/T7 amplimers respectively. Editing efficiencies, an average for triplicate transfections, for each RNA are shown beneath. Editing efficiency was determined as the number of counts in edited apoB mRNA (UAA) divided by the sum of counts in UAA plus those in unedited apoB mRNA (CAA) and multiplied by 100.

indicative of unspliced transcripts (results not shown). The editing efficiency of this splicing-defective construct was higher than that of IVS–apoB (14%, S.E.M. = 1.0%; Figure 1B). The IVS–Δ5′apoB transcript was also defective in splicing owing to deletion of the 5′ splice donor sequence (the SP6/T7 amplimer pair failed to yield PCR products corresponding to spliced RNA; results not shown), and this RNA also demonstrated markedly elevated editing compared with IVS–apoB (11%, S.E.M. = 0.1%; Figure 1B). The double-splice-site mutant IVSΔ3′5′apoB (Figure 1A) had an editing efficiency higher than either of the single-site mutants (20%, S.E.M. = 0.2%) and equivalent to the intron lacking RNA transcript, pRc-apoB (24%, S.E.M. = 0.2%;

Figure 1B). These results suggested that it is the assembly of a fully functional spliceosome and/or RNA splicing that impedes editosome assembly and/or function, and that both 5′ and 3′ splicing signals contribute to the inhibitory effect.

Each of the constructs in Figure 1 generated pre-mRNA transcripts of equivalent length, but the presence of active or inactive introns might influence expression levels of the resultant mRNAs. However, we have previously reported that the expression level of a given apoB transcript did not affect its editing efficiency [10]. Moreover, there was no competition between the editing efficiencies of exogenous and endogenous apoB transcripts, suggesting that editing factors were not made to be rate-
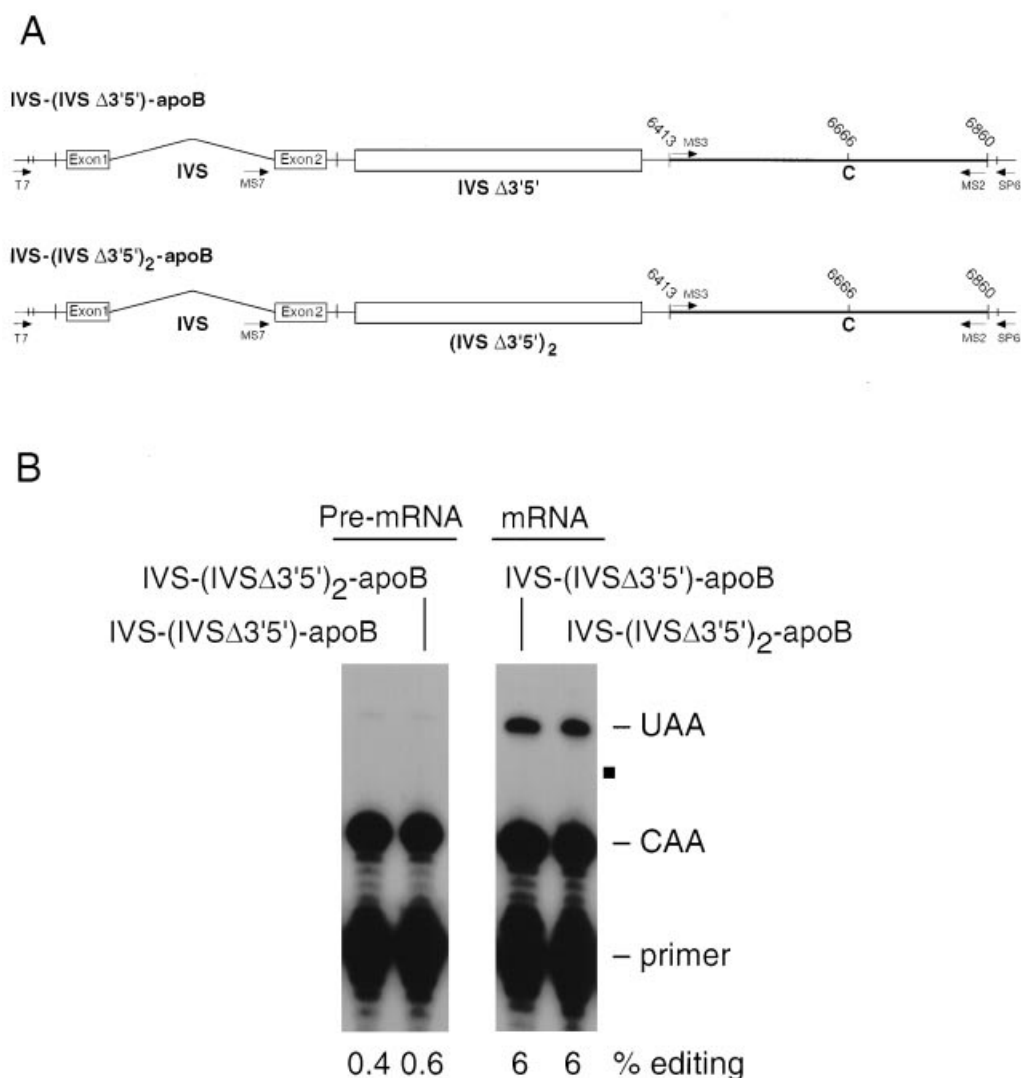
Figure 2   Effect of intron proximity on editing efficiency

(**A**) Diagram of the chimaeric apoB expression constructs. IVS–(IVSΔ3′5′)–apoB and IVS–(IVSΔ3′5′)$_2$–apoB were created by the insertion of one or two copies respectively of the IVSΔ3′5′ intron cassette into IVS–apoB. Human apoB co-ordinates and amplimer annealing sites are indicated (and see Figure 1). (**B**) Poisoned-primer-extension assays of amplified apoB RNAs. Pre-mRNA and mRNA were amplified with the MS7/MS2 or SP6/T7 amplimers respectively. Editing efficiencies, an average for duplicate transfections, for each RNA are shown beneath.

limiting by the increased concentration of apoB editing sites. These facts underscore the significance of the intron and RNA splicing on the regulation of editing efficiency.

