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A Single Editing Event Is a Prerequisite for Efficient Processing of Potato Mitochondrial Phenylalanine tRNA

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In bean, potato, and *Oenothera* plants, the C encoded at position 4 (C_4) in the mitochondrial $tRNA^{Phe}_{GAA}$ gene is converted into a U in the mature tRNA. This nucleotide change corrects a mismatched C_4-A_{69} base pair which appears when the gene sequence is folded into the cloverleaf structure. C-to-U conversions constitute the most common editing events occurring in plant mitochondrial mRNAs. While most of these conversions introduce changes in the amino acids specified by the mRNA and appear to be essential for the synthesis of functional proteins in plant mitochondria, the putative role of mitochondrial tRNA editing has not yet been defined. Since the edited form of the tRNA has the correct secondary and tertiary structures compared with the nonedited form, the two main processes which might be affected by a nucleotide conversion are aminoacylation and maturation. To test these possibilities, we determined the aminoacylation properties of unedited and edited potato mitochondrial $tRNA^{Phe}$ in vitro transcripts, as well as the processing efficiency of in vitro-synthesized potato mitochondrial $tRNA^{Phe}$ precursors. Reverse transcription-PCR amplification of natural precursors followed by cDNA sequencing was also used to investigate the influence of editing on processing. Our results show that C-to-U conversion at position 4 in the potato mitochondrial $tRNA^{Phe}_{GAA}$ is not required for aminoacylation with phenylalanine but is likely to be essential for efficient processing of this tRNA.

Editing is a posttranscriptional process which changes the primary sequence of RNAs in comparison with those of the corresponding DNA templates (for reviews, see, for instance, references 3, 9, and 14). RNA editing was first described for trypanosome mitochondria, in which extensive uridine insertion-deletion events generate translatable open reading frames. Subsequently, RNA editing has been found to be a widespread phenomenon observed mainly in mitochondria, to a lesser extent in chloroplasts, and in a few cases in transcripts of nuclear genes. In higher-plant mitochondria and in chloroplasts, RNA editing results in the conversion of cytidines into uridines. A few examples of uridine-to-cytidine changes in plant mitochondria have also been reported. Although editing was first found almost exclusively in mRNAs, a series of editing events affecting the following structural RNAs have now been described: an rRNA (34) and a few group II introns (6, 10, 18, 19, 38, 41) in plants, a mammalian cytosolic aspartic tRNA (2), several mitochondrial tRNAs of an amoeboid protozoan (20), at least three mitochondrial tRNAs of the land snail (39), the marsupial mitochondrial $tRNA^{Asp}$ (15, 28), and the platypus mitochondrial $tRNA^{Ser}_{GCU}$ (40). C-to-U editing was also shown to correct a C_4-A_{69} mismatched base pair into a classical U_4-A_{69} base pair in the acceptor stem of the potato, bean, and *Oenothera* mitochondrial $tRNA^{Phe}_{GAA}$ (5, 25). In *Oenothera* mitochondrial $tRNA^{Cys}_{GCA}$, a C_{28}-U_{42} mismatched base pair encoded by the gene in the anticodon stem is converted into U_{28}-U_{42}, another mismatched base pair (5). This supports the idea that the plant mitochondrial RNA editing system can make only C-to-U (except for rare cases of U-to-C) changes.

