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Review

Recent advances in mammalian RNA editing

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Abstract. RNA editing is a posttranscriptional modification that results in the generation of nucleotides within an RNA transcript that do not match the bases present within the genome. Mammalian RNA editing events, often represented by cytidine-to-uridine and adenosine-to-inosine conversions, are predominantly mediated by base deamination. In the past decade, important advances have been made in the understanding of editing mechanisms, the identification of RNA sequences and structures necessary for editing regulation, and the cloning and characterization of editing enzymes. It has also recently been appreciated that RNA editing within mammalian substrates can have profound functional consequences in protein function, implicating this posttranscriptional modification as important in the production of molecular diversity.

Key words. RNA editing; apolipoprotein B; glutamate receptor; serotonin receptor; hepatitis delta virus; APOBEC-1; ADAR.

Introduction

RNA editing is formally defined as any RNA-processing event (excluding RNA splicing) that generates an RNA transcript with a primary nucleotide sequence different from its gene. The editing process can occur by a variety of modifications, many of which are mechanistically unrelated. Deletion and insertion of nucleotides are common forms of the process, occurring most often in the mitochondria of primitive eukaryotes such as kinetoplastid protozoa and slime molds. In contrast to lower organisms, the most commonly identified type of editing in mammals appears to be substitutional editing, usually represented by direct nucleotide modification. This type of editing has been observed in mammalian transfer RNAs (tRNAs), precursor messenger RNAs (pre-mRNAs) and viral RNAs that have infected mammalian host cells.

With the exception of rarely described uridine-to-cytidine and uridine-to-adenosine modifications [1–3], mammalian editing events described thus far occur by nucleotide deamination. This type of editing falls into two main categories, represented by either cytidine-to-uridine (C-to-U) or adenosine-to-inosine (A-to-I) conversion. The best characterized example of C-to-U editing occurs within RNA transcripts encoding apolipoprotein B RNA [4, 5] and is mediated by the activity of a cytidine deaminase in concert with additional cellular regulatory factors. Recently described A-to-I conversions, mediated by a family of adenosine deaminases, have been observed within a growing number of RNAs, including those encoding several glutamate receptor subunits [6–8], the G protein-coupled serotonin 2C receptor [9] and the antigenome of the hepatitis delta virus [10, 11]. This review will focus on

the advancements that have been made in the definition of biochemical mechanisms responsible for substitutional RNA editing, the identification of edited mammalian RNAs, and the characterization of the cellular enzymatic and regulatory machinery responsible for mediating these editing events.

Biochemical mechanisms mediating substitutional RNA editing

One question that unites all studies of RNA editing involves elucidation of the biochemical mechanisms responsible for editing events in diverse RNA substrates. In theory, substitutional editing can be mediated by three major mechanisms, including nucleotide removal and replacement, base transglycosylation and direct nucleotide modification (reviewed in ref. 12) (fig. 1). Nucleotide replacement necessitates the cleavage of the phosphodiester backbone on each side of the target nucleotide and the subsequent religation of a new base, presumably donated from a nucleoside triphosphate. In transglycosylation, the ring of the target base is cleaved from the sugar and then replaced, maintaining the integrity of the phosphodiester bonds. Direct nucleotide modification, applicable only in conversion from purine-to-purine or pyrimidine-to-pyrimidine, involves the alteration of a portion of the parent ring moiety; for example, in the case of adenosine-to-inosine editing, this would involve enzymatic conversion of the adenosine C6 amino group to the ketone substituent characteristic of inosine.

These three possibilities can be discriminated using radiolabelling techniques and an in vitro editing system [9, 13–18]. First, a synthetic RNA message is transcribed in the presence of the appropriate [α-^{32}P] labelled ribonucleoside triphosphate (either cytidine 5′-triphosphate (CTP) or adenosine 5′-triphosphate (ATP)) to incorporate radiolabelled phosphates into the phosphodiester backbone (fig. 1, representing A-to-I editing). The synthetic RNA transcript is then incubated with in vitro editing extracts, and the reaction product is digested with nuclease P1. This nuclease cleaves single-stranded RNA into free 5′-nucleoside monophosphates, leaving the radiolabelled phosphate with its parent base. These free nucleosides can then be separated and identified using an appropriate thin-layer chromatographic system. Utilizing these steps can distinguish base excision and replacement from the two remaining mechanisms, as only base excision will remove the radiolabelled phosphate from its original base (fig. 1A).

To further distinguish between transglycosylation and base modification, the synthetic RNA message must be transcribed in the presence of a radiolabelled ring moiety (fig. 1B). In this situation, the radiolabel will remain with its parent nucleotide after cleavage only if base modification is the editing mechanism [15, 16]. Using these types of techniques, base modification has been shown to be the catalytic mechanism responsible for the majority of C-to-U and A-to-I conversions, providing clues as to the enzymatic machinery responsible for mediating these editing events [9, 13–18].

Editing events mediated by mechanisms other than deamination

While the majority of mammalian RNA editing occurs by deamination, several examples of editing deviate from this mechanism. These editing events include those occurring within diverse transcripts such as Wilms' tumour RNA [2], a mammalian tRNA [1] and an RNA encoding a human α-galactosidase [3]. While editing machinery and regulatory elements have not yet been identified and characterized for these nucleotide conversions, they encompass a full range of base alterations and represent novel mechanisms for the regulation of molecular diversity.

Site-specific U-to-C conversion in Wilms' tumour RNA

Wilms' tumour is a fairly common childhood cancer of the kidneys. *WT1* is thought to be a susceptibility gene for this disorder based on the observation that loss of the gene is associated with the development of kidney cancer [2]. The protein encoded by *WT1* is thought to be a transcriptional repressor based on its four carboxy-terminal zinc-finger domains and its ability to repress transcription from a variety of promoter sequences [2, 19–24]. New evidence also suggests that the amino terminus may act as a transcriptional activator, indicating a potential dual role for the protein [25].

RNA editing of *WT1* RNA converts a uridine to a cytidine at nucleotide 839, transforming a genomically encoded leucine into a proline [2]. The event is developmentally regulated; editing is almost undetectable during neonatal stages and rises until adulthood [2]. The leucine, or nonedited form of the protein, is a more potent transcriptional repressor than the proline-containing isoform. The physiological consequences of this difference in transcriptional efficiency are unclear, however, as the downstream effectors of *WT1* transcriptional regulation have not yet been identified. Editing of *WT1* RNA presumably occurs by amination and may be mediated by the actions of a CTP synthetase. This enzyme, known to synthesize CTP from uridine 5′-triphosphate (UTP) by transferring an amine group from glutamine [26], is the main candidate for this type of modification.

