Changing genetic information through RNA editing

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Summary

RNA editing, the post-transcriptional alteration of a gene-encoded sequence, is a widespread phenomenon in eukaryotes. As a consequence of RNA editing, functionally distinct proteins can be produced from a single gene. The molecular mechanisms involved include single or multiple base insertions or deletions as well as base substitutions. In mammals, one type of substitutional RNA editing, characterized by site-specific base-modification, was shown to modulate important physiological processes. The underlying reaction mechanism of substitutional RNA editing involves hydrolytic deamination of cytosine or adenosine bases to uracil or inosine, respectively. Protein factors have been characterized that are able to induce RNA editing in vitro. A supergene family of RNA-dependent deaminases has emerged with the recent addition of adenosine deaminases specific for tRNA. Here we review the developments that have substantially increased our understanding of base-modification RNA editing over the past few years, with an emphasis on mechanistic differences, evolutionary aspects and the first insights into the regulation of editing activity. *BioEssays* 22:790–802, 2000. © 2000 John Wiley & Sons, Inc.

Introduction

It was generally believed that the DNA-encoded, exonic sequence information stored in the genome directly predicts the amino acid composition of the resulting gene products. However, this view had to be modified following the discovery of RNA editing, a process that enables a cell to recode genomic information in a systematic and regulated manner, selectively changing the readout of a gene at single nucleotide positions within the primary RNA transcript. The type of RNA editing that was first discovered[1] works through deletion and/or insertion of bases, involving small guide RNAs complementary to the target RNA (reviewed in Refs. 2,3). This process, which is found in mitochondria of primitive eukaryotes, serves to create open reading frames where none exist. This is a fundamentally different mechanism to the other type of RNA editing, which is characterized by base substitution, in pre-mRNAs of higher eukaryotes. Here the open reading frame is altered, yielding a protein with one or more changes in its amino acid sequence. Substitution RNA editing was detected in many mitochondrial RNAs of higher plants with mostly cytidine-to-uridine (C-to-U) or U-to-C changes (for review see Ref. 4). A recent systematic analysis of *Arabidopsis* mitochondrial genes revealed that 8% of all C-containing codons are edited[5] documenting the widespread occurrence of C-to-U editing in higher plants. Table 1 lists the different types of RNA editing and some of their characteristics.

In mammals, RNA editing is mainly represented by C-to-U and adenosine-to-inosine (which functions as guanosine) (A-to-I) conversions generating single amino acid changes in the resulting protein. This often has significant consequences for protein function. Among mRNAs that were found to undergo editing, the best-characterized examples are apolipoprotein B (apoB) transcripts (C-to-U change) and messages for neuronal glutamate and serotonin receptor subunits (A-to-I changes).

Since the initial discovery of mRNA editing in mammals[6,7] more than a decade ago this process of post-transcriptional modification is now recognized as an important mechanism for generating molecular diversity.[8–10] Inosine in mRNAs was detected only after RNA editing had predicted its presence. However, the occurrence of inosine in tRNA has been known for a long time.[11] A prominent example is the inosine at the wobble position of several transfer RNAs,[12] which is the main molecular determinant for the degeneracy of the genetic code. This review will focus on the considerable progress that has been made towards the understanding of mRNA and tRNA editing in mammals involving deamination of C and A. Even though the biochemical mechanism is similar in both cases, the molecular machinery differs significantly. While cytidine

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Abbreviations: 5-HT_{2C}, 5-hydroxy-tryptamine receptor subunit 2C; ADA, adenosine deaminase; ADAR, adenosine deaminase acting on RNA; ADAT, adenosine deaminase acting on tRNA; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid; apoB, apolipoprotein B; apobec 1, apoB editing enzyme catalytic subunit 1; CDAR, cytidine deaminase acting on RNA; CNS, central nervous system; dsRNA, double-stranded RNA; ECCDA, E. coli cytidine deaminase; ECS, editing-site complementary sequence; GluR, glutamate receptor; HDV, hepatitis delta virus; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; m^1I, 1-methyl inosine; NLS, nuclear localization sequence; VLDL, very low density lipoprotein.

Table 1. Different forms of RNA editing

Type	Organism	Substrate(s)	Mechanism
Insertion/deletion			
U,ΔU	kinetoplastids	mRNA	cleavage/ligation
C, U, G, A	*P. polycephalum*	*mRNA, tRNA, rRNA*	*?*
Conversion/substitution			
C-to-U	higher plants,	mRNA	deamination
	P. polycephalum	mRNA, tRNA, rRNA	?
	mammals	mRNA	deamination
U-to-C	mammals[a]	mRNA	?
	higher plants	mRNA	?
A-to-I	mammals	mRNA	deamination

ΔU, deletion of U; [a]see reference 13.

deamination requires a multisubunit protein complex (also termed the 'editosome') recognizing primary sequence motifs in its target, adenosine deamination can be accomplished by a single enzymatic polypeptide that specifically binds to a tertiary RNA structure with partial double-stranded (ds) RNA character. Recently, there has been identification, cloning and sequence comparison of several eukaryotic mRNA-specific adenosine deaminases, cytidine deaminases and tRNA-specific adenosine deaminases. Here we will address the questions of how these enzymatic activities might be evolutionary related and what is the significance of the phenomenon of substitutional RNA editing in evolution.

One gene → many proteins

Eukaryotic organisms use several mechanisms to increase the number of functionally different proteins produced from a single gene. This includes alternative splicing of the exons, use of different promoters, translational frameshifting or post-translational modification. As an additional level of variation, RNA editing allows for the expression of several protein variants at the same time, depending on the number and combination of modifications introduced into individual mRNAs. Fig. 1 shows two thoroughly studied examples of such targets and how editing increases diversity. In the very large apolipoprotein B mRNA molecule, a single deamination event, involving C6666, changes an arginine-specifying CAA codon into a premature UAA stop signal.[13] As a result, translation of the edited RNAs produces a truncated apoB protein (apoB48), whereas unedited transcripts generate a protein of much larger size (apoB100) through translation of the uninterrupted open reading frame (Fig. 1A). In this case, the involvement of a single nonsense mutation yields two protein variants from one gene.

