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REVIEW

A-to-I and C-to-U Editing within Transfer RNAs

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Abstract—A significant number of post-transcriptional changes occur during the generation of mature transfer RNAs (tRNAs). These changes within precursor-tRNA molecules include the processing of 5′ and 3′ termini, the introduction of modifications, and also RNA editing. In this review, we will detail the reported cases of A-to-I and C-to-U tRNA editing. The most widespread example is the A-to-I conversion of the tRNA anticodon wobble base mediated by TadA in prokaryotes and the heterodimeric ADAT2–ADAT3 complex in eukaryotes. Recently, the plant chloroplast adenosine-to-inosine tRNA editing enzyme has been discovered. The editing of C-to-U is much less prevalent within tRNA and is currently only known to occur in few organellar tRNA species and the cytoplasmic threonyl-tRNA in trypanosomatids. The responsible editing enzyme remains to be identified. Finally, an unusually widespread C-to-U editing scenario was discovered in the archaeon *Methanopyrus kandleri*. This editing is mediated by CDAT8, which is responsible for the restoration of the proper folding of thirty different tRNA species. The evolution of CDAT8 will be discussed.

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EDITING OF TRANSFER RNA

Transfer RNAs (tRNA) are short, ubiquitous adaptor molecules that play a central role in protein biosynthesis. They fold into a characteristic cloverleaf-shaped secondary structure (Fig. 1) and an L-shaped tertiary structure [1, 2]. At one end of this L-shaped molecule are the three nucleotides that make up the anticodon, which can base pair to one or several codons within a messenger RNA. The other end of the L-shaped tRNA is defined by the 3′-terminal CCA end, which is aminoacylated with the amino acid to be added to the growing peptide chain during ribosomal protein biosynthesis. For each amino acid, there is a set of tRNAs (termed isoacceptors) that bind to the codons that specify an individual amino acid. An aminoacyl-tRNA synthetase exists for each amino acid that ensures that only the cognate tRNA is recognized and only the correct amino acid is attached to the tRNA [3]. Several aminoacyl-tRNA synthetases contain quality control mechanisms that recognize and correct mischarging of their tRNA [4]. However, not every codon requires its own tRNA as certain anticodon sequences can bind to multiple codons due to a mechanism called wobble base pairing [5]. Here, often the first anticodon nucleotide (position 34) is modified or edited from A-to-I (adenosine to inosine) to enable hydrogen bond formation with more than one base in the corresponding codon position.

The primary transcripts of the tRNA genes, the tRNA precursors, are heavily processed before they fold into functional mature tRNAs. These processing steps involve the removal of intronic sequences [6], the trimming of both 5′ and 3′ termini [7], as well as the modification of a large number of tRNA bases [8]. A surprisingly large degree of processing is required to remove disruptive sequences from archaeal tRNA precursors. These can contain multiple tRNA introns [9], can be split into two or three fragments [9-11], or contain permuted tRNA genes with the 3′ end of the tRNA preceding its own 5′ end [12, 13].

The editing of tRNA describes the alteration of a particular nucleotide sequence in the mature transcript from those encoded in the gene. For example, tRNA genes with imperfectly paired or incomplete acceptor stems can be transcribed into tRNA precursors whose acceptor stem is reconstructed in a template dependent editing pathway [14, 15]. Other instances of editing involve the deletion or insertion of individual bases or short sequences in the tRNA precursor [16]. In this review, we will focus on the known instances of hydrolytic deamination of tRNA bases, which effectively leads to the conversion of one base encoded in the tRNA gene to

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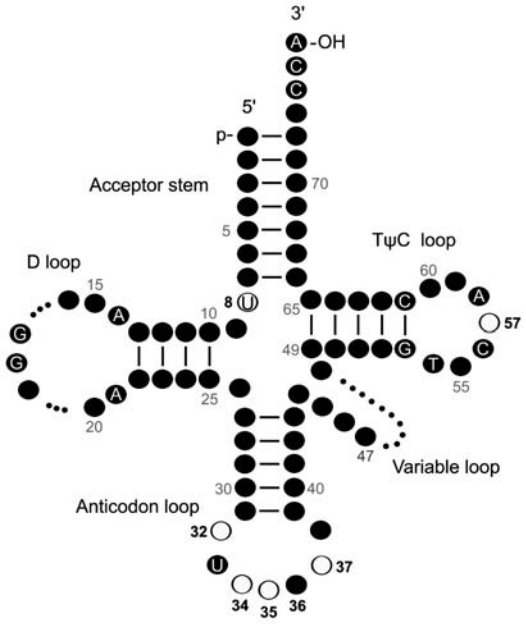
a different one present in the mature tRNA. The described nucleotide exchanges are the conversion of adenosine to inosine (A-to-I editing) and cytidine to uridine (C-to-U editing) by deaminase enzymes.