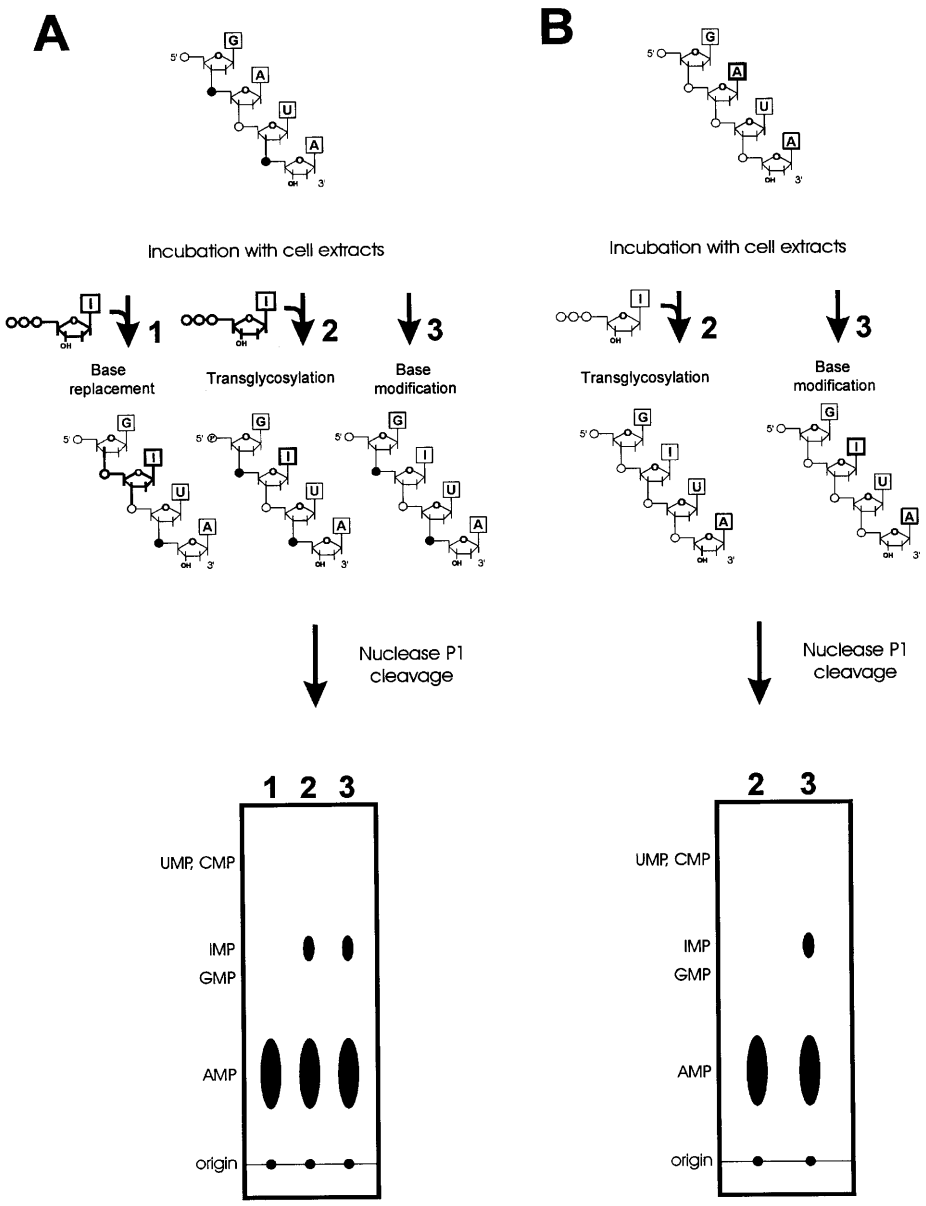

Figure 1. Techniques for determining the biochemical mechanism of substitutional RNA editing. (*A*) A synthetic RNA is transcribed in vitro using [α^{32}P]-ATP, incorporating radiolabelled phosphates into the RNA phosphodiester backbone (white circles, nonlabelled phosphate; black circles, labelled phosphate; A, adenosine; G, guanosine; U, uridine; I, inosine). The RNA is then incubated with editing-competent cellular extracts. Mechanism 1 represents nucleotide replacement; an unlabelled inosine 5′-triphosphate (ITP) molecule is inserted into the RNA backbone at the editing site accompanied by excision of the preexisting adenosine residue, removing the 5′-radiolabelled phosphate from the phosphodiester backbone (this substitution is represented by a thick black line around the new sugar and base and the conversion of a circle from black to white). In mechanism 2, representing transglycosylation, the bond between the adenine base and sugar is cleaved and only the hypoxanthine base of the ITP is incorporated, leaving the position of the radiolabelled phosphate in the backbone intact (represented by thick black line surrounding base only and retention of black circle). In the third possibility, base modification, the base is deaminated from adenosine to inosine, again resulting in the retention of the radiolabelled phosphate. Separation of these products using thin layer chromatographic (TLC) analyses reveals that both transglycosylation and base modification will result in similar patterns. Note that if the conversion were actually adenosine to guanosine, the correct TLC solvent system would resolve the difference in mobility between guanosine 5′-monophosphate (GMP) and inosine 5′-monophosphate (IMP) and ensure accurate determination of the base substitution. (*B*) To further distinguish between transglycosylation and base modification, an RNA molecule is transcribed in vitro using [2,8]^{3}H-ATP, a compound that results in labelling of the adenine ring (represented by a thick black line). If transglycosylation (mechanism 2) is the mechanism for catalysis, the unlabelled hypoxanthine base of inosine will be incorporated during the editing reaction. If editing occurs by base modification, the radiolabel will remain within the RNA strand. Cleavage and separation of these products will result in migration of label with inosine only if base modification is the correct mechanism. CMP = cytidine 5′-monophosphate; AMP = adenosine 5′-monophosphate; UMP = uridine 5′-monophosphate.

Pyrimidine conversions in mammalian transfer RNA

RNA editing in tRNA has been reported within the anticodon region of $tRNA^{Asp}$ from rats [1]. The rat gene for $tRNA^{Asp}$ occurs in a repeated gene cluster that includes $tRNA^{Leu}$, $tRNA^{Asp}$, $tRNA^{Gly}$ and $tRNA^{Glu}$. Sequencing and single-strand conformation polymorphism analyses of the multiple rat $tRNA^{Asp}$ genes have revealed that nucleotides 32 and 33 are cytidine and uridine residues, respectively [1, 27, 28]. Beier et al. [1] reexamined the sequence of cytoplasmic, processed $tRNA^{Asp}$, which had previously been reported to contain a U-C combination of bases at these positions rather than the C-U sequence dictated by the gene [29]. RNase cleavage and mobility shift assays have indicated that there are two isoforms of this cytoplasmic tRNA in rat liver, one containing the genomically encoded bases, and the other containing a uridine at position 32 and a cytidine at site 33 [1].

The putative editing sites are predicted to reside adjacent to the anticodon loop, the region of tRNA that recognizes codons of mRNA during translation to ensure addition of the appropriate amino acid to the nascent polypeptide chain [1]. If the anticodon recognizes a codon that differs from the amino acid with which the tRNA has been 'charged', the incorrect amino acid will be inserted. It has been postulated that editing at these positions may serve to maintain tRNA structure, thereby ensuring appropriate codon recognition [1].

Site-specific U-to-A conversion in α-galactosidase

α-Galactosidase is a lysosomal enzyme that is deficient in Fabry's disease, an X-linked disorder that results in defective metabolism of glycosphingolipids [30]. A uridine-to-adenosine conversion, changing a codon for phenylalanine to a tyrosine triplet, has been described within human RNA encoding α-galactosidase [3]. This conservative amino acid substitution occurs within the predicted C-terminus of the enzyme, but the functional consequences of editing at this position are unclear. The mechanism responsible for mediating this type of editing event is also unknown; to date, there are no identified enzymes that directly convert uridine to adenosine. The authors identified putative editing events from three different tissues including cerebellum, muscle and a human fibroblast cell line, suggesting that enzymatic and regulatory machinery mediating this putative U-to-A editing might be relatively widespread [3].

Editing events mediated by deamination

Site-specific C-to-U editing in apolipoprotein B RNA

The C-to-U conversion that occurs within RNA transcripts encoding apolipoprotein B was the first identified mammalian RNA-editing event. Apolipoprotein B-100 (apoB-100) is a protein synthesized in the liver that contains two domains important for cholesterol metabolism. The amino terminal half of the protein is necessary for the assembly of low density lipoproteins (LDLs), very low density lipoproteins (VLDLs) and intermediate density lipoproteins (IDLs) that deliver triglycerides and cholesterol to peripheral tissues (reviewed in ref. 31). When it exists as part of an LDL molecule, the C-terminus of apoB-100 interacts with the LDL receptor to allow lipoproteins to be endocytosed and utilized by peripheral cells [32]. When apoB-100 is a component of VLDLs, however, it lacks the ability to bind the LDL receptor, suggesting different conformations of the molecule depending upon the attached triglyceride [31].