The picture is more complex in the case of the glutamate receptor subunit GluR-6 in the central nervous system (CNS; Fig. 1B). These transcripts harbor three exonic editing sites (I/V, Y/C, Q/R) within the open reading frame giving rise to a mixture of eight alternative sequence configurations.[14,15] Since the extent of editing at the three individual sites varies, between 10 and 80% of all of the eight theoretical combinations are detectable in vivo.[15]

Table 2 lists presently known and well-characterized mRNAs that are subject to deamination editing in eukar-

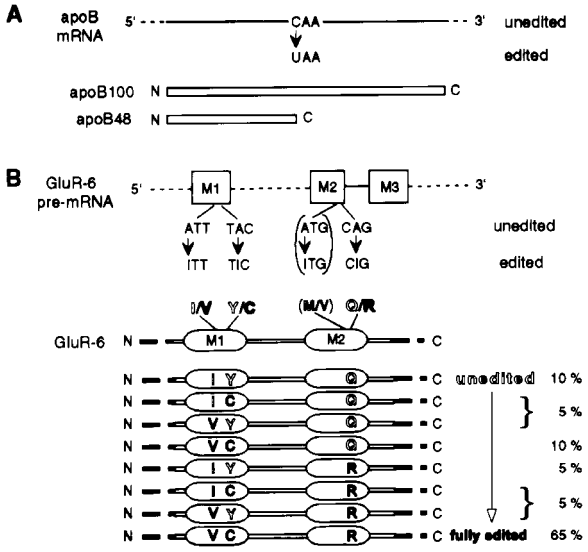

Figure 1. Diversity through RNA editing. **A:** Single site C-to-U editing of apoB mRNA results in the expression of two proteins from one gene, apoB100 and apoB48. **B:** The kainate receptor subunit GluR-6 pre-mRNA undergoes RNA editing at three major positions, two located in membrane region M1 and one in M2. The codon and ensuing amino acid changes are indicated. A fourth editing site in M2, shown in brackets, has not been further characterized.[83] All resulting GluR-6 isoforms are shown at the bottom with relative percentages as expressed in rat total brain.[14]

yotes. Some editing substrates undergo one or a few deamination event(s), but a number of RNAs have been identified that are modified at multiple positions within a defined region of the primary transcript.[8] These cases of so-called hyperediting, or hypermutation, predominantly involve viral RNA transcripts such as measles virus and polyoma virus.[8,10] A well-documented non-viral example of hyper-modification editing is a voltage-dependent potassium channel RNA (sqKv2) from squid where, in a segment of 360 nucleotides, up to 17 adenosine modifications occur within a single transcript.[16]

Functional consequences of RNA editing

Apolipoprotein B C-to-U editing

Apolipoproteins are essential components of plasma lipoproteins that serve as transport vehicles of lipid nutrients in the circulation.[17] The unedited apolipoprotein B transcript gives rise to full-length apoB (apoB100), which is synthesized in the liver and represents the major protein component of very low density lipoproteins (VLDL) and their maturation products intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). In the small intestine of most mammals, a truncated apolipoprotein B (apoB48) is produced as a result of tissue-specific base modification editing at C6666 and is incorporated into chylomicrons and their remnants (Fig. 2A). The unedited (apoB100) and edited (apoB48) variants of apolipoprotein B have quite different functions in lipid metabolism. Notably, all apoB100-associated lipoproteins are highly atherogenic, so that high plasma levels of VLDL, IDL and LDL particles increase the susceptability to atherosclerosis. However, apoB48 is a constituent of lipoproteins of much less atherogenic potential (chylomicrons and chylomicron remnants). Later we discuss how understanding the RNA editing mechanism and its regulation might eventually be used therapeutically to decrease the risk of atherosclerosis in humans.

A-to-I editing

The functional consequences of A-to-I editing have been studied most extensively in the glutamate receptor subunit GluR-B, which was the first A-to-I editing substrate identified.[18] As the major excitatory neurotransmitter in the CNS, L-glutamate activates three pharmacologically and electrophysiologically distinct receptor families referred to as NMDA (N-methyl-D-aspartate), AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid) and kainate receptors. Each receptor is assembled from a subset of evolutionary related protein subunits. Fast excitatory neurotransmission is mediated by the AMPA receptor channels composed of GluR subunits A to D. In principal neurons, the putatively pentameric receptors are characterized by a low Ca^{2+}-permeability.[19] Through functional studies with recombinantly expressed wild-type and mutant AMPA receptor channels, this crucial property was demonstrated to be dependent upon the GluR-B subunit in heteromeric receptors.[19] The molecular determinant for the dominant effect of GluR-B was traced to a single arginine (R) residue located in the channel-pore lining segment M2 (Fig. 2B).[18] The other subunits, GluR-A, GluR-C and GluR-D carry a glutamine at the homologous position.

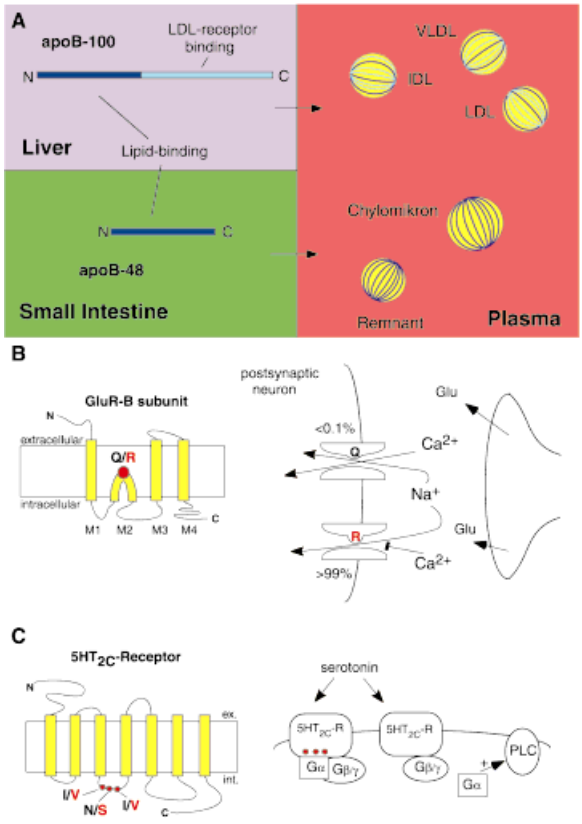

Figure 2. Functional consequences from RNA editing. **A:** ApoB100 is produced in the liver and incorporated into VLDL, IDL and LDL particles. ApoB48 is generated from edited apoB mRNA in the small intestine and is a major component of chylomicrons and their remnants. **B:** Glutamate receptor subunit GluR-B dominantly regulates the Ca^{2+} permeability of AMPA receptors as a result of Q/R site editing. The position of the Q/R site is indicated by a red dot within the membrane topology model of GluR-B. In central synapses $>99.9\%$ of GluR-B subunits carry arginine at this position as the result of A-to-I editing leading to AMPA channels with low Ca^{2+} permeability. **C:** The 5-HT_{2C} serotonin receptor topology is shown with the location of the major A-to-I editing positions within the second intracellular loop (red dots). G-protein-coupling efficiency and consequently phospholipase C (PLC) activation is less efficient with fully edited receptors.