In human apoB mRNA, C^{6666} is located in the middle of the 7.5 kb exon 26, significantly further from a 5′ or 3′ intron than in the chimaeric constructs described above. We therefore evaluated whether the proximity of the splice donor and acceptor sites to the tripartite motif affected editing efficiency. Insertion of a monomer or a dimer of the splicing-defective intron cassette (IVSΔ3′5′) increased the distance between the active intron and the editing site by 425 and 850 nt respectively (Figure 2A). This increased the effective size of the chimaeric exon to nearly 1 kb or 1.4 kb respectively; the average size of an internal exon being only 200–300 nt in mammals [44].

ApoB pre-mRNA was amplified from each transcript expressed in McArdle cells using the MS7/MS2 amplimers and nesting with the MS2/MS3 amplimer pair. The sequence of primer MS7 is unique to the functional intron sequence and thus ensured amplification of unspliced pre-mRNA. Barely detectable levels of editing were measured on both pre-mRNA transcripts. However, a 10-fold higher level of editing was observed upon the spliced mRNA of both transcripts (6.0 %) (Figure 2B), which is 6-fold higher than the spliced mRNA derived from IVS–apoB (Figure 1B). This suggested that increasing the distance between the intron and the editing site alleviated, but was not completely capable of overcoming, the inhibitory effect of spliceosome assembly/RNA splicing on editing (i.e. compare 6 with 20 % editing of IVSΔ3′5′apoB in Figure 1).

The apoB editing site is not efficiently used within an intron

A search of GenBank® for apoB mooring-sequence similarities reveals numerous potential editing sites. However, many are located short distances from splice sites or within 5′ or 3′ untranslated regions or introns where the functional consequence(s) of a cytidine-to-uridine editing event is unclear (D.
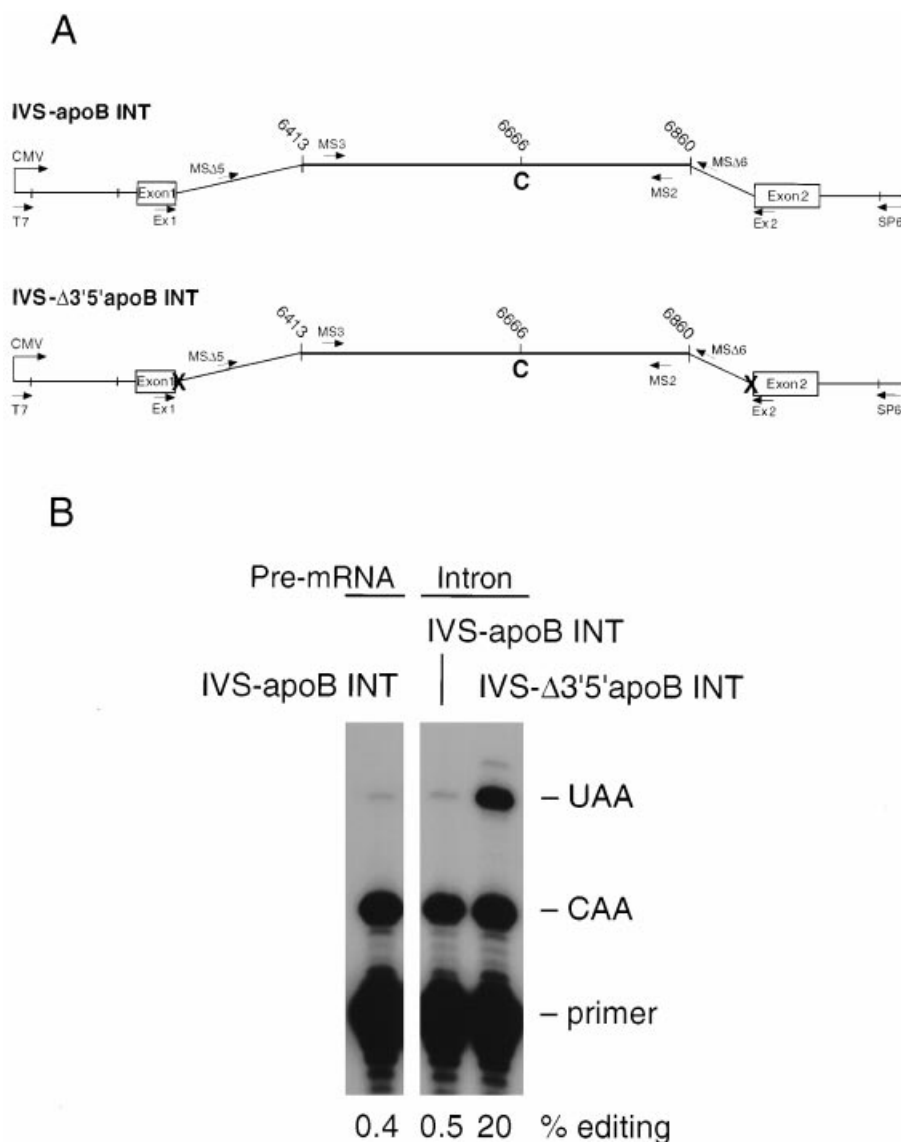
Figure 3   Editing sites within introns are poorly utilized

(**A**) Diagram of the chimaeric apoB expression constructs. The apoB editing cassette was inserted as a PCR product into a unique *Hind*III site 5′ of the polypyrimidine tract in IVS–apoB and IVS–Δ3′5′apoB (see Figure 1). Amplimer annealing sites are indicated. (**B**) Poisoned primer extension assays of amplified apoB RNAs. Unspliced pre-mRNA and intron containing RNA were amplified with the Ex1/Ex2 or MSΔ5/MSΔ6 amplimers respectively. Editing efficiencies, an average for duplicate transfections, for each RNA are shown beneath.