A truncated version of apoB-100, termed apoB-48, is generated in the small intestine of most mammals by an RNA-editing event at nucleotide position 6666 which converts a **C**AA (glutamine) triplet into a **U**AA (termination) codon [4, 5]. This short form of the protein lacks the LDL receptor-binding domain and, when complexed in VLDL predecessors, does not promote the formation of LDL particles [33]. ApoB-48 was previously thought to be essential for the formation of chylomicrons, a very early step in the cholesterol transport pathway, but recently it has been recognized that apoB-100 can share this role [31].

***Cis*-active elements involved in the regulation of site-specific apoB editing.** Substantial progress concerning the editing of apoB transcripts has been made based upon the development of in vitro editing systems from a variety of cell sources [13, 34–39]. Most of these in vitro systems have utilized extracts prepared from cellular cytosol, although evidence indicates that apoB editing is actually a nuclear event [35, 40]. For example, pre-mRNA transcripts are often found in nonedited forms, indicating that editing occurs after transcription of the relevant portion of RNA but before the nuclear event of splicing [34, 35, 40]. Editing of apoB transcripts has also been found to influence polyadenylation, another process that occurs in the nucleus [4, 5, 41].

The apoB-editing site, cytidine 6666, is centrally located within a 14-kb mRNA transcript. In vitro analyses have indicated that editing requires only a small portion of the total transcribed mRNA [34, 35, 37, 39, 41–46]. Although 26 nucleotides that surround the targeted C residue will permit low levels of editing in many extract sources, slightly longer lengths of RNA are required for editing in some cells and in studies performed in vitro [34, 41, 42, 45]. This 'bulk' RNA effect is poorly understood, but points to a potential role for additional sequences and structures in the regulation of efficient apoB editing.

Extensive site-directed mutagenesis studies by Chen et al. [35] and Shah et al. [39] have revealed that the nature of the nucleotides immediately surrounding the editing site is not critical for apoB RNA modifications. Chen et al. showed that 20 out of 22 nucleotide alterations surrounding the targeted C residue (−3 to +5 relative to the editing site) permitted editing. Further studies, however, have revealed the importance of an 11-nucleotide region downstream of the editing site (+5 to +15); many point mutations in this region, termed the mooring sequence, completely ablate or drastically reduce editing efficiency [39] (fig. 2). The placement of these 11 nucleotides relative to the targeted C is also important, as insertions or deletions that alter spacing between this element and the modified cytidine moiety affect editing. In addition to the regulatory mooring sequence, the targeted C residue is also flanked by regulatory (5′ of C 6666) and spacer regions (immediately 3′) [39, 44, 45] (fig. 2). It is hypothesized that the regulatory, spacer and mooring sequences may provide binding sites for distinct components of the cellular editing machinery; once bound, the factor(s) would be correctly positioned to edit nucleotides within a certain distance upstream from the binding site [39, 46].

Additional C residues artificially introduced into the area around cytidine 6666 are also substrates for editing, indicating that the machinery can induce multiple editing events if the C residues are correctly positioned [47]. Introduction of the mooring sequence 3–5 bases downstream of a C within a heterologous RNA transcript can promote site-specific editing [35]. Editing of a second C residue at position 6802 within the 3′ untranslated region of human apoB mRNA has also been demonstrated, although the function of editing at this postion is unclear [38]. The importance of the mooring sequence is also reflected in the editing of this distal C residue; 7 of the 11 target nucleotides downstream from this second editing site are identical to those seen in the mooring sequence associated with C 6666 [38] (fig. 2). This conservation of sequence within the mooring region also provides a potential strategy by which to identify new C-to-U edited substrates; indeed, editing within neurofibromatosis type-1 RNA (next section) was discovered by homology to the apoB-mooring sequence [48] (fig. 2).

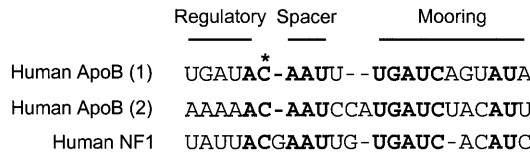

Figure 2. Comparison of the sequences surrounding human apolipoprotein B and neurofibromatosis type-1 editing sites. (1) and (2) represent the two C-to-U editing sites found within apoB RNA at either cytidine 6666 [47] or cytidine 6802 [38], respectively. The NF-1 sequence is from ref. 48. Bases that are identical between the three substrates are shown in bold. The regulatory, spacer and mooring sequences are indicated by overlined bars and the targeted C residue is marked by an asterisk.

***Trans*-acting factors involved in the regulation of site-specific apoB editing.** Using in vitro editing systems with specifically labelled RNA substrates, it has been shown that the editing of apolipoprotein B occurs by a site-specific deamination reaction that converts cytidine directly to uridine by base modification [13–15]. The machinery responsible for this editing event has recently been shown to include a catalytic, zinc-dependent, cytidine deaminase component previously termed RNA-editing protein (REPR) and now called apoB mRNA-editing enzyme catalytic polypeptide-1 (APOBEC-1) [49–55]. While this protein does not demonstrate editing activity alone, editing will occur when APOBEC-1 is coincubated with chicken enterocyte extracts or human liver cytosolic extracts, sources that do not have intrinsic editing activity [49, 56]. Additional proteins of 60 and 43 kd have been found to cross-link to the mooring sequence [43, 57–60], and a 240 kd complex has been shown to be involved in the maintenance of efficient editing [61]. These observations have led to the hypothesis that the editing of apolipoprotein B is accomplished by a large complex termed the 'editosome', with APOBEC-1 acting as the catalytic component and other cellular factors aiding in RNA recognition [57].

While the amino acid sequence of the APOBEC-1 protein is not highly conserved among species [62], there are several regions of sequence conservation that point to important functional areas of the protein. These areas include an amino-terminal nuclear localization signal and residues important for catalysis and zinc coordination. The requirement for zinc is corroborated by experiments revealing that incubation of in vitro editing extracts with *o*-phenanthroline, a zinc-chelating reagent, abolished apoB editing [50, 63, 64]. Mutagenesis of the potential zinc-coordinating residues (His^{61}, Cys^{93}, Cys^{96}) of the rabbit [54], human [64] and rat [52, 65] APOBEC-1 proteins also showed a strict requirement for zinc in the regulation of editing efficiency. A glutamic acid at position 63 is also required for catalytic activity [54, 64, 65]. While several of these mutations also result in proteins that fail to bind apoB RNA, this is not always the case and suggests that RNA binding and editing events can be separated [64]. This separation of binding and editing has also been shown by Anant et al. [66]; mutations that abolish APOBEC-1 editing activity do not always lead to an inability of the enzyme to bind substrate. Another conserved motif, consisting of a leucine-rich region interrupted by two prolines, has been hypothesized to be important for protein-protein inter-

actions either between two APOBEC-1 molecules [53, 67, 68] or APOBEC-1 and other editing factors [51–54, 65, 69]. Oka et al. [68] have recently generated transgenic animals overexpressing an APOBEC-1 molecule that can dimerize but fails to edit; these mice exhibit lower levels of editing than wild-type animals, a result that suggests that dimerization between APOBEC-1 molecules may be necessary for activity.

Regulation of apoB editing activity. In addition to the potential for dimerization and the putative role of cellular factors in the mediation of apoB editing, there are a number of other regulatory mechanisms that determine apoB-editing efficiency. For example, the editing of apoB exhibits tissue, developmental and species specificity. In humans, hepatic apoB is found exclusively in the apoB-100 form, while the small intestine expresses predominantly the apoB-48 isoform generated by RNA editing [4, 5, 70]. Human apoB transcripts are also found in both edited and nonedited forms in the colon, kidney and stomach [70]. ApoB-100 is the only form of the protein produced in the early human foetus; editing progresses throughout development, and apoB-48 is the major protein isoform produced in the adult tissues capable of editing these transcripts [71, 72]. In contrast to humans and other mammals, where apoB is found only in the nonedited form in the liver, rats and mice edit approximately 65% of hepatic apoB messages [73]. ApoB-48 has not been identified in avian species, indicating that editing of these RNAs probably does not occur in birds [74].