Whereas in plant mitochondrial mRNAs most editing events introduce changes in the specified amino acids and contribute to the conservation of functional proteins, the precise role of editing in mitochondrial tRNAs is not known. The only clear example is that of the marsupial mitochondrial $tRNA^{Asp}$ (15, 28), which obviously needs editing to acquire its aspartic acid anticodon. In plants, the various recombinations and mutations the mitochondrial DNA has undergone during evolution led to the inactivation or complete loss of a number of the native tRNA genes deriving from the genome of the ancestral endosymbiont. To ensure the complete tRNA set needed for mitochondrial translation, the missing species are either transcribed from chloroplast-originating genes which have been acquired by the mitochondrial genome or imported from the cytosol (for a review, see, for instance, reference 27). Higher plants might also make use of editing as a strategy to rescue some native mitochondrial tRNAs after sequence divergence in their genes. In the case of plant group II introns, editing increases the similarity between the secondary structure of the RNA and the consensus model of group II introns and is a prerequisite for splicing (7). It can be speculated that, by repairing a mismatched base pair, C_4-to-U_4 editing improves the secondary structure of the mitochondrial $tRNA^{Phe}$ of dicotyledonous plants and is therefore necessary for either aminoacylation or maturation, the two main processes requiring correct folding of the tRNA. We report here the different strategies we have used to check these two possibilities. Our results strongly support the hypothesis that editing at position 4 of the potato mitochondrial $tRNA^{Phe}_{GAA}$ is required for efficient processing of this tRNA.

MATERIALS AND METHODS

Preparation of a potato mitochondrial fraction containing precursor RNAs. Mitochondria purified from potato tubers as described by Neuburger et al. (29)

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were used for RNA extraction. This extraction was performed as described by Remacle and Maréchal-Drouard (32). To remove possible DNA contamination, RNA was treated three times with RNase-free DNase under conditions recommended by the manufacturer (Pharmacia).

In vitro transcription and aminoacylation of full-length tRNA transcripts. The previously cloned 2.8-kbp potato mitochondrial DNA fragment containing the *trnS-trnF-trnP* gene cluster (25) was used as a template for standard PCR amplification using oligonucleotide 1 and either oligonucleotide 2 or oligonucleotide 3 (to obtain a G_{69} mutation) as primers. The PCR products were cloned into the *Eco*RI site of pUC18 (Boehringer) according to standard protocols (33) and sequenced by the dideoxyribonucleotide chain termination method using a simplified protocol (11). In vitro runoff transcripts were prepared from these constructs as described by Perret et al. (31) after digestion with *Bst*NI. Aminoacylation was conducted under optimal conditions (8) in the presence of a potato mitochondrion or an *Escherichia coli* enzymatic extract (24).

cDNA synthesis and amplification by PCR. For cDNA synthesis, 5 μg of the potato mitochondrial fraction containing precursor RNAs (see above) was used for reverse transcription (32) with oligonucleotide 4 or 5. One-fifth of the reaction mixture was used for PCR amplification (32) in the presence of oligonucleotide 4 or 5 and of oligonucleotide 6. PCR products were cloned into the *Bam*HI site of plasmid Bluescript KS(+) (pKS) and sequenced. To produce a DNA fragment coding for the mutated C_4-G_{69} $tRNA^{Phe}$ precursor sequence, a PCR was done with oligonucleotides 6 and 7 (complementary to the 3′ end of the potato mitochondrial $tRNA^{Phe}_{GAA}$ gene and to the adjacent downstream flanking region) with a cDNA clone corresponding to an unedited $tRNA^{Phe}$ precursor used as a template.

Preparation of RNA transcripts. Uniformly labeled RNA transcripts were synthesized by standard in vitro transcription with T7 RNA polymerase in the presence of 10 μCi of [α-^{32}P]UTP or [α-^{32}P]CTP and 1 μg of linearized template plasmid DNA. The synthesized RNAs were fractionated by electrophoresis on a 15% polyacrylamide gel in the presence of 7 M urea. After identification by autoradiography, the bands corresponding to the expected products were excised from the gel and the labeled RNAs were eluted (26).

To perform primer extension analysis of the processing products and to analyze incorporation of [α-^{32}P]CTP by tRNA nucleotidyltransferase during in vitro processing of tRNA precursors, unlabeled transcripts were prepared as described above except that [α-^{32}P]UTP was replaced by 250 μM UTP.