Surprisingly, the arginine in GluR-B is not encoded on the GluR-B gene. Instead, the genomic sequence predicts a glutamine (Q) for the corresponding position.[18] However, more than 99% of the GluR-B primary transcripts undergo RNA editing, changing the glutamine-coding CAG triplet to a CIG codon specifying arginine. This single editing event thus tightly controls the Ca^{2+}-permeability of AMPA receptors.[18] The physiological significance of this editing site, termed the Q/R position, became dramatically evident when a mutant mouse line was generated that expressed some GluR-B with glutamine at the Q/R site.[20] Heterozygous animals for GluR-B(Q) displayed a severe epileptic phenotype and died within three weeks of birth as a result of the increased Ca^{2+}-permeability of AMPA receptors.[20] Interestingly, a mutant mouse line engineered to express only GluR-B(R) does not show any phenotypic aberration.[21] This raises the question: why is GluR-B(R) not already specified on the genome, if GluR-B(Q) is not needed (or even detrimental when expressed, see Ref. 20) and the GluR-B pre-mRNA is nearly quantitatively converted to yield GluR-B(R)? Presumably, GluR-B(Q) serves a non-essential but advantageous function in the CNS, which is as yet unknown.

Later, additional sites of RNA editing were discovered involving GluR subunits of AMPA and kainate receptors (see Table 2; and Refs. 14,22). In most cases, they could also be linked to functional differences between the edited and unedited receptor channels such as the R/G position of GluR-B, which was found to modulate channel gating kinetics.[22]

Recently another class of neuron-specific RNA transcripts coding for the 5-HT_{2C}-serotonin receptor were shown to undergo A-to-I editing at five major nucleotide positions (see Table 2 and Fig. 2C).[23] The G-protein-coupled receptor is involved in a variety of physiological processes such as the production of cerebrospinal fluid and the regulation of feeding behavior, and it is believed to be involved in the etiology of a number of human psychiatric and behavioral disorders.[10] RNA editing of the 5-HT_{2C}-receptor transcripts changes a region within the second intracellular loop of the membrane molecule that is known to be important for receptor:G-protein coupling (Fig. 2C).[23,24] In functional assays with recombinant channels, the unedited receptor variant displayed a 10- to 15-fold higher potency to stimulate phospholipase C than the fully edited version.[23] In the mammalian brain, the expression of partially or fully edited RNAs was found to differ in various brain regions and also between species,[25] indicating that differentially edited 5-HT_{2C} receptors may serve distinct physiological functions in those regions in which they are expressed.

For the subviral human pathogen Hepatitis Delta Virus (HDV), A-to-I editing within its antigenomic RNA controls the production of two protein variants, each of which fulfills an essential function during the viral life cycle. In the unedited form, the only open reading frame of HDV produces a protein necessary for replication (HDAg-p24) after transcription and translation of the HDV genome. The deamination event in the HDV antigenomic RNA converts an amber stop codon (UAG) to a UIG tryptophane codon.[26] As a result, transcription and translation of the edited RNA generates a protein extended at the C terminus (HDAg-p27), which has new functions. It represses replication and promotes packaging of the hepatitis delta genome. Thus RNA editing is a prerequisite for completing the viral life cycle.

Table 2. Substrates of substitutional mRNA editing

mRNA	Organism	Modification	Reference
apoB	mammals	C-to-U; CAA to Stop	6, 7
cox1	*P. polycephalum*, mito.	C-to-U	85
several	mito. of higher plants	C-to-U, U-to-C	4, 5
GluR-B	mammals	A-to-I; Q/R, R/G	18, 22
GluR-C, -D	mammals	A-to-I; R/G	22
GluR-5, -6	mammals	A-to-I; Q/R	14
GluR-6	mammals	A-to-I;I/V, Y/C	14
5-HT_{2C}R	mammals	A-to-I; I/V, N/S, I/V	23
ADAR2	mammals	A-to-I; splice acceptor	54
sialyltransferase[a]	mammals	A-to-I; Y/C	86
HDV antigenome	HDV host cells	A-to-I; amber/W	26
para[b]	*D. melanogaster*	A-to-I; Q/R, N/D, N/S	87
4f-rnp[c]	*D. melanogaster*	A-to-I; hyperediting	88
sqKv2[d]	*Loligo peali*	A-to-I; hyperediting	16
IR elements[e]	*C. elegans*	A-to-I; 3′-UTR, 5′-UTR	89
RNA viruses[f]	viral host cells	A-to-I; hyperediting	8

The type of base modification as well as the associated amino acid change(s) is indicated. Mito, mitochondriaj. [a]α-2, 6-sialyltransferase; [b]voltage-gated Na^+ channel; [c]RNA-binding protein of unknown function; [d]voltage-dependent potassium channel; [e]inverted repeat elements in mRNAs of unknown function; [f]measles virus, parainfluenza virus, vesicular stomatitis virus, avian leukosis virus.

Biochemical mechanism of C-to-U and A-to-I modification in mRNA

Characterization of C-to-U and A-to-I RNA editing reactions and elucidation of the underlying biochemical mechanisms proceeded rapidly once in vitro editing systems were developed. The C-to-U editing mechanism involving apolipoprotein B mRNA was first explained[27,28] and later the A-to-I editing of glutamate receptor pre-mRNAs.[29–31]

For GluR-B editing at the Q/R and the R/G positions editing site complementary sequences (ECS) residing within the adjacent introns of the Q/R- and R/G-site exons proved to be essential for the formation of partially base-paired RNA structures that were required for site-specific, efficient editing.[22,32] In vitro transcribed minimal RNA substrates were used in the in vitro editing systems to further delineate the cis-acting elements in apoB and GluR pre-mRNAs. These studies also helped determine the nature of the base modifications. In both cases, hydrolytic deamination of C-to-U or A-to-I was identified as the underlying reaction mechanism. Biochemical experiments suggested the involvement of zinc-dependent proteinacious deaminase activities (reviewed in Refs. 9,10,13).