Hepatic apoB editing, as assessed in mice and rats, can be regulated through a variety of dietary and pharmacological manipulations (reviewed in ref. 31). For example, previous studies have shown that in fasting animals editing levels decrease from approximately 60–70% to 30–40%. Feeding the animals a diet high in carbohydrates leads to an increase in editing, such that up to 80 to 90% of mature RNA transcripts are modified [75, 76]. Ethanol has also been shown to increase editing levels of hepatic apoB in rats when included in the diet. For example, Lau et al. [77] observed that high dietary levels of ethanol (roughly one-third of the total caloric intake) could push editing levels to 100%, an effect that was represented proportionally at the protein level by increases in apoB-48 expression. Editing of hepatic apoB also appears to be hormonally regulated in rats. Oestrogen, thryoid hormone and insulin have been shown in various model systems to regulate the ratio of apoB-100 to apoB-48 [78–81]. From the standpoint of human apoB physiology, the functional consequences of hepatic apoB-editing regulation are unclear, as apoB RNA has not been demonstrated to undergo editing in the human liver; the possibility exists, however, that these dietary and pharmacological manipulations might affect editing levels in other tissues such as the colon or kidney.

Regulation of apoB editing is also thought to occur by modulation of *apobec-1* gene transcription and APOBEC-1 protein expression. For example, the mouse *apobec-1* gene has been demonstrated to use alternate promoters and undergo alternative splicing to generate tissue-specific RNA isoforms [62]. APOBEC-1 mRNA and protein expression also correlate with the presence or absence of apoB editing in different tissues, suggesting the protein is a limiting factor for editing [31]. For example, the human liver, which does not express APOBEC-1, only produces apoB-100-encoding transcripts, suggesting that APOBEC-1 plays a crucial role in apoB editing in vivo [82].

To further characterize the role of APOBEC-1 in apoB editing, APOBEC-1-deficient mice have recently been generated using homologous recombination techniques [83–85]. RNA transcripts isolated from these animals were completely nonedited, demonstrating that APOBEC-1 is the enzyme responsible for apoB editing and revealing a lack of redundance in the editing system. Furthermore, editing could be restored by adenovirus-mediated expression of APOBEC-1 [84]. Due to the postulated roles for apoB-100 and apoB-48 in distinct functions of fat absorption and metabolism, it was somewhat surprising that these animals exhibited relatively normal fat biology. These results were further confirmed by the generation of mice expressing only apoB-100; these mice also show relatively little perturbation of intestinal fat transport and metabolism [84, 86]. Intercrossing *apobec-1-l-* knockout mice with transgenic apoB-100 animals enhanced exclusive overexpression of the apoB-100 protein; these animals did exhibit alterations in plasma cholesterol and triglyceride levels that were reflected in increased levels of VLDL and LDL [84].

Transgenic or adenovirus-mediated overexpression of APOBEC-1 in mice and rabbits has been found to increase the editing of apoB RNA and reduce the concentrations of circulating LDLs and lipoprotein(a) [64, 84, 87, 88]. These observations have led to the hypothesis that transgenic expression of APOBEC-1 could serve as a novel therapy to lower LDLs, a major contributor to coronary artery disease. However, transgenic animals overexpressing APOBEC-1 were found to exhibit liver dysplasia and carcinoma [87]. Analysis of potential C-to-U editing substrates revealed that a protein tyrosine kinase was edited exclusively in the transgenic animals, indicating that overexpression of APOBEC-1 can induce inappropriate editing of certain substrates and has the potential to act as an oncogene [87]. This observation also raises the possibility that a number of other RNA molecules might be substrates for APOBEC-1 under both normal and pathophysiologic conditions.

Site-selective C-to-U editing in neurofibromatosis type-1 RNA

The neurofibromatosis type-1 gene product neurofibromin is thought to serve as a tumour suppressor due to its homology to mammalian and yeast Ras GTPase-activating proteins (GAPs) [89, 90]. The disorder associated with this gene, an increased risk of cancer, is termed von Recklinghausen neurofibromatosis or neurofibromatosis type 1 (NF1) [91]. The disease occurs sporadically in approximately half of the documented cases [91], suggesting that a large number of mutations within the NF1 gene might be observed. This has not, however, been the case, and the nature of the NF1 defect is unclear.

Recently, Skuse et al. [48] identified an apolipoprotein B-like mooring sequence within the mRNA that encodes human neurofibromin (fig. 2) and have shown that editing of cytidine 2914 occurs at low levels ($<2\%$) in control patients. Patients with NF1, however, show almost eight times the level of editing at this position. While the functional consequences of this editing event are unknown, editing at this site converts a **C**GA (arginine) codon into a **U**GA (stop) codon, suggesting that NF1 patients lack sufficient quantities of neurofibromin. The authors suggest that a reduction in the amount of this potential tumour suppressor may prevent appropriate regulation of the Ras signalling pathway, leading to unchecked cellular proliferation and cancer.

Skuse et al. [48] also suggest that although human neurofibromin RNA contains an apoB-mooring sequence, the factors responsible for editing of this RNA may be distinct from those mediating the C-to-U conversion within apoB. These results were based primarily upon studies involving transfection of a rat APOBEC-1 complementary DNA (cDNA) construct into human neurofibromin-expressing cell lines; no editing of the NF1 message was seen under these conditions. These conclusions, however, are complicated by the fact that rat neurofibromin RNA, in contrast to the human form, does not contain an apoB-like mooring motif and suggests that the lack of editing the authors observe might be due to species differences between enzyme and substrate. Nonetheless, the intriguing possibility exists that distinct catalytic or regulatory subunits may be responsible for diverse C-to-U editing events.

Hypermutation A-to-I editing

The majority of A-to-I editing events studied to date in mammals are site-specific in nature; that is, only one or a few of the adenosines within a large stretch of RNA undergo modification. The converse class of A-to-I editing events is called hypermutation and involves multiple modifications, occasionally resulting in the conversion of up to half of the adenosines within a given RNA molecule [92]. These types of editing events have only been shown to occur within viral RNAs that have infected mammalian host cells, although recently a hypermutation-like pattern of editing has been found within a *Drosophila* RNA-binding protein called 4f-rnp [93] and a potassium channel from the squid *Loligo peali* [94].

Hypermutation was originally observed within the measles virus matrix (M) protein, where A-to-I modifications within the negative strand of the viral RNA genome result in the appearance of complementary U-to-C conversions within the positive RNA strand [95, 96]. In patients with persistent measles virus, extensive A-to-I modifications within the M transcript inactivate the M protein, preventing the virus from moving into a lytic state [96]. Failure to remove the virus from the intracellular environment can lead to long-lasting infection and death from a condition termed subacute sclerosing panencephalitis [97, 98], indicating a role for RNA editing as a cellular antiviral mechanism.

Subsequent studies have revealed that a number of viral RNAs are subject to hypermutation, including vesicular stomatitis virus [99], parainfluenza virus 3 [100], avian leukosis virus [101] and polyomavirus [102]. RNA from the the polyoma virus genome is transcribed in early and late phases, resulting in the generation of early- and late-strand RNA transcripts that correspond to certain phases of the viral life cycle [102 and references therein]. Early-strand transcripts appear to undergo downregulation by antisense RNA molecules transcribed late in the infection process. Antisense RNA molecules hybridize to early-strand transcripts, causing them to become substrates for A-to-I modification. These modified RNAs accumulate in the cell nucleus, remaining hidden from the translational machinery. The authors suggest that these modifications are regulated by the activity of the ADAR (Adenosine Deaminase that Acts on RNA) family of A-to-I editing enzymes (to be discussed in detail below). Kumar and Carmicheal [102] further propose that the concerted generation of antisense RNA molecules and expression of an ADAR family member might provide a general eukaryotic mechanism of regulating gene expression.