Preparation of potato mitochondrial processing extracts and in vitro processing assays. Lysates able to perform tRNA processing were prepared as described by Hanic-Joyce and Gray (13) from potato tuber mitochondria (29). Processing assays (13) of labeled in vitro transcripts (15,000 cpm as determined by Cerenkov counting) were performed in the presence of a dialyzed 20 to 40% ammonium sulfate fraction (10 μg of proteins). After a 30-min incubation at 26°C, the reaction mixtures were brought to 0.3 M sodium acetate (pH 4.8) and 2 μg of carrier tRNA was added. The samples were extracted once with phenol and precipitated with ethanol. RNA processing products were analyzed by electrophoresis on a 15% sequencing gel. To determine the size of the processing products, the products of a sequencing reaction were used as a ladder.

To test for incorporation of the CCA end into the processed products, processing assays were performed as described above but with unlabeled precursor RNAs as substrates and in the presence of 40 μCi of [α-^{32}P]CTP and 40 μM CTP.

Primer extension analysis. The mature tRNA-sized product obtained from unlabeled edited $tRNA^{Phe}$ precursor was recovered from a sequencing gel (26) by using as a reference the migration of the same product derived from the corresponding labeled $tRNA^{Phe}$ precursor. Primer extension was done as described by Remacle and Maréchal-Drouard (32), using one-fifth of the gel-purified processing product as a template and oligonucleotide 8, complementary to the 3′ end of potato mitochondrial $tRNA^{Phe}$, as a primer.

Oligonucleotides. The following oligonucleotides were used (*Eco*RI or *Bam*HI sequences added for cloning are in italics): 1, AGCAA*GAATTC*GAATTGTAATACGACTCACTATAGTT(C/T)AGGTAGCTCAGCTGGTTAG (T7 promoter sequence underlined); 2, GTACA*GAATTC*CCTGGTGCTTAGAAGTGGATTCGAACC (*Bst*NI site underlined); 3, GTACA*GAATTC*CCTGGTGCT***C***AGAAGTGGATTCGAACC (*Bst*NI site underlined, mutated position in boldface italics); 4, AGTAC*GGATCC*TGCTTAGAAGTGGATTCGAACCACTG; 5, GTACT*GGATCC*CCCCGGCTACGTTTGGACCTTTCGCC; 6, AGTAG*GATCC*GGGCGCTCTTCGATGAAGAAAACAG; 7, TACT*GGATCC*CCCCG GCTACGTTTGGACCTTTCGCCCGCT***C***AGA (mutated position in boldface italics); and 8, TGCTTAGAAGTGGATTCGAA.

RESULTS

Aminoacylation of in vitro-synthesized potato mitochondrial $tRNA^{Phe}_{GAA}$. In vitro transcription of constructs containing the potato mitochondrial $tRNA^{Phe}_{GAA}$ gene directly fused to the T7 RNA polymerase promoter at the 5′ end and to a *Bst*NI site at the 3′ end yielded full-length tRNA transcripts. Aminoacylation of these transcripts with either an edited or a nonedited sequence was tested in the presence of phenylalanine and of a homologous potato mitochondrial enzymatic extract. As shown in Fig. 1, identical kinetics were obtained with the edited and nonedited sequences. These data demonstrate that the stability and the capacity for aminoacylation with phenylalanine do not differ significantly between the edited and nonedited forms of the potato mitochondrial $tRNA^{Phe}_{GAA}$ in vitro transcript. The same kinetics were also obtained with a transcript possessing a doubly modified U_4-G_{69} base pair in the acceptor stem (because of the mutation of position 69) and with an *E. coli* enzymatic extract (data not shown).