A major mechanistic dissimilarity between these two types of substitutional RNA editing is suggested from the differing substrate requirements. For apoB mRNA editing,[13] a tripartite primary sequence motif (including a spacer, regulator and mooring sequence) is needed. However, a partially double-stranded RNA structure is recognized by the editing machinery during modification of GluR-B primary transcripts.[15] In addition, further evidence from cellular fractionation and other biochemical studies led to a model for apoB editing postulating a multicomponent protein complex, termed the editosome,[13] while similar studies with A-to-I conversion catalyzing activities indicated the action of a single monomeric polypeptide (Fig. 3).[10]

Expression cloning, utilizing the in vitro apoB editing system led to the isolation of a cytidine deaminase activity termed apobec-1 (apoB mRNA editing enzyme catalytic subunit 1).[33] This 27 kD protein does not display editing activity alone but catalyzes specific cytidine deamination in apoB mRNA when coincubated in extracts from human liver cells that lack endogenous editing activity.[33] As part of the postulated apoB editosome, a dimer of apobec-1 is associated with complementation factors that recognize and bind the mooring sequence (Fig. 3A). Recently, a 65 kD protein, termed ACF (apobec-1 complementation factor)[34] or ASF (apobec-1 stimulating factor)[35] has been molecularly cloned. It complements apobec-1 in an in vitro reconstitution assay and binds apoB mRNA in vitro.[34,35] However, it cannot be ruled out that additional auxiliary proteins might be involved in vivo as indicated by several studies.[35–38]

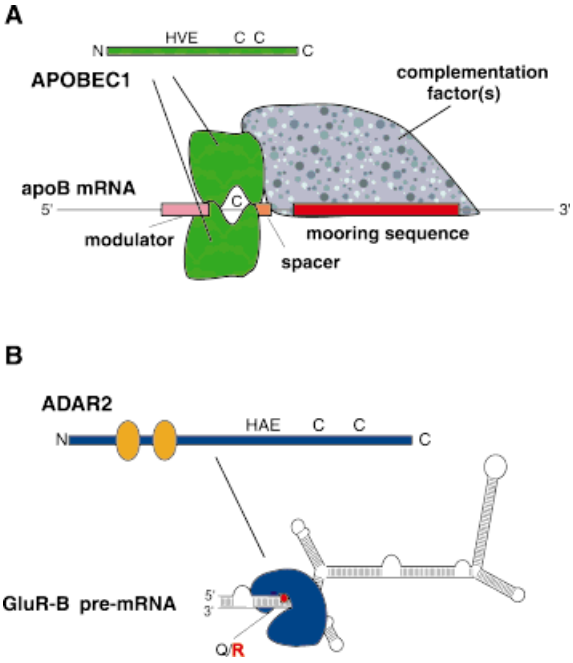

Figure 3. Mechanism of nuclear C-to-U and A-to-I editing. **A:** The apoB mRNA editosome. A schematic drawing of the apobec-1 primary structure is shown with the relative positions of critical amino acid residues of the catalytic core. For editing, an apobec-1-dimer (green) assembles with unknown auxiliary factors (complementation factors) on the apoB mRNA. The primary sequence motifs important for specific editing and contacted by the protein machinery are depicted as boxes termed regulator, spacer and mooring sequence. **B:** The ADAR2 domain architecture is shown with the two dsRBDs (orange) and the deaminase domain including the conserved residues of the catalytic center. The predicted secondary RNA structure formed by GluR-B pre-mRNA is shown with the Q/R-site adenosine located within a dsRNA region.[32] It is a presumed substrate for ADAR2, which deaminates the targeted adenosine specifically and with high efficiency.

Apobec-1 also has an RNA-binding activity and the region involved in RNA binding overlaps with the catalytic deaminase domain.[39] The presence of an N-terminal nuclear localization signal (NLS) in apobec-1 supports the notion that apoB editing is a nuclear event. Recently, two related cytidine deaminases, termed APOBEC-2[40] and AID,[41] were cloned and found to display high sequence similarity to apobec-1. However, both proteins are inactive on apoB transcripts. They have a tissue-specific expression profile with APOBEC-2 expressed exclusively in cardiac and skeletal muscle and AID in germinal center B cells.[40,41] Their substrates are as yet unidentified.

The editing machinery for adenosine deamination of GluR-B pre-mRNA is also believed to be localized within

the nucleus since the requirement for intronic sequences in the substrate argues that the editing event precedes splicing.[32] After recognizing that the targeted adenosine is part of a dsRNA structure essential for site-specific editing, it was suggested that a previously identified dsRNA-specific adenosine deaminase (DRADA/dsRAD, renamed ADAR1), might be responsible for GluR-B editing. This enzyme was subsequently isolated, cloned and tested for specific RNA editing activity in vitro.[42–44] Three double-stranded RNA binding motifs (dsRBDs), a deaminase domain and a bipartite NLS were evident from sequence analysis. However, the recombinant protein deaminated the adenosine at the Q/R site of GluR-B pre-mRNA weakly and also lacked the required site selectivity in vitro.[45] Using synthetic, extended dsRNA molecules ADAR1 unselectively converts up to 60% of the adenosines to inosine.[46]

As for apoB editing, further studies suggested that other cellular cofactors might be required to direct and regulate the enzymatic activity of the deaminase.[47] At the same time, several groups reported that, with biochemical fractionation of nuclear extracts from HeLa cells, a second dsRNA-specific deaminase activity could be separated from the ADAR1-containing fractions.[29,45] Cloning strategies based on the predicted sequence homology between the adenosine deaminases led to the isolation of a second enzyme ADAR2. When recombinantly expressed, ADAR2 edited the Q/R-site adenosine of GluR-B specifically and with high efficiency.[48] ADAR2 is a widely expressed nuclear protein of 78 kDa. It shares only 31% overall identity with ADAR1 at the amino acid level, but it has high homology within the deaminase domain. In vitro, both ADAR1 and ADAR2 convert the R/G-site adenosine in GluR-B pre-mRNA equally well.[48] Furthermore, they display distinct but overlapping activities on the 5-HT_{2C} serotonin receptor editing sites A to F.[23]

In summary, the editases involved in A-to-I editing are active as single enzyme molecules (Fig. 3), but their RNA-binding and catalytic activity is likely to be modulated by other regulatory factors in vivo. A third putative adenosine deaminase (RED2) was subsequently cloned and is expressed solely in brain.[49] It lacks detectable adenosine deaminase activity on any known RNA editing substrate despite its high sequence similarity to ADAR2.