Site-specific A-to-I editing in glutamate receptor subunit RNA

The majority of studied mammalian editing events are site-specific, resulting in the modification of relatively few adenosines within a given RNA transcript. Editing within the RNA encoding the GluR-B subunit of glutamate receptor is currently the best-studied example of site-selective A-to-I RNA editing. L-Glutamate, the ma-

jor excitatory neurotransmitter in the vertebrate central nervous system, acts as an agonist at three pharmaco-logically distinct ion channels referred to as the NMDA (*N*-methyl-D-aspartate), AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors [103]. Glutamate activation of these receptors results in neuronal cation entry and leads to the membrane depolarization critical for fast excitatory neurotransmission. It is the ability of the glutamatergic ion channels to conduct calcium, however, that underlies many important physiological functions. Many of these calcium-dependent mechanisms are critical for normal neurophysiological processes and are thought to contribute to situations such as long-term potentiation, a phenomenon in which glutamate receptor activation leads to long-lasting increases in synaptic efficacy and may be involved in the initiation and maintenance of memory [104].

While NMDA, AMPA and kainate channels are all known to be permeable to sodium and potassium, the ability to conduct calcium was previously attributed solely to the NMDA receptor [103, 105, 106]. Recently, however, molecular cloning techniques have revealed that all three of these receptor subtypes are multisubunit in nature [107–120], and current studies examining the function of these cloned proteins expressed in vitro have indicated that certain subunit combinations of AMPA and kainate receptors can be permeable to calcium ions [121, 122]. The observation that non-NMDA receptor subtypes are capable of permitting calcium entry into neurons has prompted a need to redefine the biological role(s) of AMPA and kainate channels and to examine the regulation of their subunit composition.

AMPA-subtype glutamate receptors are composed of combinations of four subunits, GluR-A, -B, -C and -D [107, 108]. Structure-function analyses of the GluR-B protein have revealed that channels containing the B subunit exhibit a very low to negligible calcium permeability; this phenotype can be traced to a specific arginine residue in the second hydrophobic domain of the protein [121, 123, 124]. This critical arginine is unique to GluR-B; the remaining subunits (GluR-A, GluR-C, GluR-D) contain a glutamine in the analogous position [108].

Interestingly, while the messenger RNA (mRNA) sequences of the GluR-B subunit encode an arginine (C**G**G) at this regulatory position, analyses of genomic DNA sequences have indicated the presence of a glu tamine codon (C**A**G) [6, 125]. Using genomic Southern and other appropriate analyses, Sommer et al. [6] excluded the possibilities that multiple genes or alternative splicing were sources of the arginine codon, proposing instead that RNA editing generated the altered base at this position termed the Q/R site (fig. 3, table 1). It has subsequently been shown, using the techniques outlined in a previous section, that the editing of GluR-B results from an A-to-I conversion (reflected as an A-to-G discrepancy between genomic and cDNA sequences due to the similar base-pairing properties of inosine and guanosine) [16–18]. The presence of this crucial arginine residue within the GluR-B protein and the dominant role of this subunit in regulating ion selectively of the AMPA receptor have suggested that maintenance of the RNA-editing process and GluR-B subunit expression may represent critical regulatory mechanisms controlling normal neuronal calcium homoeostasis.

Recently, it has been demonstrated that mice defective in their ability to completely edit GluR-B transcripts synthesize AMPA receptors that exhibit inappropriate permeability to calcium in principal neurons and interneurons of the central nervous system [130]. Mice heterozygous for the editing-incompetent allele develop acute neuronal degeneration in the CA-3 region of the hippocampus and an epileptic phenotype characterized by recurrent seizures and death by 3 weeks of age. This lethal phenotype indicates a critical role for GluR-B RNA editing in the maintenance of normal brain function and neuronal calcium permeability [130].

Similar RNA-editing events have been identified within other subunits of the glutamate receptor family. Within GluR-B, -C, and -D subunit RNA, an A-to-I conversion changes an arginine codon (**A**GA) to a glycine triplet (**I**GA) at a position called the R/G site [8] (fig. 3, table 1). The function of this editing event is to shorten the recovery time of the receptor after desensitization, presumably enhancing receptor activity within the brain. In contrast to editing at the Q/R site, which occurs with $>99\%$ efficiency throughout development, editing at

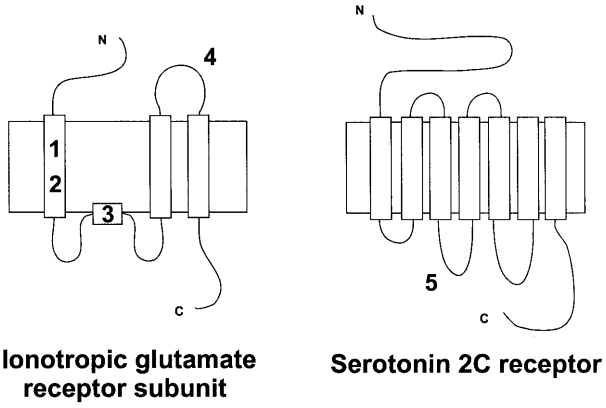

Figure 3. Topological location of glutamate and serotonin receptor A-to-I editing sites. The predicted topology of an ionotropic AMPA/kainate cation channel subunit and the G protein-coupled serotonin 2C receptor are shown as well as the predicted positions of amino acids altered by A-to-I RNA editing. See table 1 for further details.

Table 1. Location and functional consequences of identified mammalian A-to-I editing events.

Edited substrate	Editing site* cDNA/amino acid	Amino acid transition nonedited/edited	Protein region	Function consequence	Ref.
GluR-B	1820/607	Q/R	3	↓ calcium permeability	6, 125
GluR-B	2290/764	R/G	4	↑ recovery from	8
GluR-C	2305/769	R/G	4	desensitization	
GluR-D	2293/765	R/G	4		
GluR-5	1973/658	Q/R	3		6, 7
GluR-6	2012/671	Q/R	3	↑ calcium permeability†	
GluR-6	1699/567	I/V	1	modulate calcium	7
	1712/571	Y/C	2	permeability†	
5-HT_{2C}R	469 or 469 & 471/157	I/V	5	G protein-coupling	9, 127
	471/157	I/M	5	regulation	
	475/159	N/D	5		
	476/159	N/S	5		
	475 & 476/159	N/G	5		
	481/161	I/V	5		
HDV	587/196	Amber/W		Promotes viral packaging	11, 128, 129

The table lists the edited substrate, the position of the editing sites within the published cDNA and protein sequences, the region of the protein from figure 3 that encompasses the editing site and the known functional consequences of editing at each position. *The rat GluR-B, C, D and 5-HT_{2C}R, mouse GluR-5 and 6, and HDV sequences were compared from sequences found in GenBank (accession nos.: GluR-B M36419 [107]; GluR-C M36420 [107]; GluR-D M36421 [107]; 5-HT_{2C}R M21410 [127]; GluR-5 X66118 [126]; GluR-6 X66117 [126]; HDV M58629/M34325 [129]). †The consequences of editing at the GluR-6 Q/R site are dependent upon the editing status at the I/V and Y/C sites. See text for details.

the R/G site increases during maturation in mammals and indicates potentially different functions of edited AMPA receptors in the central nervous system at various developmental time points [8].

Within the kainate receptor family, two subunit RNAs undergo A-to-I editing events [6, 7, 131]. RNAs encoding the GluR-5 and GluR-6 subunits exhibit 40 and 80% editing, respectively, at a site analogous to the GluR-B Q/R site [6, 7, 126] (fig. 3, table 1). GluR-6 subunit RNA also undergoes editing at two additional positions within the predicted first transmembrane domain (TMI). Editing at these positions converts an isoleucine (**A**TT) to a valine codon (**I**TT) (I/V site) and a tyrosine (T**A**C) to a cysteine triplet (T**G**C) (Y/C site). The calcium permeability of kainate receptors containing the GluR-6 subunit is dependent upon the combinatorial pattern of editing at these three positions [7]. For example, if the I/V and Y/C sites are nonedited, both edited (arginine) and nonedited (glutamine) versions of the subunit at the Q/R site exhibit relatively high calcium permeability when assembled into homomeric channels [7]. When both TMI sites are in the edited configuration, the arginine form of the receptor is more permeable to calcium. Coassembly of calcium-permeable and calcium-impermeable GluR-6 subunits generates receptors with low calcium conductance, indicating a functional dominance of the calcium-impermeable subunits [7].