A-to-I EDITING OF THE tRNA ANTICODON IN EUKARYOTES

A-to-I editing was found to occur at two positions in eukaryotic tRNAs. Inosine is present in seven or eight tRNAs of higher eukaryotes as the first anticodon nucleotide, I34 (Fig. 1). In yeast, I34 is found in $tRNA^{Ala}$(AGC), $tRNA^{Arg}$(ACG), $tRNA^{Ile}$(AAU), $tRNA^{Pro}$(AGG), $tRNA^{Ser}$(AGA), $tRNA^{Thr}$(AGU), and $tRNA^{Val}$(AAC) [17, 18]. These tRNA isoacceptors all belong to tRNA species whose amino acid is represented by four codons in the universal genetic code. The second position of A-to-I editing is the nucleotide following the anticodon, A37. The $tRNA^{Ala}$(AGC) of yeast was shown to contain a N1-methylinosine (m^1I) at position 37, which is generated by a methylation step following the A-to-I deamination of base 37 [19]. Thus, yeast $tRNA^{Ala}$(AGC) maturation involves two different A-to-I editing events in its anticodon loop. Analysis of the proteins responsible for these two A-to-I editing events revealed the activity of two different enzymes. The deamination of base 37 is catalyzed by an enzyme termed ADAT1 (adenosine deaminase acting on tRNA). The knockout yeast strain of the gene encoding ADAT1 is viable and produced tRNAs that were not edited at base 37 but only at base 34 [20]. The editing activity of ADAT1 relies on the proper folding of the $tRNA^{Ala}$ substrate as well as the local conformation of the anticodon loop [20]. A functional homolog of yeast ADAT1 was identified and characterized in humans [21]. The ADAT1 enzymes display a close relation to other members of the ADAR (adenosine deaminases acting on RNA) family members that edit A-to-I in nuclear precursor-messenger RNAs [21, 22]. However, ADAT1 is specific for A37 in $tRNA^{Ala}$ and does not edit double-stranded RNA substrates of other ADAR enzymes. ADAT1 shares an unusual feature with human ADAR2, as both enzymes were shown to require a highly negatively charged metabolite, inositol hexakisphosphate (IP6), for editing activity [23]. IP6 binds in the core of the catalytic domain of ADAT1 and is buried in a highly basic cavity.

The anticodon wobble base A34 is deaminated by a different deaminase, the heterodimeric ADAT2/ADAT3 enzyme [24]. The knockout of the gene encoding for either ADAT2 or ADAT3 resulted in a lethal phenotype in yeast, which stresses the essentiality of inosine as the wobble base. Both subunits contain a deaminase domain that is similar to those of cytidine deaminases and ADARs/ADAT1. However, only ADAT2 harbors an important, conserved glutamate residue required for proton shuttling, which identifies it as the catalytic subunit of the heteromeric deaminase complex.

The crystal structure of a candidate human tRNA-specific adenosine-34 deaminase subunit ADAT2 has



tRNA base	tRNA editing	Organisms	tRNA	Deaminase
8	C8 to U8	*Methanopyrus kandleri*	30 tRNAs	CDAT8
32	C32 to U32	Trypanosomatids	$tRNA^{Thr}$(AGU)	ADAT2?
34	A34 to I34	some Eubacteria	$tRNA^{Arg}$(ACG)	TadA
		chloroplasts		TadA
		Eukaryotes	7-8 tRNAs	ADAT2/ADAT3
	C34 to U34	*Leishmania* mitochondria	$tRNA^{Trp}$(CCA)	?
35	C35 to U35	Marsupial mitochondria	$tRNA^{Asp}$(GCC)	?
37	A37 to m^1I37	Eukaryotes	$tRNA^{Ala}$(AGC)	ADAT1
57	A57 to m^1I57	Archaea	several tRNAs	?