Regulation of RNA editing

Several regulatory mechanisms are known that influence apoB editing efficiency. Tissue-specific expression of apobec-1 through the use of alternative promoters and alternative splicing was demonstrated in mice.[50] Further, it is known, that in mice and rats, the extent of editing can be modulated by a variety of dietary and pharmacological manipulations.[13] For example, a diet high in carbohydrates increases apoB editing whereas, in fasting animals, the extent of editing is decreased by up to 50%. It is a conceptually attractive possibility, therefore, that increasing apoB editing therapeutically might lower the atherogenic factor in humans. This was explored by generating transgenic mice that overexpress apobec-1.[51] The primary goal of lowering the plasma levels of VLDL, IDL and LDL particles was met; however, the animals developed liver dysplasia and hepatocellular tumors due to hyperactivity of the editing enzyme, which resulted in modification of RNAs that are not physiological substrates for apobec-1.[51] Such gene-therapy strategies will only be successful if the apobec-1 expression level can be precisely adjusted with increased editing of the physiological substrate but without hyperediting.

Using gene targeting, apobec-1 knock-out mice have also been generated and these mice are completely deficient in apoB editing.[52,53] However, no phenotypic differences to wild-type animals were observed.

The regulatory features of ADAR proteins are only beginning to be explored. An intriguing example was reported recently affecting ADAR2 expression.[54] An auto-regulatory feed-back mechanism involving RNA editing of the ADAR2 primary transcript was suggested from the observation that, in cells with high deaminase activity, a splice acceptor site is generated in an intron by adenosine deamination. This leads to the accumulation of an ADAR2 splice variant, which produces a truncated, inactive protein. It could be demonstrated that ADAR2 targets this editing site in vitro and that a predicted partially dsRNA structure can form in this region of the primary transcript.[54]

Another mechanism for regulation of editase activity arises from alternative splicing of both ADAR1 and ADAR2 RNAs.[10] Differences in editing activity of the resulting protein isoforms on GluR-B substrates in vitro has been demonstrated[55,56] and it is suggested that as yet unidentified substrates might be the specific targets for individual splice variants.

Furthermore, human ADAR1 is expressed from two alternative promoters one of which is interferon inducible, leading to the expression of an N-terminally truncated protein of indistinguishable activity in vitro but altered subcellular localization in vivo.[57] A unique high-affinity binding domain for DNA in the left-handed Z-conformation has been localized in the ADAR1 N-terminus.[58] This has led to a model of editing by ADAR1 where the enzyme is localized on its target genes by binding to transiently forming Z-DNA, which is generated within these genes during transcription.[59] The three-dimensional structure of a fragment of ADAR1 has been determined bound to a small fragment of Z-DNA.[60] The coupling of transcription and editing would allow RNA editing to be complete before removal of the involved intronic sequences through splicing.

The processes determining the selection of editing sites and regulating the extent of editing are unknown at present. For example, at the GluR-B R/G position, the extent of

editing increases during ontogenesis from 20%–80%.[22] There is also evidence from studies in mammalian cells that maturation-induced increase of editing ratios does not simply correlate with changes in ADAR expression.[61] Possible scenarios for regulation of editing activities include regulated nuclear localization (the editing enzymes need to be in the nucleus to exert their function), post-translational modifications (for example phosphorylation, arginine dimethylation) and association with modulating cofactors. The close proximity of characterized editing sites to the exon/intron splice junction raises the question of whether the machineries for editing and splicing might influence each other (see below).

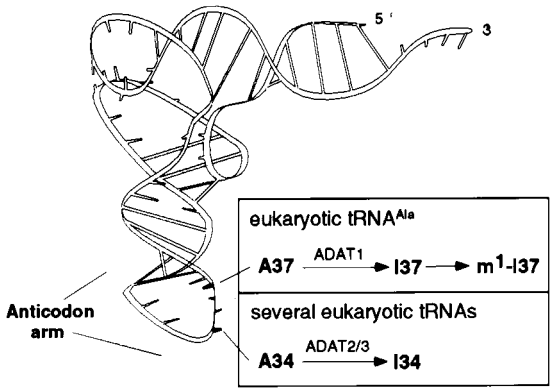

Figure 4. A-to-I editing of tRNA. The characteristic L-shaped three-dimensional structure of a tRNA molecule is shown schematically with the anticodon arm highlighted as well as the adenosines that undergo deamination. In case of A_{37} the conversion product I_{37} is further modified by a methylase yielding m^1-I_{37}. The two enzymatic activities responsible for the site-selective editing events are ADAT1 (A_{37} exclusively in tRNA^{Ala}) and the heterodimer ADAT2/3 (A_{34} in several eukaryotic tRNAs).

Editing of tRNA

The numerous nucleotide modifications occurring in eukaryotic tRNAs also include inosine and 1-methyl-inosine (m^1I).[12] Inosine is found in position 34 (the wobble position of the anticodon) of seven (yeast) or eight (higher eukaryote) tRNAs, whereas m^1I is present exclusively in alanyl tRNA at position 37 (see Fig. 4).[62] Biochemical studies had shown that the modifications proceed through hydrolytic deamination of A-to-I (and subsequent methylation at position 37 to m^1I).[63] The previous cloning of adenosine deaminases active on mRNA intensified attempts to characterize the enzymatic activities involved in tRNA-specific A-to-I conversion. Initial experiments ruled out the possibility that ADAR1 or ADAR2 are responsible for these modifications, which was also supported by the lack of detectable dsRNA-specific adenosine deaminase activity in extracts from yeast cells.[64]