Site-specific A-to-I editing within 5-HT_{2C}R RNA

Serotonin (5-HT) is a monoamine neurotransmitter that mediates its activity by interaction with a large family of receptor subtypes. There are at least 13 members of the 5-HT receptor family, which are classified according to their pharmacological profile, molecular structure and signal transduction properties [132, 133]. The 5-HT_{2C} class of receptors includes three members termed the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} subtypes. These proteins are coupled to the stimulation of phospho-ipase C (PLC) by a G_q/G_{11}-like G protein, leading to the generation of diacylglycerol and inositol phosphates. 5-HT_{2C} receptors (5-HT_{2C}Rs) are found in the highest density in the choroid plexus and in lower levels in the hippocampus, olfactory bulbs, striatum and cortex (reviewed in ref. 134). Physiological functions of the 5-HT_{2C}R include production of cerebrospinal fluid, regulation of feeding behaviour and involvement in the aetiology of affective disorders such as schizophrenia and depression [135, 136]. The 5-HT_{2C}R is also known to be a hallucinogenic drug target [137]. 'Knockout' of the protein causes feeding abnormalities and death from spontaneous seizures, indicating that this receptor is also involved in the regulation of synaptic transmission [138].

RNA editing of the 5-HT_{2C}R occurs within a region of RNA predicted to encode the second intracellular loop of the protein [9, 127]. Editing events at four

major positions, termed A, B, C and D, and one minor site, C′, are predicted to alter the coding potential of this RNA region (fig. 3, table 1). Sequence analyses of RNA transcripts derived from dissected rat brain regions have indicated the generation of 7 major protein isoforms that were encoded by 11 predominant RNA species [9]. The expression of edited RNAs was also found to differ in various brain regions, predicting a unique repertoire of receptors in different brain areas and suggesting tissue- or cell-specific regulation of editing [9].

The editing sites within 5-HT_{2C}R RNA are predicted to alter amino acids within a region of the receptor known to be important in receptor:G-protein coupling [139–141] (fig. 3, table 1). To examine the possible functional consequences of editing in this region, edited 5-HT_{2C}R isoforms were transiently expressed in NIH-3T3 cells and tested for their ability to interact with the phospholipase C signal transduction pathway [9]. Comparison of the dose-response curves for the fully edited (VSV) versus the nonedited (INI) forms of the receptor revealed that serotonin was 10- to 15-fold less potent in its ability to stimulate the production of inositol phosphates when interacting with the VSV receptor variant [9]. This rightward dose-response shift is suggestive of a reduced G-protein coupling efficiency, possibly due to an alteration in receptor structure or regulatory phosphorylation at the introduced serine residue. While partially edited 5-HT_{2C}R isoforms exhibited phenotypes similar to the INI receptor in their ability to stimulate phosphoinositide hydrolysis [9], it is possible that distinct receptor isoforms might regulate as yet unexamined signal transduction cascades such as the modulation of cyclic AMP levels.

Site-specific A-to-I editing in hepatitis delta virus

The hepatitis delta virus (HDV) is a subviral human pathogen that requires concurrent expression with the hepatitis B virus for packaging and propagation [142–145]. Simultaneous infection with these two agents also increases the risk of liver complications. Transcription and translation of the delta virus genome produce the only protein encoded by the HDV genome, termed hepatitis delta antigen-p24 (HDAg-p24); this protein is essential for the replication of HDV by RNA polymerase II [146]. It had previously been observed that, although the genome of HDV encoded only one predicted protein species, two forms of the protein were often observed in infected livers and when the cloned HDV sequence was expressed in tissue culture cells (discussed in [128]). It has subsequently been shown that genomic replication of HDV generates an RNA intermediate, termed the antigenome, that is edited by an A-to-I transition that converts an amber stop codon (U**A**G) to a tryptophan codon (U**I**G) (amber/W site) [10, 11, 128, 129]. The functional consequence of this conversion appears after subsequent replication of antigenomic RNA to generate multiple copies of genomic RNA for packaging and release from the cell to complete the viral life cycle (table 1). Transcription and translation of this edited RNA produce an altered HDAg-p24 protein that contains a 19-amino acid carboxy-terminal extension. The larger form of the protein, called HDAg-p27, represses replication and is necessary for packaging of the hepatitis delta genome in conjunction with the hepatitis B virus [144, 145, 147, 148]. Therefore, this RNA editing event produces two distinct protein isoforms, each one serving a critical function in the life cycle of the virus.

***Cis*-active elements involved in the regulation of site-specific A-to-I editing.** Work from many laboratories has revealed that regulation of glutamate receptor editing occurs by the interaction of a double-stranded RNA substrate with a family of editing enzymes. Best studied for editing of the Q/R site of GluR-B, Higuchi et al. [149] defined a duplex RNA region formed by base pairing between exonic and intronic sequences that regulates editing efficiency. The secondary structure in this region is unique to GluR-B; GluR-A, -C and -D, which do not undergo editing at the Q/R site, do not possess this secondary structure. Ten nucleotides with exact complementarity to the Q/R site, termed the editing site complementary sequence (ECS), reside approximately 350 bases into the proximal region of intron 11 within an inverted repeat duplex RNA structure. Disruption of base pairing between the ECS and the nucleotides surrounding the Q/R site has been shown to drastically reduce editing efficiency [149, 150]. Further mutations within the duplex RNA of the inverted repeat region also affect editing, revealing that the global structure of the RNA is important for editing regulation [149, 150].

Other adenosines located within the duplex RNA region necessary for GluR-B editing also undergo modification [16, 149], indicating that these regions might provide important contacts for editing factors. These sites, often called 'hotspots', reside both within the GluR-B exon and within the intronic inverted repeat region [16, 149]. The extent of editing at these hotspot positions is lower than the editing observed at the GluR-B Q/R site, indicating that these sites may be in a less favourable structural context. A contrasting explanation is that these alternate sites are substrates for a distinct editing enzyme that does not exhibit the same editing efficiency as the enzyme responsible for Q/R site modification.

A double-stranded RNA structure has also been found to be a requirement for editing at the R/G sites of GluR-B, -C and -D RNA [8] the Q/R sites of GluR-5 and GluR-6 RNA [151], the amber/W site of HDV RNA [11], and the sites within 5-HT_{2C}R RNA [9, 152] (for a comparison of these RNA structures, the reader is referred to Rueter and Emeson, 1998 [141]). In general,

these secondary structures are formed by base pairing between nucleotides of the intron that are immediately downstream of the edited exon [9, 11, 149, 150]. An exception is the editing of the Q/R positions of the GluR-5 and GluR-6 kainate receptor subunit RNAs; in each case, the region required for editing is located approximately 1900 nucleotides downstream of the targeted adenosines [151]. The *cis*-active elements required for editing at the I/V and Y/C sites of GluR-6 RNA have not yet been identified, suggesting that their locations reside at some distance from the editing sites.

As described for GluR-B editing, mutations that disrupt double-stranded areas of each of these structures dramatically reduce editing efficiency, again indicating the importance of double-stranded RNA in the mediation of A-to-I editing [8, 9,11, 151]. Unlike the editing within glutamate receptor transcripts, however, sequence analyses of 5-HT_{2C}R pre-mRNA messages derived from whole brain did not reveal any additional significant editing hotspots [152], suggesting that the modification of intronic sites may not be shared among editing substrates. While these studies indicate that similarities do exist between structures required for diverse editing events, they also reveal that the length of the structure, the position within the downstream intron and the base pairing characteristics of each structure are distinct for different editing sites, suggesting mechanisms by which specific adenosine residues can be targeted for modification.