Fig. 1. Positions of A-to-I editing and C-to-U editing in tRNA. The secondary structure of a tRNA molecule is schematically depicted and numbered according to [17]. The known positions of A-to-I editing and C-to-U editing in tRNAs are indicated by white circles. Restrictions of these editing events to certain organisms, groups or domains, as well as available information about the editing enzymes are listed.

been solved by the structural genomics consortium (SCG) and is available under PDB ID: 3DH1 [25].

A-to-I EDITING OF THE tRNA ANTICODON IN PROKARYOTES

There are generally fewer instances of tRNA A-to-I editing found in prokaryotes. The only known tRNA to contain an inosine as anticodon nucleotide 34 is the $tRNA^{Arg}$(ACG) present in eubacteria and plant chloroplasts. All other codons ending with C are read by tRNAs that contain a G34 as the first anticodon base [17]. Archaeal and mitochondrial tRNAs are not known to contain inosine in their anticodons. However, in several archaeal tRNAs, position 57 (in the middle of the T-loop) contains a N1-methylinosine residue [26]. Here, in contrast to the generation of m^1I37 in eukaryotic tRNAs, the methylation of A57 occurs before the deamination step [19].

The prokaryotic A-to-I editing of position 34 was studied in *Escherichia coli*, where in 2002 the essential tRNA-specific adenosine deaminase TadA was identified [27]. TadA displays 24% identity towards the eukaryotic ADAT2 subunit and can deaminate A34 of $tRNA^{Arg}$(ACG) transcripts from both *E. coli* and yeast. Furthermore, it can utilize mini-substrates containing only the anticodon stem and loop, which indicates that the nucleotides at positions 33-36 are sufficient for inosine formation by TadA [27]. Thus, the anticodon is defined as the major RNA recognition element. Crystal structures of the dimeric TadA have been solved from *Agrobacterium tumefaciens* [28], *Aquifex aeolicus* [29], *Streptococcus pyogenes* [30], and *E. coli* [30] and in complex with RNA from *Staphylococcus aureus* (Fig. 2; see color insert) [31]. The latter structure revealed insights into the sequence- and structure-specific interactions of an anticodon stem-loop of $tRNA^{Arg}$(ACG) with TadA and the active site architecture necessary for efficient hydrolytic deamination. The conformation of the anticodon loop bases is drastically altered to allow maximal access for TadA (Fig. 2). The nucleotides 33, 34, 35 and 37 are splayed outwards to facilitate specific protein contacts (Fig. 2). The protein surface of TadA contains discrete recognition pockets for each of the loop nucleotides including an intricate and deep pocket for A34 [31].

Recently, the identity of the A-to-I tRNA editing enzyme in plant chloroplast has been revealed. A protein, termed AtTadA, was identified in *Arabidopsis thaliana* and shown to catalyze A-to-I editing in the anticodon of the plastid $tRNA^{Arg}$(ACG) transcript [32]. The protein contains a C-terminal region with significant sequence similarity to the bacterial TadA protein, but also a large N-terminal domain of over 1000 amino acids that is dispensable for catalytic activity. The disruption of the *A. thaliana* TadA gene severely affects chloroplast translation efficiency and results in impaired photosynthetic function [33].

C-to-U EDITING OF THE tRNA ANTICODON

C-to-U editing of tRNAs is mainly found in eukaryotic organelles. In plant mitochondria, C-to-U changes have been identified in miscellaneous parts of the tRNA molecule [34, 35]. Some examples include the C-to-U editing of C4 (acceptor stem) of $tRNA^{Phe}$ and C28 (anticodon stem) of $tRNA^{Cys}$ in dicotyledons, as well as three C-to-U changes of C7, C12, and C41 (acceptor stem, D-arm, and anticodon stem) in a single mitochondrial $tRNA^{His}$ molecule from larch [35, 36]. Editing is required for the tRNA precursors to be processed into mature tRNAs by RNase P and RNase Z. The exact role of these editing events and the involved enzymes are still unclear. Other organellar C-to-U editing events are mainly found in the anticodon region, where they have a direct influence on the recognition of different codons. One studied example is the mitochondrial $tRNA^{Asp}$(GCC) in marsupials. The anticodon is edited from GCC to GUC to enable the recognition of aspartate codons instead of glycine codons [37]. However, a significant portion of this tRNA remains unedited, which raises the possibility that one tRNA is used for different codons specifying different amino acids [38]. Similarly, C-to-U editing of the anticodon of mitochondrial $tRNA^{Trp}$(CCA) allows decoding of the mitochondrial UGA stop codon in *Leishmania tarentolae* [39].

