C to U editing and modifications during the maturation of the mitochondrial tRNA\textsubscript{Asp} in marsupials

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ABSTRACT

In marsupial mitochondria, the nucleotide residue at the second position of the anticodon of the tRNA for aspartic acid is changed post-transcriptionally such that the translational machinery recognizes it as a uracil rather than the cytosine residue encoded in the gene. By postlabeling nucleotide analysis, we show here that the cytosine residue is converted to a conventional uracil residue in an RNA editing event that affects approximately half of the tRNA molecules under steady state conditions. Furthermore, we have identified three different tRNA\textsubscript{Asp} species which all carry three pseudouridines and two methylations but have the anticodons GCC, GUC and QUC respectively, the latter representing a rare example of queuine incorporation into a mitochondrial tRNA. This allows us to describe a likely sequential order of modification of the tRNA\textsubscript{Asp}, where methylations and conversions of uridines to pseudouridines precede the editing event, while the exchange of guanine by queuine takes place after the C to U editing event.

INTRODUCTION

RNA editing is a group of heterogeneous processes that result in that RNAs come to differing in primary sequence from what is conventionally predicted from their gene sequence. While insertional/deletional editing leads to the incorporation or excision of individual bases, substitutional editing replaces one nucleotide by another (I). The insertional/deletional type of editing occurs in the kinetoplast of trypanosomes, where many mRNAs are edited by extensive addition or removal of uracil residues (2) and in some mitochondrial transcripts of \textit{Phyosarum polycephalum}, where mainly C and, rarely, U and A residues are inserted (3,4). Substitutional editing occurs in mRNA transcripts of plant chloroplasts (5) and mitochondria (6-8) as well as in four mitochondrial tRNAs of \textit{Acanthamoeba} (9), in three mitochondrial tRNAs of a land snail (10) and in one mitochondrial tRNA of potato and bean (11). Among mammals, substitutional editing has been described for the cytoplasmic mRNAs for apolipoprotein B (12) and a glutamate gated ion channel (13), as well as one cytoplasmic (14) and one mitochondrial tRNA (15). In the two cases of mammalian mRNA editing, the chemical nature of the resultant bases have been determined. The transcript encoding apolipoprotein B in humans contains a cytosine residue that is deaminated to a uracil residue (16) whereas in the mRNA encoding the glutamate gated ion channel, an adenosine residue is deaminated to an inosine residue (17-19). Thus, in both cases, deamination is the mechanism by which the bases are altered. However, in the case of \textit{Acanthamoeba}, substitutional editing events occur that involve changes between purines and pyrimidines (9). Therefore, these reactions cannot be explained by deamination.

We have previously shown by cDNA analysis that editing of the mitochondrial tRNA for aspartic acid in the North American opossum (\textit{Didelphis virginiana}) changes its anticodon from GCC to a form that is recognized as GUC by reverse transcriptase (15). However, since this analysis did not allow the determination of the chemical nature of the edited base, a cytosine modification such as lysidine, that has the base pairing properties of a uracil (20), could not be excluded. We have now purified the aspartyl tRNA and show that the nucleotide residue at the second position of the anticodon of the processed tRNA contains the conventional base uracil. Thus, this substitutional C to U RNA editing is responsible for the creation of the aspartate identity of this tRNA. Additionally, the analysis of three different isolates of this tRNA\textsubscript{Asp} allows us to describe a putative modification pathway of a mitochondrial tRNA.

MATERIALS AND METHODS

Preparation of total tRNA

Total RNA was isolated from ~170 g of frozen liver of an American opossum by the guanidinium thiocyanate method (21). High molecular weight RNA was precipitated by the addition of NaCl to a final concentration of 1 M at 4°C. After centrifugation, the tRNA fraction in the supernatant was ethanol precipitated, redissolved in water and analyzed by electrophoresis through a 10% polyacrylamide gel containing 7 M urea.

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Isolation of the mitochondrial tRNA for aspartic acid

An oligonucleotide (5'-TAAGATATAGGAGTTAACCTATAT-3') complementary to the 3' half of the tRNAAsp and biotinylated at the 5'-end was immobilized either on streptavidin-coated magnetic beads (Dynal, Norway) or agarose beads (Gibco BRL, USA). The beads carrying the oligonucleotide were incubated with RNA preparations for 5 min at 70°C in 6× SSC and subsequently for 1 h at room temperature under gentle agitation. The beads were collected and washed several times with 3× SSC until the optical density at 260 nm of the supernatant was <0.02. The RNA bound to the beads was collected by heating to 70°C in 0.1× SSC for 5 min and subsequent collection of the supernatant. After concentration by ethanol precipitation, the tRNAs were further purified by electrophoresis in two successive polyacrylamide gels containing 7 M urea (first gel: 5% polyacrylamide stacking gel/pH 6.5, 10% separation gel/pH 8.3; second gel: 10% stacking gel/pH 6.5, 20% separation gel/pH 8.3) (23). Gels were stained with 0.03% toluidine blue in water. Bands were cut out with a sterile blade, the tRNA eluted by incubation in 500 mM ammonium acetate, pH 5.7, 0.1 mM EDTA, 0.1% SDS at 4°C overnight (24) and concentrated by ethanol precipitation.