While no mooring sequence has been identified for A-to-I editing events, sequences surrounding a targeted nucleotide may play a role editing site selection and efficiency (table 2). For example, ADAR1, a candidate A-to-I editing enzyme, prefers adenosines with certain 5′ neighbours (A = U > C > G) [153]. An examination of the targeted adenosines within GluR-B RNA indicates that all editing sites within the predicted duplex RNA structure have a 5′ adenosine or uridine neighbour except for the Q/R site; the nucleotide 5′ of this editing target is a cytidine. These observations suggest that specific adenosines may be preferred targets for a particular enzymatic activity due to the influence of surrounding nucleotides.

In addition to sequence preferences, the structural features of the RNA immediately surrounding an editing target may also determine enzymatic specificity. For example, the Q/R site of GluR-B and the B, C and D sites of 5-HT_{2C}R RNA are predicted to exist within perfect duplexes [141, 149, 150, 152] (table 2). Other editing

Table 2. A-to-I editing site *cis*- and *trans*-acting factor characteristics.

Substrate	5′ Neighbour	Predicted base pairing characteristics of targeted A	Putative deaminase
GluR-B			
Q/R	C	base-paired	ADAR2
4	A	base-paired	ADAR2
60 (hotspot 1)	U	base-paired*	ADAR1
262–265	U/A/I	base-paired*	ADAR2
GluR-B			
R/G	C	C mismatch	ADAR1&2
GluR-5			
Q/R	C	bulge mismatch	?
GluR-6			
Q/R	C	bulge mismatch	?
I/V	U	?	?
Y/C	U	?	?
Amber/W	U	C mismatch	ADAR1
5-HT_2CR			
I/V157			
A site	A	bulge mismatch	ADAR1 > 2
B site	U	base-paired	ADAR1
N/S/D159			
C site	U	base-paired	ADAR1&2
C′ site	A/I	base-paired	?
I/V161	U	base-paired	ADAR2

The editing substrate, the 5′ neighbouring base of the editing site, the putative base-pairing characteristics of the RNA immediately surrounding the adenosine target and the ADAR family member that has been observed to edit the site are compared. Putative deaminases have been determined using either recombinant enzyme and substrate incubated in vitro or by coexpression of substrate and enzyme in tissue culture cells. * Indicates that a directly neighbouring nucleotide is not base-paired. ? Indicates that these characteristics are unknown. From refs 8, 149, 150, 173 (GluR-B); 151 (GluR-5, GluR-6); 11 (HDV); 9 (5-HT_{2C}R).

sites, such as the R/G site of GluR-B, -C and -D and the amber/W position of HDV [8, 11, 141], are predicted to reside across from a nonpairing cytidine residue. In the cases of the Q/R sites of the GluR-5 and GluR-6 subunits and the A site of 5-HT_{2C}R RNA, the editing side is predicted to exist within a small loop region [141, 151, 152]. The combination of these RNA sequence and structural features may play a role in determining the selectivity and efficiency of editing at a given position.

***Trans*-acting factors involved in the regulation of site-specific A-to-I editing.** The prototype A-to-I editing enzyme was described several years prior to the actual identification of a mammalian edited RNA substrate. This enzyme was originally characterized as an 'unwindase' activity found first in *Xenopus* oocytes and then shown to exist within and purified from the nucleus of mammalian cells [154–161]. The term *unwindase* referred to the ability of this enzymatic activity to unwind RNA:RNA duplexes by adenosine-to-inosine deamination. The actual unwinding phenotype was shown to result from the loss of stable A:U base pairs, resulting in destabilizing bulges in the structure [162, 163]. This enzyme, cloned from a variety of sources [164–167], is now termed ADAR1 (previously DRADA or dsRAD) [168]. ADAR1 has the ability to deaminate up to 50% of the adenosines within both strands of a double-stranded RNA substrate [162, 163]. Editing efficiency is related to the nucleotides immediately surrounding the targeted adenosines and RNA duplex length [153, 169]. The presumed indiscriminant deamination properties of ADAR1 were previously considered to exclude this protein as a candidate enzyme for site-selective editing. Recent studies, however, have shown that ADAR1 can act very specifically on short RNA duplexes [153], similar to those found within the glutamate, HDV and 5-HT_{2C}R RNA substrates, suggesting that it might mediate the editing of these RNA molecules.

Incubation of ADAR1 with a synthetic GluR-B substrate, however, indicated that ADAR1 did not possess strong deamination activity at the Q/R site and suggested the potential existence of additional enzymes with discrete site selectivity [170, 171]. In further support of this hypothesis, in vitro editing studies monitoring the deamination of a synthetic double-stranded RNA substrate had previously indicated that at least two A-to-I editing activities could be separated during the biochemical fractionation of nuclear extracts from HeLa cells [17, 171, 172]. Cloning strategies soon isolated ADAR2 (previously termed RED1), a protein related in structural similarity to ADAR1 [173–175]. In the rat, ADAR1 and ADAR2 are 130 and 80 kDa, respectively, and share only 31% identity at the amino acid level [165–173]. Both proteins are predicted to contain a bipartite nuclear localization signal [164–167, 173–175] (fig. 4), emphasizing that A-to-I editing is a nuclear event occurring before or during RNA splicing. ADAR1 contains three double-stranded RNA-binding domains (DRBMs), while ADAR2 contains only two that are most closely related in amino acid sequence to DRBMs 1 and 3 of ADAR1 [164–167, 173–175] (fig. 4). The enzymes share the highest degree of homology within the deaminase domain and, in similarity to important functional residues within the cytidine deaminases, ADAR1 and 2 share amino acids presumably involved in zinc coordination. Mutation of these residues within ADAR1 has been shown to result in a loss of catalytic activity [177], suggesting a shared dependence upon zinc for editing activity of deaminases acting on both adenosine and cytidine.

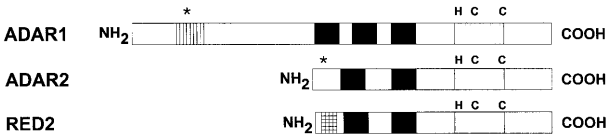

Figure 4. Structural characteristics of the ADAR family. A schematic diagram is presented indicating the major functional domains within ADAR family members. The deaminase domain is indicated by a gray rectangle and the putative zinc-chelating residues [H (histidine) and C (cysteine)] are shown. The double-stranded RNA-binding domains are represented as black rectangles, and the bipartite nuclear localization signals of ADAR1 and 2 are indicated by an asterisk. The Z DNA-binding domain of ADAR1 is shown as a striped rectangle, and the arginine-rich domain of RED2 is indicated by a checkered box. From refs 164–167, 179, 180 (ADAR1); 173–175 (ADAR2); 168, 176 (RED2/ADARB2).

Deamination by the ADAR family occurs at the C-6 position of the adenine ring, a region that is buried deep within the major groove of duplex RNA when it is in its normal A-form conformation. Sequence similarities between the C-terminus of ADAR1 and type II DNA methyltransferases have led investigators [153, 166] to suggest that ADAR1 may flip the target adenosine from the RNA duplex, similar to the mechanism of action described for the type II methyltransferase enzymes [178]. This mechanism may also explain the preference of ADAR1 for certain neighbouring nucleotides, as some bases may permit easier access to the target. ADAR1 also contains a sequence within its amino terminus that constitutes a Z DNA-binding domain (fig. 4). The Z conformation of DNA is thought to occur during transcription and has been hypothesized to target ADAR1 to newly synthesized, unspliced RNAs [179, 180]. ADAR2 lacks this sequence; therefore, a general role for this region in mediating the RNA-editing activity of the ADAR family is unclear.

Incubation of recombinant or purified ADAR1 and 2 with potential substrates transcribed in vitro has revealed that, together, these enzymes have the ability to modify

Table 3. Characteristics of alternatively spliced ADAR protein isoforms.