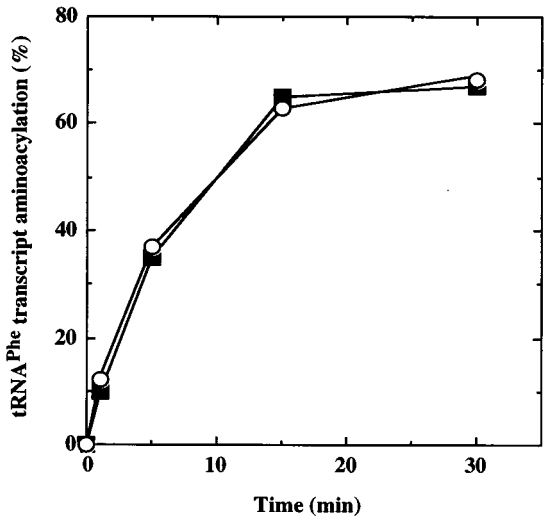
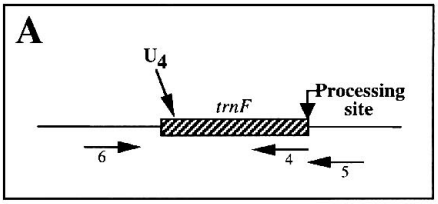
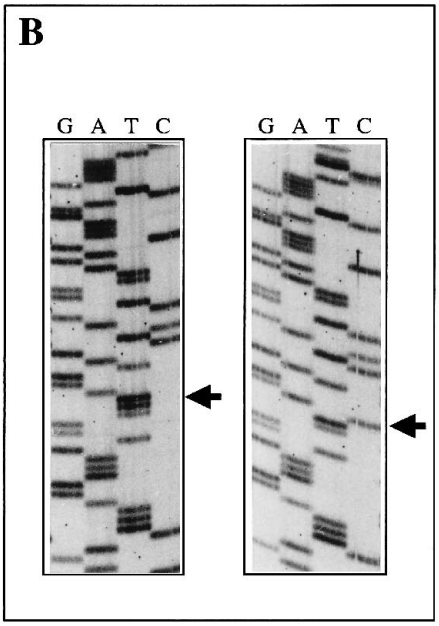


FIG. 1. Aminoacylation kinetics of in vitro-synthesized potato mitochondrial $tRNA^{Phe}_{GAA}$ (3 μM) in the presence of a saturating amount of a homologous mitochondrial enzymatic extract. ○, edited form; ■, nonedited form.

Occurrence of $tRNA^{Phe}$ editing at the precursor level. Since C_4 editing did not seem to affect $tRNA^{Phe}$ recognition by the corresponding mitochondrial phenylalanyl-tRNA synthetase and since only the edited form could be detected as a mature $tRNA^{Phe}$ in potato plants (25), we speculated that editing might have an influence on processing. If this is the case, the sequence change has to occur at a very early stage after transcription and, unless editing is the rate-limiting step, it might be possible to detect edited $tRNA^{Phe}$ precursor molecules. To test such a hypothesis, cDNAs representative of the $tRNA^{Phe}$ sequence in potato mitochondrial precursor RNAs were synthesized. The strategy used is schematically presented in Fig. 2A. A potato mitochondrial RNA fraction was used as a template for reverse transcriptase (RT) cDNA synthesis in the presence of a primer complementary either to the N_{49}-N_{73} sequence of the tRNA (oligonucleotide 4) or to the downstream region of the gene (oligonucleotide 5). The synthesized cDNAs were amplified by PCR in the presence of a primer corresponding to the upstream flanking region of the gene (oligonucleotide 6) and one of the two primers used for the reverse transcription. As an example, Fig. 2B shows the sequences of two PCR clones obtained with oligonucleotides 5 and 6. With either combination of primers (oligonucleotides 4 and 6 or 5 and 6), the amplified $tRNA^{Phe}$ sequence was in its edited form, with a U at position 4, in 50% of the 34 clones sequenced (Fig. 2C), while the other 50% retained the original C. Editing of $tRNA^{Phe}$ therefore appears to occur already at the precursor level in potato mitochondria.