However, homology searches with amino acid sequences located around the putative catalytic center and conserved among ADARs led to the identification of ADAT1, first from yeast (scADAT1/Tad1p)[64] and then from human,[65] mouse[66] and *Drosophila melanogaster*.[67] The protein specifically and exclusively targets adenosine A37 in tRNA^{Ala} in vitro[64–66,68] and in vivo.[64] The sequence homology between ADAT1 and the ADARs is substantial within the deaminase domain but ADAT1 lacks dsRBDs and no cross reactivity was detected between the tRNA- and mRNA-specific enzymes. This suggested a different mode of substrate recognition.[64,65,68]

The enzymatic activity responsible for editing A34 in the wobble position of the anticodon of several tRNAs was recently cloned.[69] Surprisingly, it revealed a heterodimer of two related polypeptides termed ADAT2/Tad2p and ADAT3/Tad3p.[69] Even though active as adenosine deaminase, the sequence composition and architecture of the catalytic domain of ADAT2/3 more closely resembles that of cytidine deaminases than adenosine deaminases.[69] As discussed below, these findings are of interest in understanding the evolution of substitutional RNA editing. The functional significance of inosine in tRNAs at position 34 is evident from its role in protein synthesis, where it allows for the alternative (wobble) pairing of U, C or A at the third codon position. The importance is further underscored by the observation that in yeast, ADAT2 and ADAT3 are essential genes.[69]

We can only speculate on the functional role of the m^1I_{37} modification present only in tRNA^{Ala}. It is located immediately adjacent to the anticodon, so it might help to maintain translational accuracy, possibly by preventing ribosomal frameshifting.[12] However, ablation of the ADAT1 gene in yeast is not lethal and mutant strains appear normal even though there is no A37-modification in these cells.[64] Additional genetic and biochemical experimentation is required to uncover the functional implications of A-to-I editing at this position in tRNA^{Ala}, which has apparently been perpetuated throughout eukaryotic evolution.

The superfamily of RNA dependent deaminases and the evolution of base-modification editing

In order to examine the evolutionary aspects of substitutional RNA editing, it is interesting to compare the biochemical mechanisms of the editing reactions as well as the sequences and modular architecture of the involved molecules. The most significant features conserved in all RNA-dependent deaminases are the amino acid residues essential for catalytic activity; i.e. cysteine and histidine residues involved in zinc-coordination and a glutamine residue utilized

for transition-state stabilization and proton shuttling.[9,13,68] All of these enzymes use zinc to activate water to form a hydroxide nucleophile. However, the spacing between Zn-coordinating residues in cytidine deamination (CDARs) and adenosine deamination (ADARs) is characteristically different. The second and third cysteines are separated by only two amino acids in CDARs, whereas in ADARs at least 60 residues are inserted. Of the ADAT proteins acting on tRNA, ADAT1 is organized like ADARs in this respect, however, ADAT2 and ADAT3 share the configuration of CDARs. A crystal structure is available for the *E. coli* cytidine deaminase (ECCDA).[70] Sequence alignments of the *E. coli* enzyme with apobec-1 have resulted in a structural model for apobec-1 based on the *E. coli* structure. Using results from mutational analysis, sequences have been identified that are functionally as well as structurally equivalent in these enzymes[71] (Fig. 5). In contrast, comparison of the crystal structures of the *E. coli* cytidine deaminase and the adenosine deaminase acting on mononucleotides (ADA),[72] showed that the secondary and tertiary structure motifs of the two deaminases are completely unrelated. Even though they are both zinc-containing enzymes and share some mechanistic features, the active sites are formed by entirely different polypeptide folds.[73]

To carry out a phylogenetic analysis including all deaminases, we focus only on the catalytic domain since the different mode of substrate recognition has led to great sequence diversity in the respective specialized regions outside of the deaminase domain. We have aligned sequences from the deaminase domain of all presently characterized deaminases from different species using the Clustal W1.8 program. Guided by the sequence motifs that were shown to be crucial for deaminase function, the structural information from the *E. coli* enzyme ECCDA and the modeling for apobec-1, we chose a region of 34 amino acids from the alignment for prediction of a phylogenetic tree (Table 3). The used algorithm[74] produces an evolutionary distance matrix involving the maximum likelihood method.[74,75] The predicted phylogenetic tree is presented in Fig. 6, obtained with the Phylodendron tree printer program.[75] It shows the superfamily of RNA-dependent deaminases as evolutionary related subfamilies. For comparison, we have also tested longer sequences for tree generation and have obtained similar results (not shown). The addition of flanking sequence regions that are less conserved among the deaminases and are pointed away from the catalytic center, (according to the ECCDA crystal structure) leads to a tree topology with lower resolution. The internodal distances in the tree depend on the rate of evolution. Thus, only sequence regions with similar substitution rates should be combined for phylogenetic analysis. Furthermore, the consistency of the constructed tree increases when the sequence divergence is low or moderate and the amount of data is large.[75] In our view, the sequences used here for