Landsman, M. Forsythe and H. C. Smith, unpublished work). The release of the entire human, mouse and rat genome sequences will likely reveal more mooring-sequence similarities, although their location in introns or exons may be uncertain until these genomes are annotated. In this regard, our results suggested that mooring-sequence-dependent editing sites may not be biologically active if they are positioned too close to splice junctions.

In an attempt to be able to predict functional cytidine-to-uridine editing sites from these transcriptomes, we also investigated whether the apoB editing site is recognized when positioned within an intron. A 450 nt section of the apoB RNA transcript containing the editing site was placed within the intron of the adenovirus late leader sequence (IVS–apoB INT) and this construct was expressed in transfected McArdle cells. Pre-mRNA transcripts were amplified using the Ex1/Ex2 amplimers followed by nested PCR with the MSΔ5/MSΔ6 amplimer pair and were

edited at an efficiency of 0.4 % (Figure 3B). Intron-containing transcripts were amplified using the MSΔ5/MSΔ6 amplimers followed by nested PCR with the MS2/MS3 amplimer pair and were edited at an efficiency of 0.5 % (Figure 3B). The use of the MSΔ5/MSΔ6 amplimer pair in the initial PCR would not distinguish between unspliced pre-mRNA or spliced-out lariat RNA, but given the rapid degradation of lariat RNA, it is unlikely that the amplified PCR products represent lariat RNA species. If, however, there were amplified lariat species present, the difference of 0.1 % between intron-containing and unspliced pre-mRNA suggests that lariat RNAs containing apoB editing sites are not efficient editing substrates.

Mutation of the 5′ and 3′ splicing signals of the above construct to generate IVS–Δ3′5′apoB INT restored editing efficiency (20 %; Figure 3B) to a level equal to that of IVS–Δ3′5′apoB construct (20 %; Figure 1C). A minor additional primer extension product
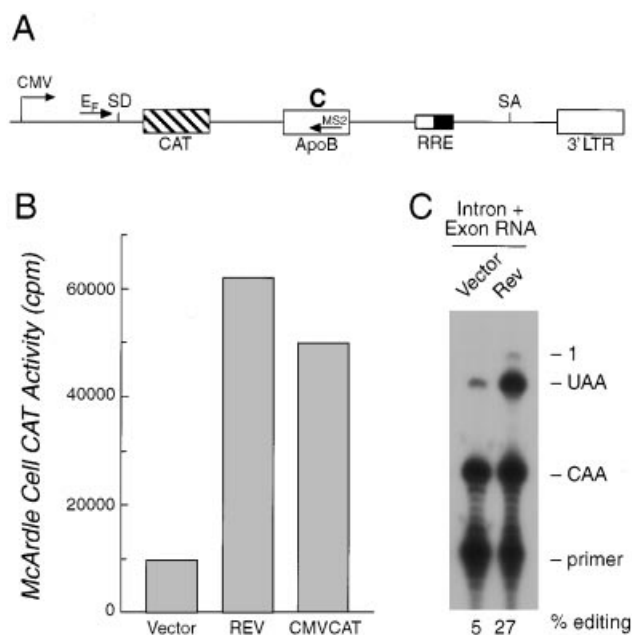
Figure 4   Editing is regulated by RNA splicing

(**A**) Diagram of the modified CAT reporter construct (CMV128) used in the Rev complementation assay; a gift from Dr Thomas J. Hope of the Salk Institute. The splice donor (SD), splice acceptor (SA), RRE, intron and 3′ long tandem repeat (LTR) are from the HIV-1 genome. CMV128 was modified by insertion of the apoB editing cassette as a PCR product into the *Bam*HI site 3′ of the CAT gene. Amplimer annealing sites are indicated. (**B**) McArdle cell CAT activity in the absence (Vector) or presence of the Rev transactivator. Values are averages for duplicate experiments. CMVCAT was an assay control transfection. (**C**) Poisoned-primer-extension assays of amplified apoB RNAs. 'Intron and exon RNA' was amplified using the EF/MS2 amplimers. Editing efficiencies for each RNA are shown beneath. Promiscuous editing is indicated by '1'.

indicative of promiscuous editing was also apparent. These results support the hypothesis that pre-mRNA is not an effective substrate for cytidine-to-uridine editing and that this likely results from interference by spliceosome assembly/RNA splicing or potentially the rapid nuclear export of spliced mRNAs into the cytoplasm.

Blocking the commitment of transcripts to the splicing pathway alleviates splice-site inhibition of editing

Most apoB mRNA editing substrate studies have employed cDNA transcripts which lack introns [16,45,46]. Wild-type apoB cDNA transcripts expressed in wild-type McArdle cells edit 2–3-fold more efficiently than the endogenous transcript [10,16]. We have demonstrated that chimaeric splicing–editing reporter RNAs (IVS–apoB) had low editing efficiency as nuclear transcripts, which did not change once spliced mRNAs had entered the cytoplasm (Figure 1; [9]). Hence the window of opportunity for a transcript to be edited in wild-type cells was confined to the nucleus, and when introns are proximal to the editing site, its utilization was impaired. We therefore reasoned that if spliceosome assembly was involved in the inhibition of editing, then by-passing the spliceosome assembly commitment step may alleviate the inhibition (in a manner similar to intronless cDNA transcripts).