The only known non-organellar C-to-U editing event is found in the cytoplasmic $tRNA^{Thr}$(AGU) of *Trypanosoma brucei*. This tRNA is edited twice, with a C-to-U conversion of base 32 in addition to an A-to-I conversion of the wobble base 34 [40]. Here, the C-to-U editing event stimulates the subsequent A-to-I editing and also precedes the removal of the leader sequence during 5′ tRNA processing by RNase P [40, 41]. The enzyme(s) responsible for the deamination of cytidine residues in tRNAs remain to be identified. For the $tRNA^{Thr}$(AGU) from *T. brucei*, it was found that ADAT2 (as the catalytic subunit in the ADAT2/ADAT3 heterodimer) catalyzes the conversion of A-to-I at position 32. In addition, the knock-down of this enzyme also reduced the amount of C-to-U editing found in the same tRNA [42]. It was hypothesized that ADAT enzymes were derived from a gene duplication of a cytidine deaminase ancestor [24] with shared core domains between ADATs and cytidine deaminases. Therefore, it might be possible that a single deaminase could catalyze both A-to-I and C-to-U conversions in the same tRNA. However, currently C-to-U editing by ADAT2/ADAT3 could not be performed on tRNA, but only on single-stranded DNA [42]. The biologically relevant implications of these results await further analysis.

C-to-U EDITING AT tRNA POSITION 8 RESTORES THE FOLDING AND FUNCTIONALITY OF ARCHAEAL tRNAs

In 2009, a different case of C-to-U editing within tRNA molecules was discovered for 30 different tRNA species of the hyperthermophilic archaeon *Methanopyrus kandleri* [43]. *Methanopyrus kandleri* is found in fluid of black smokers in the deep sea, where some strains can survive temperatures of up to 122°C [44]. The complete genome of *M. kandleri* strain AV19 was determined in 2002 [45]. Subsequent analysis of the *M. kandleri* tRNA genes revealed an unusual feature as the otherwise conserved base U8 (T8 in the tRNA gene) appeared to be mutated to C8 in 30 out of 34 cases. This feature was puzzling for several reasons. (i) It is known that the U8 forms a conserved reverse Hoogsteen base-pair with an equally conserved A14 base that is required to define and stabilize the functional angle of the two segments (T-arm/acceptor stem and D-arm/anticodon stem) of the L-shaped tRNA molecule. (ii) A C8:A14 base variation was found to significantly decrease tRNA suppression efficiency [46]. (iii) A U8C mutation in human mitochondrial tRNA was found to lead to tRNA misfolding and defects in translational initiation and elongation [47]. (iv) U8 is also suggested to be a contact point for the tRNA splicing endonuclease [48]. Taken together, *M. kandleri* is currently the only sequenced organism with this apparently deleterious U8C point mutation present in the majority of its tRNA genes. It has been suggested that the contribution of the U8–A14 interaction might be less critical for *M. kandleri* tRNA molecules [49]. However, the solution to this problem turned out to be RNA editing, as the unusual C8 in these tRNA genes was shown to be post-transcriptionally converted to the standard U8, thus guaranteeing a functionally folded tRNA molecule with a tertiary U8–A14 base pair [43]. A cytidine deaminase was identified that edits specifically C to U at position 8 of tRNAs and termed CDAT8 (for cytidine deaminase acting on tRNA base C8). CDAT8 is composed of three fused domains: an N-terminal cytidine deaminase domain, a central ferredoxin-like domain, and a notable C-terminal THUMP RNA binding domain (Fig. 3; see color insert). This THUMP domain has been identified to mediate tRNA core binding in other enzymes including ThiI, which is responsible for the thiolation modification of base U8 to 4-thiouridine. It appears that this domain was "reused" in evolution to direct a different active enzyme center (cytidine deamination instead of thiolation) to its site of action. The cytidine deaminase domain of CDAT8 itself displays a similar overall tertiary structure as other members of the cytidine deaminase superfamily. However, superimposition with available cytidine deaminase crystal structures reveals that it displays an unusually compact cytidine deaminase fold.

CDAT8 is also unique in its number of substrates. While ADAT enzymes edit a maximum number of eight different tRNAs, CDAT8 has to function on 30 different tRNAs with a C8 base. How does CDAT8 recognize all its different tRNA substrates? The crystal structure of CDAT8 with a modeled tRNA substrate suggests a plausible solution. The acceptor arm of the tRNA fits snugly into a basic groove of CDAT8, which suggested that the universal 3′ terminal CCA end and the length of the acceptor stem might be recognized. Indeed, it could be shown that other large portions of the tRNA molecule, including the D-arm and the anticodon arm, can be deleted without abolishing C-to-U editing activity at base 8 [43].