Quantitation of tRNA editing

An oligonucleotide (5'-AACCTATAATTTACACTAT-3') that recognizes the sequence immediately downstream of the second position of the anticodon was extended by 10 U of AMV reverse transcriptase (Pharmacia, Sweden) in the presence of either 10 μCi [32P]dGTP and 20 μM each of ddATP, ddCTP, ddTTP (for detection of the non-edited base) or 10 μCi [32P]dATP and 20 μM each of ddCTP, ddGTP, ddTTP (for detection of the edited base). The resulting extension products were analyzed by polyacrylamide gel electrophoresis and quantified in a Fuji phosphorimager using PCBAS software (Raytest, Germany).

Sequence determination

The purified tRNA was sequenced by a postlabeling method (25) with some modifications (26). tRNA (1 μg) was partially hydrolyzed by boiling for 15 s in 2 μl water and labeled with 10 U polynucleotide kinase (Stratagene) and 250 μCi [32P]γ-ATP. The labeled fragments were separated on a preparative polyacrylamide gel, individually isolated and digested to completion with 1 μg of nuclease P1 (Pharmacia) in 10 μl 50 mM ammonium acetate, pH 5.3 at 55°C for 2 h. The P1 digest was dried under vacuum and redissolved in water containing 20 mg/ml of each of the four conventional nucleotide-5'-monophosphates. The latter served as internal markers for the migration in the subsequent thin layer chromatography (TLC), which was carried out overnight on CEL 300-10 plates (20 × 20 cm; Macherey and Nagel, Düren, Germany) using the following solvent systems:

Solvant system A: iso-propanol:32%HCl:H2O (70:17.5:12.5)
Solvant system B: iso-butric acid:NH4OH:H2O (57.5:4:38.5)

Marker nucleotides were detected by UV shadowing whereas the radioactively labeled nucleotides derived from the tRNA were visualized by autoradiography.

Base catalyzed rearrangement of N1-methyladenosine

Radioactively labeled nucleotides were isolated from the TLC plate using a scalpel blade, washed twice with 400 μl ethanol and dried under vacuum. Subsequently they were redissolved in 200 μl water and separated from the TLC-powder by centrifugation. The supernatant was dried down, redissolved in 200 μl concentrated ammonia (33%) and incubated for 2 h at 60°C. The ammonia was removed by centrifugation under vacuum, the nucleotide redissolved in 2 μl TLC marker solution and subjected to a second TLC using solvent system A.

RESULTS AND DISCUSSION

The mitochondrial tRNAAsp was isolated from the liver of a North American opossum by hybridization to an oligonucleotide immobilized on a solid support (22). Further purification by electrophoresis in two consecutive polyacrylamide gels resulted in the separation of several different species of tRNAAsp. The four species that had the highest apparent molecular weights could be isolated from the gel in quantities that allowed the determination of the extent to which they were edited by a minisequencing procedure. In this assay, a primer was annealed to the base immediately downstream of the editing site and extended in the presence of either radioactively labeled guanosine or adenosine. In the former case, a labeled extension product is produced by the non-edited tRNA whereas in the latter case, a labeled product is formed by the edited tRNA. Figure 1 shows that the different species (a-d) of the tRNA were, in the order of decreasing apparent molecular weights, edited to 65% (species a), 52% (species b), 35% (species c) and 50% (species d) respectively. Thus, the different RNAs isolated represent different versions of tRNAAsp that are all heterogeneous with respect to the extent to which they are edited. Their different apparent molecular weights are probably due to differences in their modifications as well as differences in secondary and tertiary structure that may remain even in the presence of denaturing agents. In other preparations, a similar situation was observed.

In order to determine the nature of the edited base, as well as other modifications present in the tRNA, the electrophoretic tRNA species of the highest apparent molecular weight, which was edited...
to 65% (Fig. 1, species a), was sequenced by postlabeling nucleotide analysis. Briefly, the band was isolated from the acrylamide gel, partially hydrolyzed by heating to 100°C, end-labeled and subjected to polyacrylamide gel electrophoresis. The bands, resulting from hydrolysis events at each of the nucleotide residues in the tRNA, were isolated. After enzymatic digestion to mononucleotides, each fraction was divided in two aliquots that were each separated by TLC using different solvent systems. The radioactive nucleotides representing the residues present at each position in the tRNA were visualized by autoradiography and their identity determined by comparison to standards (27).