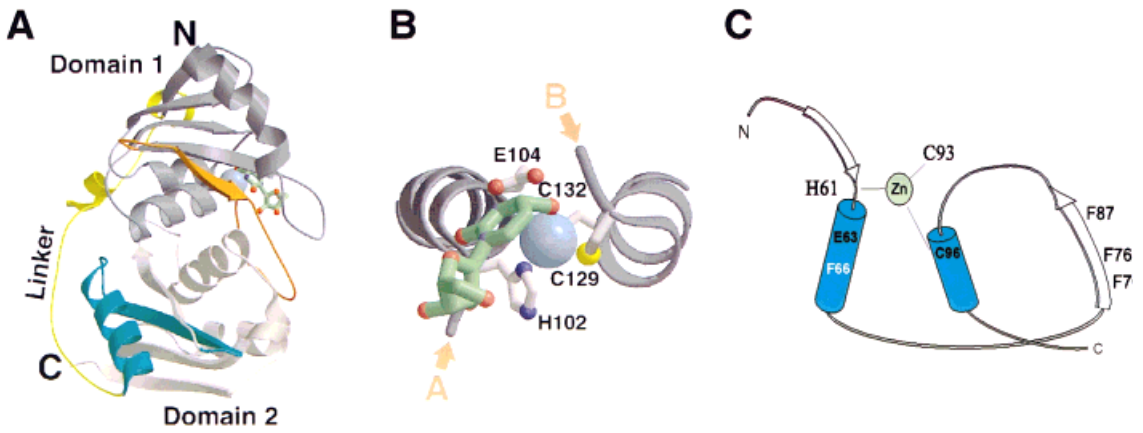

Figure 5. Catalytic core structure of the *E. coli* cytidine deaminase (ECCDA) and a homology model for apobec-1. **A:** The core domain fragment of ECCDA. The amino and carboxy termini are identified by N and C, together with the respective core domains 1 and 2, which are shaded dark and light gray, respectively, and connected by a linker peptide (yellow). The chelated zinc atom is indicated by a blue circle and the transition state analog inhibitor zebularine is shown in a light-green ball-and-stick representation. **B:** Configuration of ECCDA active site. The location of the catalytic residues H102 and E104 contributed by α-helix A, and C129 and C132 by α-helix B, are depicted. The zinc and zebularine are colored as in A. **C:** Schematic representation of the structural model for the apobec-1 active site as obtained from sequence alignments, mutational analysis and molecular modeling. The residue H61 is equivalent to H102, C93 to C129 and C96 to C132 in ECCDA. (A and B are adapted from Navaratnam et al., 1998,[71] with permission; C is based on a diagram in Navaratnam et al., 1995—Ref 84).

Table 3. Alignment of catalytic core domain of cytidine and adenosine deaminases

Gene	Species	GenBank Accession #	Sequence alignment with clustal W
APOBEC1	mouse	U22262	SNHVEVNFLEKFTTERY-FRPTWFLSWSPCGECSR
APOBEC1	human	L26234	TNHVEVNFIKKFTSERD-FHPTWFLSWSPCWECSQ
APOBEC1	rabbit	OCU10695	TNHVEVNFLEKLTSEGR-LGPTWFLSWSPCWECSM
APOBEC2	human	AF161698	AAHAEEAFFNTILPAFD---PTWYVSSSPCAACAD
AID	mouse	AF132979	GCHVELLFLRYISD-WD-LDPTWFTSWSPCYDCAR
ADAR1	human	HSU10439	DCHAEIISRRGFIRFLY-SELHLYISTAPCGDGAL
ADAR1	X.laevis	XLU88065	DCHAEVVSRRGFIRFLY-SQLHLYISTAPCGDGAL
ADAR2	human	HSU76420	DCHAEIISRRSLLRFLY-TQLHLYISTSPCGDARI
ADAR2	rat	RNU43534	DCHAEIISRRSLLRFLY-AQLHLYISTSPCGDARI
RED2	rat	RNU74586	DCHAEIVARRAFLHFLY-TQLHLYVSTSPCGDARL
ADAR	*C. elegans*	AF051275	DCHAEILARRGLLRFLY-SEVHLFINTAPCGVARI
ADAR	*D. melanogaster*	DMBN35H14[a]	DSHAEIVSRRCLLKYLY-AQFHLYINTAPCGDARI
dCMP/CMP	Human	L12136	VCHAELNAIMN-KNSTDVKGCSMYVALFPCNECAK
dCMP/CMP	Yeast	YSCDCD1	CLHAEENALLEAGRDRVGQNATLYCDTCPCLTCSV
CDA	Human	L27943	GICAERTAIQKAVSEGY-KDFMQDDFISPCGACRQ
CDA	*E. coli*	ECCDD	TVHAEQSAISHAWLSGE-KALAITVNYTPCGHCRQ
hypCDA	*C. elegans*	P30648	VVHAEMNAIIN-KRCTTLHDCTVYVTLFPCNKCAQ
hypCDA	*E. coli*	P30134	TAHAEIMALRQGGLVMQ-NYRTLYVTLEPCVMCAG
hypCDA	*H. influenza*	P44931	TAHAEIIALRNGAKNIQ-NYRTLYVTLEPCTMCAG
ADAT1	Human	AF125188	DSHAEVIARRSFQRYLL-HQLVFFSSHTPCGDASI
ADAT1	Yeast	SCE7297	DCHAEILALRGANTVLL-NRIALYISRLPCGDASM
ADAT1	*D. melanogaster*	AF192530	DSHAEVLARRGFLRFLY-QELHFLSTQTPCGDACI
ADAT2	Human	AL031320.6[a]	TRHAEMVAIDQVLDWCRQSGTVLYVTVEPCIMCAA
ADAT2	Yeast	SCE242667	VAHAEFMGIDQIKAMLG-SRGTLYVTVEPCIMCAS
ADAT3	Human	AC012615.1[a]	LLHAVMVCVDLVARGQGRGGYDLYVTREPCAMCAM
ADAT3	Yeast	SCE242668	IDHSVMVGIRAVGERLR-EGVDVYLTHEPCSMCSM

[a]Deduced amino acid sequence from genomic DNA; AID, activation induced cytidine deaminase; hypCDA, hypothetical cytidine deaminase.

tree prediction are the most suitable for analysis of the evolutionary relationship between the deaminases. However, because of the rather low number of residues involved some artificial bias may exist in the resultant tree topology.

The main conclusion from the available data and phylogenetic tree is that all RNA editing enzymes probably evolved from a common ancestor, as proposed.[76] The close relationship between the tRNA-specific adenosine deaminases ADAT2/3 and the cytidine deaminases supports the view that cytidine and adenosine deaminases are homologues.[69,76] This also reconfirms the notion that RNA-specific adenosine deaminases are not evolutionary related to the mononucleotide specific adenosine deaminase (ADA), but that these functionally related proteins are rather the result of convergent evolution. However, since we do not know the ancestor of these sequences, it is not possible to distinguish whether the cytidine deaminases constitute the ancestral group of the whole supergene family, as suggested,[40] or only of the C-to-U editases. As has been suggested before[64,66,68], an ADAT1-like protein might represent the ancestor of ADARs since they are related in sequence and the ADAR proteins have only been found in higher eukaryotes whereas ADAT1 is present also in yeast and no ADAT1-like sequences exist in prokaryotes. The question remains as to when the subfamilies of ADAT1/ADARs and CDA/CDARs diverged. Interestingly, the sequence data and phylogenetic analysis implies that the ADAT2/3 enzymes evolved directly from cytidine deaminases. One possible scenario for the evolution of the deaminases is that CDAs gave rise to ADAT2/3-like activities and these to ADAT1, which in turn was the precursor of ADARs (see also Refs. 64,66,68). Information from additional crystal structures of ADARs, ADATs and CDARs, as well as the identification of more gene family members, may make it possible to answer this question.