Species/enzyme	Amino acid changes generated by alternative splicing	In vitro deaminase activity (ds RNA substrate)	Ref.
hADAR1a	insertion 694–712, 807–832	yes	
hADAR1b	insertion 694–712	yes	
hADAR1c		yes	164, 165 167
rADAR1		yes	
hADAR2a	long C-terminus*	yes	
hADAR2b	long C-terminus, insertion 466–505	yes	174, 175
hADAR2c	short C-terminus, insertion 466–505	5–10-fold decrease	
hADAR2d	short C-terminus	5–10-fold decrease	
rADAR2a		yes	172, 183
rADAR2b	insertion 466–475	yes	
RED2/ADARB2		no	176

A comparison of ADAR protein variants is presented (r, rat; h, human). The amino acid changes generated by alternative splicing and the ability of each of these modified proteins to deaminate a synthetic double-stranded RNA substrate are shown. * Indicates an alternative splicing event at the C-terminus of hADAR2 that results in the inclusion of either a 29-amino acid peptide (long C-terminus) or a peptide of 2 amino acids (short C-terminus).

many positions that have been identified to undergo A-to-I editing in vivo (table 2). For example, ADAR2 has been shown to be highly selective for the Q/R-editing site of GluR-B RNA, while ADAR2 exhibits a much greater ability to edit the +60 position (also called hotspot 1) of GluR-B RNA [173–175]. ADAR1 and 2 may have overlapping specificities at some positions, however, as it has been shown that both enzymes are capable of editing the R/G site of GluR-B transcripts to roughly equivalent extents in vitro [171, 173]. Importantly, it has been observed that editing patterns seen after an RNA substrate is edited in vitro are occasionally different than the pattern identified in vivo. This is particularly true for in vitro incubations performed with ADAR1 and has been observed for editing both of GluR-6 and HDV substrate RNAs [11, 151, 153]. Similar discrepancies between in vivo and in vitro editing specificities are seen when 5-HT_{2C}R RNA is used as a substrate [9]. Editing of the A and C positions of the 5-HT_{2C}R was observed to migrate exclusively with rat ADAR1 (rADAR1) activity in fractionated rat brain extracts. These sites could be efficiently edited by rADAR2, however, when the 5-HT_{2C}R substrate was coexpressed with this enzyme in tissue culture cells [9]. These studies suggest that overexpression of editing enzymes may result in inappropriate levels of editing or in the induction of editing at novel positions, such as that observed for the overexpression of APOBEC-1. Alternatively, accessory factors may exist in vivo that provide editing site specificity, either by enhancing or prohibiting editing at certain sites.

A-to-I enzyme regulation and diversity. ADAR1 is expressed in almost every cell and tissue type examined [157]. The expression pattern of ADAR2 may be slightly more restricted; for example, expression of this enzyme appears low in skeletal muscle [173]. ADAR2 is also expressed at higher levels than ADAR1 in certain rat brain regions, most notably the olfactory bulb, thalamus, hippocampus and cerebellum [173]. This widespread distribution of the ADAR enzymes both within and outside of the central nervous system suggests that many potential substrate RNAs may exist. In addition to enzymatic regulation inherently controlled by expression patterns, editing activity also appears to be regulated throughout development. For example, editing at the R/G site of GluR-B, -C and -D increases throughout developmental time, suggesting potential modulation of enzyme levels or activity specific to certain developmental time points [8].

Human ADAR1 (hADAR1) expression has been shown to be induced by the presence of interferon [181, 182]. Two forms of the hADAR1 protein exist in human cells, termed p150 and p110 [182]. The p110 isoform is constitutively expressed exclusively in the nucleus, while the p150 variant appears to exist in both cytoplasmic and nuclear compartments [181]. Expression of the p150 isoform is induced after exposure to interferon α or γ in a time-dependent fashion, while levels of the p110 protein are unaffected by interferon treatment [181, 182]. The mechanism for the generation of these two isoforms is unclear, but antibody mapping studies have suggested that the p110 isoform is an amino-terminal truncated version of p150 [181].

Another mechanism of protein regulation arises from alternative splicing of the RNAs for both ADAR1 and ADAR2 (table 3). There are now three published hADAR1 isoforms, termed 1a, 1b and 1c. hADAR1a represents the full-length protein isoform [164, 165, 167], while in hADAR1b, 26 amino acids are deleted between the second and third DRBMs [167] (table 3). In hADAR1c, an additional 19 amino acids between the third DRBM and deaminase domain are deleted [167]

(table 3). While these enzyme isoforms appear to have similar levels of activity when incubated with a perfect RNA duplex, it is possible that these variants will act selectively on or be specifically coexpressed with a given substrate molecule in vivo [167].

Alternatively spliced isoforms of ADAR2 have been identified in both human and rat. In human RNA transcripts, 120 nucleotides can be inserted within the deaminase domain to produce the hADAR2b isoform [174, 175] (table 3). This insertion contains a high degree of homology to an Alu-J sequence, a conserved sequence found within a number of human genes [174, 175]. An insertion of 40 amino acids at this position would alter the linear spacing of two of the zinc-coordinating residues, suggesting a potential alteration in function. It appears that the hADAR2b isoform may have reduced catalytic activity for substrates such as the GluR-B Q/R and R/G sites as well as a synthetic double-stranded RNA molecule, suggesting that alternative splicing may represent a cellular mechanism for controlling ADAR2 enzymatic activity [174, 175]. Within rADAR2 transcripts, a 30-nucleotide insertion occurs at a position homologous to the human Alu J insertion [168, 176, 183], but the functional consequences of the generation of multiple rat isoforms have not yet been examined in detail.

hADAR2 RNA has also been found to be alternatively spliced at the 3′ end of the coding region, generating proteins with distinct carboxyl termini and 3′ untranslated regions [174, 175]. These alternative splicing events generate four potential hADAR2 (hADAR2a–d) protein isoforms, depending upon the combinatorial pattern of splicing at each alternative site. Two of these isoforms, hADAR2c and 2d, are truncated at the C-terminus and are unable to edit the GluR-B Q/R and R/G sites that are normal substrates for hADAR2a and b in vitro [175]. These isoforms may represent enzymes that are specific for currently unidentified editing positions; alternatively, they may modulate editing by preventing access of functional hADAR2 variants to target sites [175].

Subsequent searches for additional members of the mammalian adenosine deaminase family have isolated RED2 (or ADARB2), a protein sharing 60% amino acid identity with ADAR2 [176]. RED2 appears to be expressed only in the mammalian brain and, in contrast to ADAR2, possesses a 54-amino acid extension at its amino terminus that is arginine-rich (fig. 4), the function of which is unknown. While RED2 shares the major structural features characteristic of the ADAR family (table 3), it does not possess measurable deamination activity towards any ADAR1 or -2 substrate. For this reason, RED2 has not been formally placed into the ADAR family [168]. RED2 appears to have a dysfunctional adenosine deaminase domain; chimeras replacing this region with the corresponding area of rADAR2 exhibit weak A-to-I editing activity at appropriate sites [176]. In contrast, the converse chimera was inactive using in vitro RNA-editing analyses [176]. While no RNA molecules have been identified as substrates for RED2, the possibility exists that this protein is responsible for editing substrates that await identification.

Summary

The identification of RNA-editing events within diverse mammalian substrates indicates that these posttranscriptional modifications are important for the generation of molecular diversity. The determination of an increasing number of modified RNA substrates, as well as the cloning of putative editing enzymes, has vastly increased our knowledge of both *cis*- and *trans*-acting factors necessary for the catalysis of substitutional RNA-editing events. Future studies are certain to provide insights into the regulation of editing, both by modulatory cellular factors as well as by environmental stimuli. Further knowledge of the molecular mechanisms involved in mammalian RNA editing will most certainly lead to the identification of other editing substrates and to an improved understanding of the roles of proteins encoded by edited RNAs in normal and pathological cellular function.

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