To investigate this hypothesis, we have separated the processes of RNA splicing and RNA nuclear export by utilizing a modification of the Rev complementation assay that has been

employed to identify HIV-1 Rev-like nuclear export sequences [41]. Rev functions, by interaction with an RRE, to export unspliced RNA out of the nucleus. A reporter plasmid was constructed which contained an intron interrupted by the CAT gene and a functional apoB RNA editing cassette (Figure 4A). CAT activity could only be expressed if unspliced RNA was exported to the cytoplasm, a process wholly dependent upon an active Rev protein expressed from a co-transfected plasmid. In the presence of Rev, spliceosome assembly on the transcript does not occur and therefore should not interfere with the utilization of the apoB editing site contained with the intron.

McArdle cells were co-transfected with the modified reporter construct, together with either a control vector or a Rev expression vector. CAT activity was determined 48 h later (Figure 4B). In the presence of the control vector, very low levels of CAT activity were expressed, presumed to be due to splicing and degradation of the CAT transcript as a lariat RNA. Expression of the Rev protein resulted in nuclear export of unspliced intronic RNA and translation of the CAT protein, as evident in the 7-fold higher level of CAT activity in these cell extracts. These findings demonstrated that, in McArdle cells, HIV-1 Rev protein successfully diverted RNAs from the spliceosome assembly pathway and transported them into the cytoplasm.

Total cellular RNA was harvested from parallel transfections, the apoB sequence amplified, and the editing efficiencies were determined (Figure 4C). Consistent with our findings described above, editing of apoB RNA within an intron of the RRE construct in the absence of Rev expression was very low ('intron + exon' amplified with EF/MS2). However, the editing efficiency was enhanced 5-fold when the Rev protein was co-expressed. Given that editing in the cytoplasm has never been demonstrated in wild-type McArdle cells [9], nor would it be driven by an increase in apoB RNA abundance in the cytoplasm [10], we propose that the enhanced editing occurred in the nucleus as a consequence of pre-mRNAs by-passing commitment to the spliceosome assembly and/or RNA export pathways. We cannot formally exclude the alternative explanation that unspliced CAT–apoB chimaeric RNAs were edited in the cytoplasm. However, this would necessitate the activation of cytoplasmically localized editing factors by Rev, for which there is no precedent.

In addition to an enhanced editing efficiency, the unspliced CAT–apoB RNA was also promiscuously edited (additional primer extension stop labelled '1', Figure 4C). Promiscuous editing does not occur under physiological expression levels of APOBEC-1 in McArdle cells [10,40,47], in rat tissues or under biological conditions where editing efficiencies are greater than 90%, e.g. rat intestine [48]. Nor does it occur when rat hepatic editing efficiencies are stimulated by metabolic or hormonal manipulations [49,50]. Promiscuous editing appears to be unique to cells in which APOBEC-1 has been artificially overexpressed [10,40,47] and is observed under these conditions on both nuclear and cytoplasmic transcripts [9]. The results presented in Figures 3 and 4 are therefore the first demonstration of promiscuous editing in the nucleus without the exogenous overexpression of APOBEC-1.

DISCUSSION

ApoB mRNA editing, while conceptually a simple process of hydrolytic cytidine deamination to uridine [51], has turned out to have surprising complexities in both the number of proteins involved and the cell biology involved in its regulation. It is well established that a sequence element consisting of three proximal components (enhancer, spacer and mooring sequence) com-

prise the *cis*-acting sequences required for efficient site-specific editing of C6666 in apoB mRNA [11–15,45]. A multiple protein editosome catalyses and regulates editing of C^{6666} [11,22,26]. The components of the minimal editosome from defined *in vitro* system analyses are APOBEC-1 as a homodimeric cytidine deaminase [28] bound to the auxiliary protein ACF/ASP that serves as the editing-site recognition factor through its mooring-sequence-selective RNA-binding activity [30,31]. Several other auxiliary protein candidates have also been described that had binding affinities for APOBEC-1 and/or apoB mRNA and that demonstrated the ability to modulate editing efficiency [23,24,26,31,33,34,37,38]. Although, under biological conditions, editing occurs only in the nucleus [8,9], nuclear and cytoplasmic distributions have been described for both APOBEC-1 and ACF [9,52,53]. Nuclear editing has been characterized as occurring coincident with, or immediately after, pre-mRNA splicing [8–10]. Prior to splicing, pre-mRNA was not efficiently edited [8]. It was not apparent, given the size of exon 26 and the nature of the *cis*-acting RNA sequence requirements, why there was a lag in editing activity during pre-mRNA maturation. We have addressed this question in studies suggesting that spliceosome assembly and/or nuclear RNA export pathways regulate the utilization of cytidine-to-uridine editing sites.

In reporter RNA constructs, introns within 350–1000 nt of the apoB editing site suppressed editing efficiency. This inhibition was dependent on an active 5' splice site and/or 3' splice donor site and was partially alleviated after the reporter RNA had been spliced. This suggests that the process of spliceosome assembly functionally interfered with editosome assembly and/or function. This is supported by the distance dependence of this inhibition. When the splice sites were located more distal to the editing site, editing efficiencies were increased albeit not to levels seen on RNAs that do not contain introns. The gating hypothesis [10] proposed that each apoB RNA had a temporal 'window of opportunity' to become edited during its splicing and export from the nucleus. In this model, factors involved in spliceosome and editosome assembly are thought to compete for access to the mRNA. Consequently it is predicted that there will be less steric hindrance between the spliceosome and the editosome, and editing efficiency will improve the more distal an intron is located relative to the editing site [e.g. IVS–(IVSΔ3'5')–apoB or IVS–(IVSΔ3'5')$_2$–apoB compared with IVS–apoB]. This phenomenon might explain the lower editing efficiency of native apoB editing prior to splicing, because the native editing site is only three times further away from the 5' or 3' splice junctions than that used in our reporter RNA constructs.