THE EVOLUTION OF CDAT8

The evolution of CDAT8 and the necessity for C-to-U editing at tRNA base 8 is puzzling. The universality, as well as structural and functional importance of base U8 is evident within all organisms from all three domains of life. This is not different for *M. kandleri* as the mature tRNA still requires the U8 base and the tertiary U8:A14 base pair. Only the presence of an enzyme like CDAT8 allows the mutation of base 8 to C in the tRNA gene. A proposed timescale of evolution would start with the introduction of a single U8C mutation in the *M. kandleri* genome. Apparently the hypothetical benefit of such mutation must have outweighed the folding problems of this tRNA. The invention of CDAT8 would then restore the folding problems of such tRNA while allowing further U8C point mutations in different tRNA genes to evolve. Two questions remain. First, how did CDAT8 evolve? The modular arrangement of CDAT8 implicates that it arose from the fusion of independent domains. The tRNA binding THUMP domain defines the center of action; the cytidine deaminase domain provides the actual activity. Where do these domains come from? The THUMP domain is conserved and might have been provided by different tRNA modifying enzymes. However, structural and sequence alignments with the isolated cytidine deaminase domain do not provide clear results but group CDAT8 as a unique family within the "cytidine deaminase-like" superfamily. The second question is even harder to pinpoint. Why is a U8C mutation in the tRNA gene beneficial to the cell? We can exclude that the C8 base is helpful in the mature tRNA. Therefore, this base change must provide a strong selective advantage in evolution in any of the steps that lead to the functional tRNA molecule. It could provide an advantage in the tRNA gene, in the unprocessed tRNA precursor, or in the unmodified tRNA. It will require further research to distinctively answer this question, but one can propose several ideas that make *M. kandleri* tRNA processing unique. One first obvious point is that tRNA processing and folding is required to appear at or near the highest temperatures that life is possible. This should

impose certain problems for the folding of any structural RNA and is indicated by the almost exclusive use of G:C base pairs in all stems of all *M. kandleri* tRNAs. It is possible that a C8 base might provide an additional strong G:C base contact during the folding process of the tRNAs. A second possibility is that a C8 mutation is required to prevent or temporarily delay the thiolation of base 8, which is commonly found as a 4-thiouridine in mature tRNAs. The third option is that C8 is beneficial for genome stability at tRNA genes. It is known that tRNA genes in archaea are hotspots for the integration of mobile genetic elements like viruses. These viruses utilize conserved large regions of tRNA genes as attachment sites and restore the tRNA gene upon integration. Therefore, it might be possible that the point mutation of a base that, without CDAT8, would not be allowed to change, might be able to interfere with or prevent such integration at tRNA targets. These hypotheses await further analysis. A final aspect to be considered is why only *M. kandleri* evolved such mechanism to allow U8C mutations even though other hyperthermophilic archaea would have to battle, e.g. with similar RNA folding concerns. In this regard one has to consider the debated unusual phylogenetic position of *M. kandleri*. Phylogenetic analysis of archaea based on 16S ribosomal RNA genes and elongation factor EF-1α placed *M. kandleri* deep-branched at the root of the archaeal tree, unrelated to any other known methanogen [50, 51]. On the other hand, whole-genome trees pair-group *M. kandleri* with *Methanobacteriales* and *Methanococcales* [45]. Further phylogenetic analysis using either proteins involved in transcription or in translation showed a surprising divergence between the placement of *M. kandleri* with (i) *Methanococcales* and *Methanobacteriales* in one tree and (ii) at the base of the Euryarchaeota in the other tree [52]. It was concluded that these incongruent placements in the different phylogenetic trees are an artefact caused by an unusual accelerated evolution of the *M. kandleri* transcriptional proteins in comparison to other archaeal lineages [52]. Furthermore, the high number of identified orphan proteins and gene fusions implicate that the *M. kandleri* genome allowed a high level of gene loss, gene capture, and intramolecular recombination events. These observations have been linked to the absence of transcription elongation factor TFS [52]. It appears that the evolution of CDAT8 is only one example of an orphan protein that provides a benefit for this peculiar organism.

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