In the TLC analysis, pseudouridine residues were detected at positions 27, 32 and 55 (numbering according to the nomenclature of ref. 28), where such modifications commonly exist in various tRNA isoacceptors of eukaryotes and eubacteria. At position 9, two signals were observed that migrated as a mixture of adenosine and N6-methyladenosine (m6A) in solvent systems A and B. In order to clarify whether this represents a partial conversion of N1-methyladenosine (m1A, which has migration properties identical to those of adenosine) to m6A, induced by the experimental conditions, the spot migrating as adenosine was isolated from the TLC plate and incubated under alkaline conditions, which leads to a conversion of m1A to m6A (Dimroth rearrangement). Reanalysis by TLC showed that the nucleotide had been almost completely converted to m6A (data not shown). Thus, at this position an m1A residue exists in the tRNA fraction analyzed. Furthermore, an N2-methylguanosine (m2G) was identified at position 10. Whereas m2G represents a common modification at position 10 of mitochondrial and cytoplasmic tRNAs, m1A at position 9 is present exclusively in mitochondrial tRNAs.

Additionally, two signals appeared at the second position of the anticodon (Fig. 2A). One of these migrated as a cytosine (C), while the other migrated as a uracil (U). A second TLC analysis, using solvent system B, confirmed that the edited nucleotide residue at the second position is a uracil (Fig. 2B). Therefore, while in Escherichia coli tRNA for isoleucine the modification of a C residue to lysidine changes the pairing properties (20), in this case a C to U substitutional editing event is responsible for the creation of the aspartic acid anticodon of this tRNA.

The tRNA species with the highest apparent molecular weight was similarly sequenced from another RNA preparation. It carried the same three pseudouridine residues and the two methylated bases described above as well as a uracil residue at the second anticodon position. In addition, a hypermodified base at the first position of the anticodon occurred. Based on its mobility in the two solvent systems, this nucleotide was identified as queuosine (data not shown). This is in accordance with the observation that tRNAs which have a U at the position immediately 5' of anticodons with the general sequence GUN carry queuosine residues at the first position of the anticodon (28). In E.coli (29) and in Xenopus laevis oocytes (30), the enzyme that incorporates queuine into tRNAs requires the target sequence UGU (bold letters represent the two first positions of the anticodon) in order to replace the guanine by queuine. Therefore, guanine is likely to
be exchanged for queuine only in edited tRNA molecules. Besides the tRNA\textsubscript{Asp} in rat liver mitochondria (31), this is the first example of a mitochondrial tRNA containing this hypermodification. Figure 3 summarizes the modifications observed in the tRNA\textsubscript{Asp}. As many other mitochondrial tRNA isoacceptors, the tRNA\textsubscript{Asp} shows unusual composition of the D- (no guanine at positions 20 and 21, no dihydrouridine at position 22) and the \textit{ΨC}-loop (no m\textsuperscript{5}U at position 54, no \textit{ΨC} sequence).

From a third tRNA preparation, the sequence of a band of intermediate molecular weight was determined. The two methylation positions at positions 9 and 10 were observed as well as pseudouridines at positions 27 and 32 (position 55 was not analyzed). However, in contrast to the other tRNA\textsubscript{Asp} species studied, the anticodon carried neither a uracil residue at the second position, nor a queuine at the first position. Thus this tRNA species had the genomic anticodon GCC.

In conclusion, the experiments described above revealed that at least three different forms of the tRNA\textsubscript{Asp} exist (Fig. 4), which differ both in their extent of anticodon editing and in the occurrence of other modifications. In Figure 4, a putative pathway for maturation of the tRNA is outlined. Since methylations (at positions 9 and 10) and pseudouridine conversions (at positions 27, 32 and probably 55) can be detected in the unedited tRNA, they are the first modifications that are introduced into the transcript to create Form 1. Subsequently, editing of the second position of the anticodon occurs in a reaction that can be envisioned to be either a deamination, in analogy with the editing of apolipoprotein B mRNA (16) or a transglycosylation reaction. This leads to Form 2 (sequence analysis shown in Fig. 2). Finally, to produce Form 3, the first position of the anticodon becomes converted to queuine in a reaction that is likely to require the editing of the second position of the anticodon to have occurred. Further work is needed to clarify to what extent the various modifications affect either the editing or the function of the tRNA. However, the fact that not only the fully processed and edited version of tRNA\textsubscript{Asp}, but also the non-edited form of the tRNA (anticodon GCC) may fulfill some hitherto unknown function. One possibility is that this form functions as an additional tRNA for glycine that would recognize two (GGC, GGU) out of four glycine codons. Preliminary experiments support this idea.

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