Evolutionary significance of RNA editing

Editing events can only be detected indirectly by comparing the genomic DNA sequences with that of the mature mRNA for the respective gene. In addition, the extent of RNA editing at a particular nucleotide position varies between fractions of a percent up to 100% in vivo. These are main reasons for base modification RNA editing in eukaryotes only being discovered relatively recently, and the number of identified genes being still small (see Table 1). Since the editing factors are found ubiquitously and their expression is not restricted to the tissues in which RNA substrates have been identified, it is anticipated that a substantial number of RNA editing substrates will be discovered. Furthermore, some CDARs and ADARs have been cloned for which no putative editing

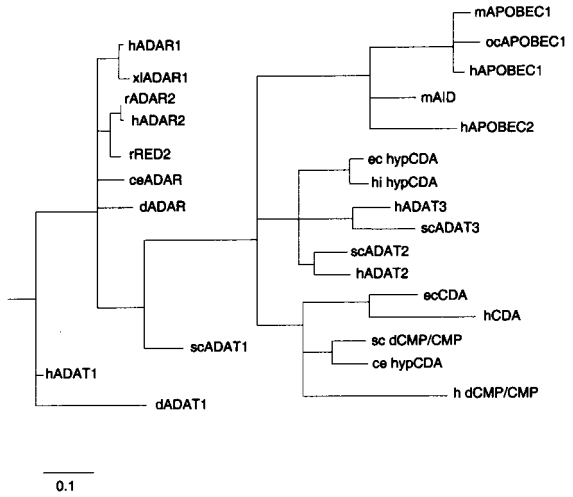

Figure 6. Phylogeny of RNA-dependent deaminases. The horizontal branch length of the phylogenetic tree reflects both the distance between sequences and the rate of evolution in each lineage. Within the CDAR subfamily (apobec-1, 2, AID) the evolutionary rate is higher than between ADARs indicated by longer branch lengths. The horizontal scale indicates the numbers of amino acid substitutions per position along tree branches. For individual sequences and GenBank accession numbers see Table 2.

substrate can be assigned. In the case of A-to-I editing, it has been shown that inosine exists in rat mRNA at tissue-specific levels and is highest in the brain with approximately 1 IMP residue every 17000 nucleotides.[77] This suggests that, on average, 5–10% of rat brain mRNAs may contain inosine.

We may ask why editing, especially A-to-I editing, has been so widely adopted, that the enzymatic activity is found in all metazoic tissues that have been examined. As cited above, an important component in selecting editing sites involves the participation of intronic sequences to form double-stranded RNA substrates for the enzyme. As is widely known, intronic sequences change rapidly during evolution because they are under very few constraints. However, exonic sequences are strongly selected for and do not change frequently. Over time, different segments of introns within pre-mRNAs form new exon complementary sites, which are substrates for this enzyme. These produce new exonic editing sites. However, only if the ensuing amino acid change results in a new function that is beneficial to the organism, is it then selected and maintained; that segment of the intron then remains constant. Thus nature has developed a mechanism for generating phenotypic diversity, creating new functions without changing exonic sequences. Furthermore, this opportunity could not be neglected by nature since it offers a way to have both the original, unedited phenotype as well as the edited phenotype with the new function without changing exonic sequences. Phenotypic diversity is then produced at a small cost, namely that of developing an editing system. The deamination by which adenosine is changed to inosine means that 60% of the amino acids can be altered, potentially giving rise to a large number of alternative functions. If one adds to this the possibility of RNA editing at splice sites[78] (for example editing the A in the downstream AG splice signal), alternative splicing could be induced as a result. Likewise, editing an exon to produce an upstream splice site would result in the elimination of a segment of an exon through normal splicing. In the future, it is likely that we will find a large number of examples of these types of editing. Indeed, it may be that the modifications in the flow of genetic information produced by editing may be of the same order of magnitude as that produced by alternative splicing.

Future directions

It will be interesting to see how the different deaminases and RNA targets interact in vivo and if the observed overlapping substrate specificities of ADARs are also maintained under physiological conditions. For several ADAR proteins, gene-targeting approaches in mice are underway and the results of ADAR2 gene inactivation have just been reported.[79] Clearly, the Q/R-site editing of GluR-B is drastically reduced but not eliminated. The ADAR2-deficient mice suffer a severe epileptic and ultimately lethal phenotype resulting from the increased CA^{2+}-permeability of AMPA receptors. Even though reduced editing rates are observed in other substrates, the Q/R site of GluR-B seems to be the only essential ADAR2 target in vivo.[79] Crossing of *ADAR2*$^{\pm}$ with *GluR-B (R)*$^{+/+}$ mice, which carry the edited Q/R-site codon in both GluR-B alleles, completely rescues the *ADAR2*$^{-/-}$ phenotype.[79] In addition, an influence from the editing status of the Q/R site on subsequent splicing of the adjacent intron was observed.[79] This supports the view that these RNA processing events do not occur independently. Reminiscent of the observed coupling between 5′-capping, splicing and 3′-polyadenylation of pre-mRNA (reviewed in Ref. 80), the molecular machines for splicing and editing might positively or negatively modulate each other's activity.

In one recently described case involving the *D. melanogaster* para transcript, it has been shown that the dsRNA structure formed by the exonic sequences surrounding the editing sites and the intronic ECS prevents splicing of the intron.[81] An RNA helicase activity (mle) is required to resolve the dsRNA structure indicated from the resulting "splicing catastrophe" in *D. melanogaster* mutants lacking mle.[81]

These findings raise the question of whether the involvement of a helicase might be a common theme of the interplay between editing and splicing,[81,82] given that, in all known A-to-I editing substrates, the 5′-splice site is near or even

involved in the exon/intron dsRNA structure. Weakening of the secondary structure through adenosine deamination might occur, but this is probably not sufficient. The duplex must open up to free the 5′-splice signal for splicing to proceed. It will be interesting to see whether the extent of RNA editing might thus be regulated through the activity of RNA helicases and/or editing might regulate splicing by facilitating the work of helicases. Certainly, the elucidation of the three-dimensional structure of editing enzymes in complex with their substrates will provide valuable insight into the mechanisms of base-modification RNA editing in the future.

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