Furthermore, a strong processing site is present at the 3′ end of the potato mitochondrial $tRNA^{Phe}$, as judged from Northern (RNA) blot and primer extension analyses, leading in vivo





C

RT-PCR	T	NE	E	P
oligos 4 + 6	10	5	5	50
oligos 5 + 6	24	12	12	50

FIG. 2. Analysis of the in vivo sequence of potato mitochondrial $tRNA^{Phe}_{GAA}$ in precursor RNAs by RT-PCR and cDNA sequencing. (A) Schematic presentation of the strategy used for the different RT-PCR amplifications. Oligonucleotide numbering is as described in Materials and Methods. Sequence data for the *trnF* gene and its flanking regions have been reported previously (25). Evidence for the strong processing site at the 3′ end of $tRNA^{Phe}$ is described elsewhere (32). RT-PCR amplification in the presence of potato mitochondrial RNA was primed with either oligonucleotides 4 and 6 or oligonucleotides 5 and 6. (B) Sequence analysis of 2 (one edited and one nonedited [arrows]) of the 24 $tRNA^{Phe}$ cDNAs amplified by RT-PCR with oligonucleotides 5 and 6. (C) Complete results of 34 cDNA sequence analyses after RT-PCR amplification of potato mitochondrial $tRNA^{Phe}_{GAA}$ from precursor RNAs. T, total number of cDNA clones analyzed; NE, number of clones with the unedited sequence; E, number of clones with the edited sequence; P, percentage of clones with the edited sequence; oligos, oligonucleotides.

to a strong predominance of the intermediate precursors yielded by the corresponding cleavage over the 3′ unprocessed precursor forms (32). Therefore, in our experiments, cDNAs obtained by using a primer located in the tRNA before this strong processing site (oligonucleotide 4 in Fig. 2A) for RT-PCR amplification could roughly be considered representative of the strongly predominant precursors processed at the 3′ end of the tRNA, whereas amplification with a primer located after this site (oligonucleotide 5 in Fig. 2) could logically occur only on 3′ unprocessed precursors. As the same percentage of edited clones was observed in both cases, these data support the idea that the C-to-U change at position 4 in the 5′-end region of $tRNA^{Phe}$ does not have a strong influence during 3′-end processing of this tRNA in potato mitochondria.

In vitro processing of potato mitochondrial $tRNA^{Phe}$ precursors. An edited and a nonedited labeled RNA spanning the flanking and coding sequences of the potato mitochondrial $tRNA^{Phe}$ gene were synthesized in vitro by T7 RNA polymerase transcription of the corresponding cDNAs obtained in the previous section by RT-PCR amplification with oligonucleotides 5 and 6. The ^{32}P-labeled transcripts produced were 202 nucleotides (nt) long, with 74 nt coming from the tRNA, 40 and 25 nt from the potato mitochondrial upstream and downstream regions, respectively, and the remaining 63 nt (50 at the 5′ end and 13 at the 3′ end) from the pKS vector (Fig. 3A). Equal amounts of these labeled in vitro precursor transcripts were incubated in the presence of a homologous potato mitochondrial lysate. Incubation of the edited precursor RNA with the mitochondrial lysate yielded a major product corresponding in size to the mature tRNA (Fig. 3B), as established by comparison with a DNA sequence ladder and with the migration of labeled full-length tRNA transcript synthesized in vitro from the gene constructs used in the aminoacylation studies. This is indicative of both processing of the edited precursor RNA and addition of the uncoded CCA end to the processed product, which would be in agreement with the fact that plant mitochondrial extracts exhibit both processing and tRNA nucleotidyltransferase activity (13, 22). Incorporation of the CCA end was further suggested by the fact that omitting CTP and ATP in the incubation medium led to the formation of a faster-migrating processing product. In processing assays with the nonedited precursor RNA, the mature tRNA-size product was either absent or present at a very low level, but, when present, it migrated at the same position as for the edited precursor. According to the reasoning mentioned above, this argues in favor of the idea that, whenever processing happens to occur with the unedited precursor, it is accurate and followed by CCA addition. Figure 3 shows the strongest signal (about 10% of that yielded by the edited precursor, as judged by scanning) obtained with the unedited precursor out of five independent experiments. It is unlikely that this absence or very low level of mature tRNA-size product observed with the nonedited precursor RNA is due to a fast turnover of these molecules, because the nonedited full-length tRNA transcript synthesized in vitro from the gene constructs used in the aminoacylation studies is as stable as the edited one when incubated with the potato mitochondrial lysate (not shown). It thus appears that although a complete processing reaction can occasionally occur with the nonedited precursor RNA, the process is inefficient, which strongly suggests that the C_4-to-U_4 change in the amino acid stem is necessary prior to processing of the potato mitochondrial $tRNA^{Phe}$. Similar processing assays were done with precursor RNAs synthesized in the presence of [α-^{32}P]CTP. Total P1 nuclease hydrolysis of the small amount of mature tRNA-size processing product obtained with the nonedited precursor followed by thin-layer chromatography (25) yielded no [^{32}P]UMP (not shown). This makes it unlikely that the low level of processing product obtained in some experiments when the nonedited precursor RNA is tested is due to editing which occurs during the incubation with the mitochondrial lysate, as C-to-U editing in plant mitochondria is believed to be due to deamination (14).