Importantly, these results have implications for the prediction of novel mooring-sequence-dependent RNA-editing sites. Not only is there a requirement for a target cytidine to be appropriately located upstream of a mooring sequence, but for efficient utilization, the editing site should not be in close proximity to an intron. Considering that the average size of an internal exon is only 200–300 nt in mammals [44], it is highly unlikely that a significant amount of mooring-sequence-dependent editing will be observed in mRNAs with standard sized exons. In fact an analysis of the human, mouse and rat expressed-sequence-tag databases by Hidden Markov modelling has confirmed that the majority of mooring-sequence identities within coding sequences are located proximal to intron/exon junctions (D. Landsman, M. Forsythe and H. C. Smith, unpublished work). An evaluation of select RNA transcripts revealed that they were in fact not edited (M. Forsythe and H. C. Smith, unpublished work). Related to these observations are results showing that editing sites located within introns were not inefficiently utilized. Taken together, the results support the hypothesis that spliceosome assembly and editosome assembly processes are communicating a temporal and spatial relationship that ultimately determines the efficiency of mooring-sequence-dependent editing. Consistent with this possibility of communication between the spliceosome and editosome is the finding that several proteins that have a role in RNA structure and/or splicing have also been implicated in RNA editing as auxiliary factors. These include hnRNP C, hnRNP D, APOBEC-1-binding protein (which has homology with hnRNP A and B) and KSRP, a protein involved in alternative splice site utilization [31,36–38].

The Rev–RRE interaction in chimaeric splicing/editing transcripts subverted pre-mRNA from the spliceosome assembly pathway and markedly alleviated the suppression of editing of pre-mRNA. Current evidence suggests that editing of all RNAs occurred in the nucleus; however, we cannot rule out the possibility that editing of the unspliced apoB chimaeric RNA occured after it was exported to the cytoplasm. Although we have reported cytoplasmic localization of APOBEC-1 and ACF [9,52], there is no precedent for them to form active editosomes in the cytoplasm *in vivo* or for there to be cytoplasmic editing when APOBEC-1 expression was under biological regulation [9]. Specifically, cytoplasmic APOBEC-1 and ACF existed within an inactive 60 S complex that only became an activated 27 S editosome in the nucleus [11,22]. The possibility of cytoplasmic editing was shown in studies in which an enhanced editing efficiency was observed upon IVS–apoB chimaeric RNA by a cytoplasmically 'tethered' chicken muscle pyruvate kinase–APOBEC-1 fusion protein [9]. This fusion protein was not capable of normal cellular localization but, when overexpressed, could form active editing complexes with cytoplasmic auxiliary proteins. We propose therefore that, under normal regulation of APOBEC-1 and ACF expression and their cellular localization, the impairment of mooring-sequence-dependent editing on intronic RNA and its rescue in the presence of the Rev protein demonstrate that spliceosome assembly can interfere with editosome assembly/function.

The significance of the promiscuous editing observed upon the intronic RNA in the presence of Rev is uncertain. It is possible that editing-site fidelity as well as utilization is maintained by the commitment to spliceosome assembly in wild-type McArdle cells. However, the promiscuous editing observed on IVS–Δ3'5'apoB INT was unexpected, given the nature of the transcript, i.e. a cDNA equivalent to IVS–Δ3'5'apoB in Figure 1 on which no promiscuous editing was observed at equivalent editing at C^{6666}. A possibility for this could be the fortuitous introduction of a pair of tandem UGAU sequences within the intronic sequence 3' of the editing site, a motif that we have previously shown to promote promiscuous editing [16].

Our description of the relationship of RNA splicing and editing is novel for apoB cytidine-to-uridine mRNA editing. However, an emerging theme in RNA processing is an interdependence of multiple steps in RNA maturation. Perhaps the most relevant to apoB editing is the adenine-to-inosine editing of glutamate and 5-hydroxytryptamine receptors. In contrast with apoB mRNA editing, mRNA substrates that undergo adenine-to-inosine editing all require the presence of a complementary intron sequence to form a partially double-stranded RNA structure that is recognized by the appropriate ADAR1 or ADAR2 enzyme [3,5,6]. The critical role of *cis*-acting intronic sequences indicates deamination is a nuclear event, and as the editing site is frequently located close to a 5' splice acceptor site [54,55], suggests that the level of editing maybe influenced by interference or interaction with RNA splicing. For example, endogenously expressed GluR2 mRNA from neuronal cell lines is always edited to 100 % at the Gln/Arg site, whereas unspliced

GluR2 transcripts are edited to only 70–90 % [54], suggesting a partial inhibition of splicing until editing has occurred. Conversely, the transcript of the *Glu-R6* gene contains three exonic editing sites (Ile/Val, Tyr/Cys and Gln/Arg) which are edited to different extents, suggesting that there must be a tightly regulated and co-ordinated action of the appropriate ADAR and the spliceosome at each editing site [56,57]. In crosses of ADAR2$^{+/-}$ with GluR-B (R)$^{+/+}$ mice, an influence from the editing status of the Gln/Arg site on subsequent splicing of the downstream intron was observed [58], suggesting that these RNA processing events do not occur independently.

The major steps in pre-mRNA processing, capping, splicing, 3′-end cleavage and polyadenylation are coupled to transcription through recruitment of the necessary processing factors to the largest subunit of the RNA polymerase II. This represents an efficient process for increasing local concentrations of related processing and transcription factors on pre-mRNAs as and when they are needed [59]. Many analyses of RNA processing have attempted to identify active versus inactive populations of processing factors and have postulated that the greatest concentration of factors may or may not correspond to sites of function, dependent upon metabolic activity [60]. Specifically, recent photobleaching studies ([59] and references cited therein) suggested that 'speckles' correspond to sites where free small nuclear RNPs transiently assemble before recruitment by the C-terminal domain of RNA polymerase II and transfer to nascent transcripts. It is easily conceivable, therefore, that the processes of RNA editing and RNA splicing should be tightly co-ordinated, and the observation of nuclear and cytoplasmically localized APOBEC-1 and ACF corresponds to active and inactive complexes respectively. These two components of the minimal editosome, together with other editosomal proteins if necessary, could be rapidly recruited to newly synthesized apoB mRNA transcripts by a co-ordinated action of RNA polymerase II and spliceosome assembly.