It should be emphasized that only a few intermediate products could be detected. In particular, no signals corresponding to the 5′ or 3′ trailers were observed. The major processing

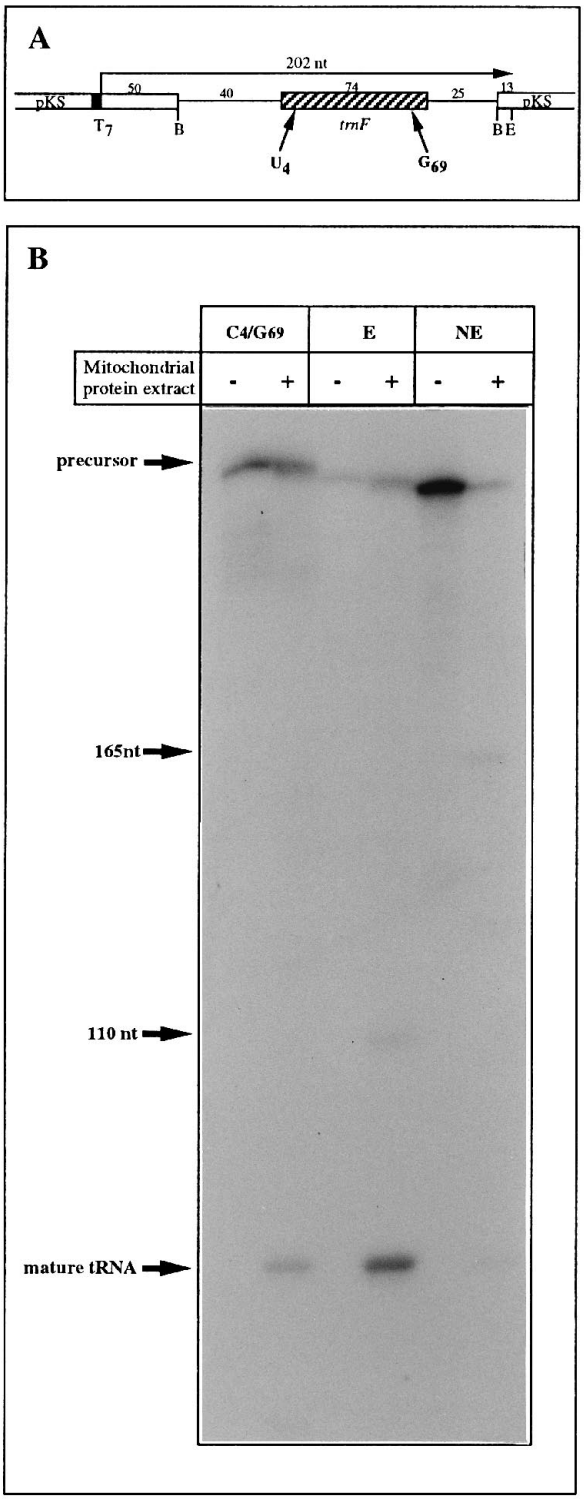


FIG. 3. In vitro processing of the nonedited, edited, and C_4-G_{69} mutated forms of potato mitochondrial $tRNA^{Phe}_{GAA}$. (A) Schematic presentation of the gene constructs used as templates for the synthesis of $tRNA^{Phe}$ precursors. The RT-PCR-amplified cDNAs comprising the potato mitochondrial $tRNA^{Phe}$ (74 nt) with its flanking sequences (40 and 25 nt) were cloned into the *Bam*HI (B) site of plasmid pKS. After digestion with *Eco*RI (E), transcription from the T7 RNA polymerase promoter (T7) yielded transcripts (202 nt) also including vector sequences (50 and 13 nt). (B) In vitro-synthesized labeled precursor RNAs corresponding to the nonedited (NE), edited (E), and mutated (C_4-G_{69}) forms of potato mitochondrial $tRNA^{Phe}_{GAA}$ were incubated in the presence (+) or absence (−) of a potato mitochondrial protein extract. Processing products were analyzed on a 15% acrylamide sequencing gel. The migration of the in vitro-synthesized mature-size potato mitochondrial $tRNA^{Phe}_{GAA}$ and the putative processing intermediates of about 110 and 165 nt (as deduced from a ladder) is indicated on the left.

intermediate obtained with the edited precursor RNA was a product of about 110 nt which might correspond to the tRNA plus the 3′ trailer (Fig. 3B). In contrast, assays with the nonedited precursor RNA yielded a product of about 165 nt, which would fit the size of the tRNA plus the 5′ trailer (Fig. 3B).

Since editing of a C_4-A_{69} mispairing into a normal U_4-A_{69} base pair in the acceptor stem of potato mitochondrial $tRNA^{Phe}$ appeared to promote efficient processing of the corresponding precursor RNA in vitro, we wondered whether the same effect would be obtained when this mismatched C_4-A_{69} was artificially changed into another conventional base pair. In order to test such a hypothesis, a C_4-G_{69} base pair was created in the tRNA, instead of the U_4-A_{69} base pair, by mutating the A_{69} into a G in the clone encoding the nonedited precursor RNA. A mature tRNA-size product was also obtained when the C_4-G_{69} in vitro transcript of this mutated gene was incubated in the presence of the mitochondrial lysate (Fig. 3B). On average, the signal obtained in this case reached about 40% of that yielded by the edited U_4-A_{69} precursor RNA.