Most, if not all, known RNA processing reactions can occur *in vitro*, but they are not as efficient as *in vivo*. This is also true for *in vitro* apoB RNA editing reactions. However, IVS–apoB RNA transcripts were edited with the same efficiency as intronless apoB transcripts *in vitro* (H. C. Smith, unpublished work). This indicates that the presence of an intron *per se* does not interfere with editing, but, as we have shown, there is a clear interdependence of splicing and editing for editing site regulation and fidelity *in vivo*. Such interdependence is also exhibited in mammalian nonsense-mediated decay ('NMD') of RNA, wherein only RNAs that contain nonsense codons and that have passed through the spliceosome are 'marked' and targeted for decay [61]. This imprinting of nuclear pre-mRNA by proteins that remain bound in the cytoplasm is a means of mRNAs 'communicating their history' [62] and/or perhaps ensuring that no further RNA processing/editing occurs in the cytoplasm [63].

In conclusion, we have demonstrated a spatial and temporal relationship between RNA splicing and apoB RNA editing. The suppression of editing-site utilization by proximal introns may explain the uniquely large size of exon 26 and/or the scarcity of other mooring-sequence-dependent cytidine-to-uridine editing sites. Moreover, these studies highlight the need to consider apoB RNA editing as an integrated process with RNA transcription and splicing, potentially expanding the number of auxiliary factors that should be considered as involved in apoB RNA editing.

REFERENCES

1 Gott, J. M. and Emeson, R. B. (2000) Functions and mechanisms of RNA editing. Annu. Rev. Genet. **34**, 499–531

2 Smith, H. C., Gott, J. M. and Hanson, M. R. (1997) A guide to RNA editing. RNA **3**, 1105–1123

3 Simpson, L. and Emeson, R. B. (1996) RNA editing. Annu. Rev. Neurosci. **19**, 27–52

4 Maas, S. and Rich, A. (2000) Changing genetic information through RNA editing. BioEssays **22**, 790–802

5 Maas, S., Melcher, T. and Seeburg, P. H. (1997) Mammalian RNA-dependent deaminases and edited mRNAs. Curr. Opin. Cell. Biol. **9**, 343–349

6 Rueter, S. M. and Emeson, R. B. (1998) Adenosine-to-inosine conversion in mRNA. In Modification and Editing of RNA (Grosjean, H. and Benne, R., eds.), pp. 343–361, American Society for Microbiology Press, Washington

7 Rueter, S. M., Dawson, T. R. and Emeson, R. B. (1999) Regulation of alternative splicing by RNA editing. Nature (London) **399**, 75–80

8 Lau, P. P., Xiong, W. J., Zhu, H. J., Chen, S. H. and Chan, L. (1991) Apolipoprotein B mRNA editing is an intranuclear event that occurs post-transcriptionally coincident with splicing and polyadenylation. J. Biol. Chem. **266**, 20550–20554

9 Yang, Y., Sowden, M. P. and Smith, H. C. (2000) Induction of cytidine to uridine editing on cytoplasmic apolipoprotein B mRNA by overexpressing APOBEC-1. J. Biol. Chem. **275**, 22663–22669

10 Sowden, M., Hamm, J. K., Spinelli, S. and Smith, H. C. (1996) Determinants involved in regulating the proportion of edited apolipoprotein B RNAs. RNA **2**, 274–288

11 Smith, H. C., Kuo, S. R., Backus, J. W., Harris, S. G., Sparks, C. E. and Sparks, J. D. (1991) In vitro mRNA editing: identification of a 27 S editing complex. Proc. Natl. Acad. Sci. U.S.A. **88**, 1489–1493

12 Backus, J. W. and Smith, H. C. (1992) Three distinct RNA sequence elements are required for efficient apolipoprotein B (apoB) RNA editing in vitro. Nucleic Acids Res. **20**, 6007–6014

13 Smith, H. C. (1993) Apolipoprotein B mRNA editing: the sequence to the event. Semin. Cell Biol. **4**, 267–278

14 Shah, R. R., Knott, T. J., Legros, J. E., Navaratnam, N., Greeve, J. C. and Scott, J. (1991) Sequence requirements for the editing of apolipoprotein B mRNA. J. Biol. Chem. **266**, 16301–16304

15 Backus, J. W. and Smith, H. C. (1991) Apolipoprotein B mRNA sequences 3′ of the editing site are necessary and sufficient for editing and editosome assembly. Nucleic Acids Res. **19**, 6781–6786

16 Sowden, M. P., Eagleton, M. and Smith, H. C. (1998) Apolipoprotein B RNA sequence 3′ of the mooring sequence and cellular sources of auxiliary factors determine the location and extent of promiscuous editing. Nucleic Acids Res. **26**, 1644–1652

17 Backus, J. W. and Smith, H. C. (1994) Specific 3′ sequences flanking a minimal apolipoprotein B (apoB) mRNA editing 'cassette' are critical for efficient editing in vitro. Biochim. Biophys. Acta **1217**, 65–73

18 Hersberger, M. and Innerarity, T. L. (1998) Two efficiency elements flanking the editing site of cytidine 6666 in the apolipoprotein B mRNA support mooring dependent editing. J. Biol. Chem. **273**, 9435–9442

19 Nakamuta, M., Tsai, A., Chan, L., Davidson, N. O. and Teng, B.-B. (1999) Sequence elements required for apolipoprotein B mRNA editing enhancement activity from chicken enterocytes. Biochem. Biophys. Res. Commun. **254**, 744–750

20 Hersberger, M., Patarroyo-White, S., Arnold, K. S. and Innerarity, T. L. (1999) Phylogenetic analysis of the apolipoprotein B mRNA editing region. Evidence for a secondary structure between the mooring sequence and the 3′ efficiency element. J. Biol. Chem. **274**, 34590–34597

21 Richardson, N., Navaratnam, N. and Scott, J. (1998) Secondary structure for the apolipoprotein B mRNA editing site. AU binding proteins interact with a stem loop. J. Biol Chem. **273**, 31707–31717

22 Harris, S. G., Sabio, I., Mayer, E., Steinburg, M. F., Backus, J. W., Sparks, J. D., Sparks, C. E. and Smith, H. C. (1993) Extract-specific heterogeneity in high-order complexes containing apolipoprotein B mRNA editing activity and RNA-binding proteins. J. Biol. Chem. **268**, 7382–7392

23 Giannoni, F., Bonen, D. K., Funahashi, T., Hadjiagapiou, C., Burant, C. F. and Davidson, N. O. (1994) Complementation of apolipoprotein B mRNA editing by human liver accompanied by secretion of apolipoprotein B48. J. Biol. Chem. **269**, 5932–5936

24 Yamanaka, S., Poksay, K. S., Balestra, M. E., Zeng, G. Q. and Innerarity, T. L. (1994) Cloning and mutagenesis of the rabbit apoB mRNA editing protein. J. Biol. Chem. **269**, 21725–21734

25 Yang, Y. and Smith, H. C. (1996) In vitro reconstitution of apolipoprotein B RNA editing activity from recombinant APOBEC-1 and McArdle cell extracts. Biochem. Biophys. Res. Commun. **218**, 797–801

26 Yang, Y., Yang, Y., Kovalski, K. and Smith, H. C. (1997) Partial characterization of the auxiliary factors involved in apolipoprotein B mRNA editing through APOBEC-1 affinity chromatography. J. Biol. Chem. **272**, 27700–27706

27 Steinburg, M. F., Schock, D., Backus, J. W. and Smith, H. C. (1999) Tissue-specific differences in the role of RNA 3′ of the apolipoprotein B mRNA mooring sequence in editosome assembly. Biochem. Biophys. Res. Commun. **263**, 81–86

28 Lau, P. P., Zhu, H. J., Baldini, A., Charnsangavej, C. and Chan, L. (1994) Dimeric structure of a human apolipoprotein B mRNA editing protein and cloning and chromosomal localization of its gene. Proc. Natl. Acad. Sci. U.S.A. **91**, 8522–8526

29 Navaratnam, N., Bhattacharya, S., Fujino, T., Patel, D., Jarmuz, A. L. and Scott, J. (1995) Evolutionary origins of apoB mRNA editing: catalysis by a cytidine deaminase that has acquired a novel RNA-binding motif at its active site. Cell **81**, 187–195

30 Mehta, A., Kinter, M., Sherman, N. and Driscoll, D. (2000) Molecular cloning of apobec-1 complementation factor, a novel RNA binding protein involved in the editing of apolipoprotein B mRNA. Mol. Cell. Biol. **20**, 1846–1854

31 Lellek, H., Kirsten, R., Diehl, I., Apostle, F., Buck, F. and Greeve, J. (2000) Purification and molecular cloning of a novel essential component of the apolipoprotein B mRNA editing enzyme-complex. J. Biol Chem. **275**, 19848–19856

32 MacGinnitie, A. J., Anant, S. and Davidson, N. O. (1995) Mutagenesis of apobec-1, the catalytic subunit of the mammalian apolipoprotein B mRNA editing enzyme, reveals distinct domains that mediate cytosine nucleoside deaminase, RNA-binding, and RNA editing activity. J. Biol. Chem., **270**, 14768–14775

33 Teng, B., Burant, C. F. and Davidson, N. O. (1993) Molecular cloning of an apolipoprotein B mRNA editing protein. Science **260**, 1816–1819

34 Inui, Y., Giannoni, F., Funahashi, T. and Davidson, N. O. (1994) REPR and complementation factor(s) interact to modulate rat apolipoprotein B mRNA editing in response to alterations in cellular cholesterol flux. J. Lipid Res. **35**, 1477–1489

35 Blanc, V., Navaratnam, N., Henderson, J. O., Anant, S., Kennedy, S., Jarmuz, A., Scott, J. and Davidson, N. O. (2001) Identification of GRY-RBP as an apo B mRNA binding protein that interacts with both apobec-1 and with apobec-1 complementation factor (ACF) to modulate cytidine to uridine editing. J. Biol. Chem. **276**, 10272–10283

36 Greeve, J., Lellek, H., Rautenberg, P. and Greten, H. (1998) Inhibition of the apolipoprotein B mRNA editing enzyme-complex by hnRNP C1 protein and 40 S hnRNP complexes. Biol. Chem. **379**, 1063–1073

37 Anant, S. G., Giannoni, F., Antic, D., DeMaria, C. T., Keene, J. D., Brewer, G. and Davidson, N. O. (1997) AU-rich RNA binding proteins Hel-N1 and AUF1 bind apolipoprotein B mRNA and inhibit posttranscriptional C to U editing. Nucleic Acids Symp. Ser. **36**, 115–118

38 Lau, P. P., Zhu, H. J., Nakamuta, M. and Chan, L. (1997) Cloning of an Apobec-1-binding protein that also interacts with apolipoprotein B mRNA and evidence for its involvement in RNA editing. J. Biol. Chem. **272**, 1452–1455

39 Fisher, C. L. and Pei, K. P. (1997) Modification of a PCR-based site-directed mutagenesis method. BioTechniques **23**, 570–574