Identification of the 5′ and 3′ processing sites. Primer extension analysis was used to check the accuracy of 5′-end in vitro processing of the $tRNA^{Phe}$ precursors. For that purpose, unlabeled and labeled edited precursor RNAs were incubated under the same conditions in the presence of the mitochondrial extract and run in parallel on a sequencing gel. The mature tRNA-size unlabeled processing product was recovered from the gel, using as a reference the migration of the same product derived from the labeled precursor RNA. Primer extension (Fig. 4A) using oligonucleotide 8 yielded a single cDNA product of 74 nt with a homogeneous 5′ end corresponding precisely to the first nucleotide of the mature $tRNA^{Phe}$.

Taking into account that tRNA nucleotidyltransferase activity has already been detected in wheat or potato mitochondrial lysates (13, 22), we checked whether the 3′-terminal CCA sequence was present in the mature tRNA-size products obtained in our in vitro assays, thereby also verifying indirectly the accuracy of 3′-end processing of the $tRNA^{Phe}$ precursors. For these experiments, unlabeled edited (U_4-A_{69}) or unedited (C_4-A_{69}) precursor RNAs were incubated in the presence of [α-^{32}P]CTP with or without potato mitochondrial lysate. A strong signal corresponding to a labeled mature tRNA-size product was obtained with the edited precursor RNA in the presence of the protein extract (Fig. 4B), whereas only a very faint signal could be observed at the same position with the nonedited pre-tRNA. These results reflect incorporation of the CCA end into the processed tRNA molecules, which both indicates accurate 3′-end processing and strongly suggests that the tRNA-size product obtained in the in vitro processing assays described in this section and the previous sections has a mature 3′ end. The latter is also supported by the fact that this tRNA-size processing product always migrated as the labeled full-length tRNA transcript synthesized in vitro from the gene constructs used in the aminoacylation studies and not as the 3-nt-shorter, CCA-deprived $tRNA^{Phe}$ encoded by the gene. The very faint signal obtained with the nonedited pre-tRNA in the [^{32}P]CTP incorporation experiment whose results are shown in Fig. 4B is consistent with the fact that no significant level of mature tRNA-size processing product was detected in