40 Sowden, M. P., Hamm, J. K. and Smith, H. C. (1996) Over-expression of APOBEC-I results in mooring sequence dependent promiscuous RNA editing. J. Biol. Chem. **271**, 3011–3017

41 Taagepera, S., McDonald, D., Loeb, J. E., Whitaker, L. L., McElroy, A. K., Wang, J. Y. J. and Hope, T. J. (1998) Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase. Proc. Natl. Acad. Sci. U.S.A. **95**, 7457–7462

42 Sowden, M. P., Harrison, S. M., Ashfield, R. A., Kingsman, A. J. and Kingsman, S. M. (1989) Multiple cooperative interactions constrain BPV-1 E2 dependent activation of transcription. Nucleic Acids Res. **17**, 2959–2972

43 Neumann, J. R., Morency, C. A. and Russian, K. O. (1987) A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5**, 444–448

44 Robberson, B. L., Cote, G. J. and Berget, S. M. (1990) Exon definition may facilitate splice site selection in RNAs with multiple exons. Mol. Cell. Biol. **10**, 1084–1094

45 Driscoll, D. M., Lakhe-Reddy, S., Oleksa, L. M. and Martinez, D. (1993) Induction of RNA editing at heterologous sites by sequences in apolipoprotein B mRNA. Mol. Cell. Biol. **13**, 7288–7294

46 Bostrom, K., Garcia, Z., Poksay, K. S., Johnson, D. F., Lusis, A. J. and Innerarity, T. L. (1990) Apolipoprotein B mRNA editing. Direct determination of the edited base and occurrence in non-apolipoprotein B producing cell lines. J. Biol. Chem. **265**, 22446–22452

47 Siddiqui, J. F. M., Van Mater, D., Sowden, M. P. and Smith, H. C. (1999) Disproportionate relationship between APOBEC-1 expression and apolipoprotein B mRNA editing activity. Exp. Cell Res. **252**, 154–164

48 Greeve, J., Altkemper, I., Dieterich, J.-H., Greten, H. and Winder, E. (1993) Apolipoprotein B mRNA editing in 12 different mammalian species: hepatic expression is reflected in low concentrations of apoB-containing plasma lipoproteins. J. Lipid Res. **34**, 1367–1383

49 Lau, P. P., Cahill, D. J., Zhu, H.-J. and Chan, L. (1995) Ethanol modulates apolipoprotein B mRNA editing in the rat. J. Lipid Res. **36**, 2069–2078

50 Baum, C. L., Teng, B.-B. and Davidson, N. O. (1990) Apolipoprotein B messenger RNA editing in the rat liver: modulation by fasting and refeeding a high carbohydrate diet. J. Biol. Chem. **265**, 19263–19270

51 Johnson, D. F., Poksay, K. S. and Innerarity, T. L. (1993) The mechanism for apo-B mRNA editing is deamination. Biochem. Biophys. Res. Commun. **195**, 1204–1210

52 Yang, Y., Yang, Y. and Smith, H. C. (1997) Multiple protein domains determine the cell type-specific nuclear distribution of the catalytic subunit required for apolipoprotein B mRNA editing. Proc. Natl. Acad. Sci. U.S.A. **94**, 13075–13080

53 Dance, G. S. C., Sowden, M. P., Yang, Y. and Smith, H. C. (2000) APOBEC-1 dependent cytidine to uridine editing of apolipoprotein B RNA in yeast. Nucleic Acids Res. **28**, 424–429

54 Higuchi, M., Single, F. N., Kohler, M., Sommer, B., Sprengel, R. and Seeburg, P. H. (1993) RNA editing of AMPA receptor subunit GluR-B: a base-paired intron–exon structure determines position and efficiency. Cell **75**, 1361–1370

55 Egebjerg, J., Kukekov, V. and Heinemann, S. F. (1994) Intron sequence directs RNA editing of the glutamate receptor subunit GluR2 coding sequence. Proc. Natl. Acad. Sci. U.S.A. **91**, 10270–10274

56 Kohler, M., Burnashev, N., Sakmann, B. and Seeburg, P. H. (1993) Determinants of Ca^{2+} permeability in both TM1 and TM2 of high affinity kainate receptor channels: diversity by RNA editing. Neuron **10**, 491–500

57 Seeburg, P. H., Higuchi, M. and Sprengel, R. (1998) RNA editing of brain glutamate receptor channels: mechanism and physiology. Brain Res. Rev. **26**, 217–229

58 Higuchi, M., Maas, S., Single, F. N., Hartner, J., Rozov, A., Burnashev, N., Feldmeyer, D., Sprengel, R. and Seeburg, P. H. (2000) Point mutation in an AMPA receptor gene rescues lethality in mice deficient in the RNA editing enzyme ADAR2. Nature (London) **405**, 78–81

59 Lewis, J. D. and Tollervey, D. (2000) Like attracts like: getting RNA processing together in the nucleus. Science **288**, 1385–1389

60 Spector, D. (1993) Macromolecular domains within the cell nucleus. Annu. Rev. Cell Biol. **9**, 265–315

61 Le Hir, H., Izaurralde, E., Maquat, L. E. and Moore, M. J. (2000) The spliceosome deposits multiple proteins 20–24 nucleotides upstream of mRNA exon–exon junctions. EMBO J. **19**, 6860–6869

62 Kataoka, N., Yong, J., Kim, V. N., Velazquez, F., Perkinson, R. A., Wang, F. and Dreyfuss, G. (2000) Pre-mRNA splicing imprints mRNA in the nucleus with a novel RNA-binding protein that persists in the cytoplasm. Mol. Cell **6**, 673–682

63 Maquat, L. and Carmichael, G. G. (2001) Quality control of mRNA function. Cell **104**, 173–176