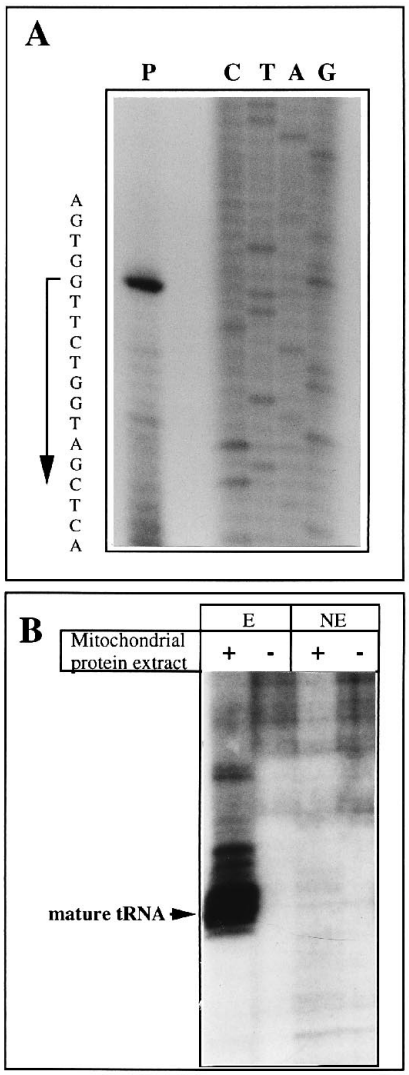


FIG. 4. In vitro analysis of 5′- and 3′-end processing of $tRNA^{Phe}$. (A) Primer extension analysis of the mature tRNA-size in vitro processing product obtained from the edited precursor (Fig. 3) by using as a primer an oligonucleotide complementary to the 3′ end of potato mitochondrial $tRNA^{Phe}$. The products of a DNA sequencing reaction performed with the potato mitochondrial $tRNA^{Phe}$ gene and the same primer were used as a ladder. This DNA sequence is shown in its complementary form (C, T, A, G) to allow direct alignment of the primer extension product (P) with the terminal sequence of the tRNA. The sequence of the region surrounding the processing site is presented on the left, with an arrow starting at the 5′-terminal nucleotide of the mature $tRNA^{Phe}$. (B) Addition of the CCA end during in vitro processing of the edited (E) and nonedited (NE) forms of potato mitochondrial $tRNA^{Phe}_{GAA}$. In vitro-synthesized unlabeled precursor RNAs were incubated with [α-^{32}P]CTP in the presence (+) or absence (−) of a potato mitochondrial protein extract. Processing products were analyzed on a 15% acrylamide sequencing gel. Migration of the in vitro-synthesized mature-size potato mitochondrial $tRNA^{Phe}_{GAA}$ is indicated (arrowhead).

this case in the processing assay of labeled unedited precursor run in parallel under the same conditions.

DISCUSSION

Editing and aminoacylation. We tested the functional role of editing in the potato mitochondrial $tRNA^{Phe}_{GAA}$. The editing site concerned in this case, namely, position 4, is located in the amino acid acceptor stem, which in all tRNAs contains identity determinants for recognition by the cognate aminoacyl-tRNA synthetase (see, for instance, reference 30). The N_4-N_{69} base pair, which is not conserved among phenylalanine tRNAs (35), has not been implicated as an identity element for $tRNA^{Phe}$, but it could be involved in the fine-tuning of the tRNA structure allowing correct positioning of the identity nucleotides for recognition by phenylalanyl-tRNA synthetase. We show here that the same aminoacylation efficiency of potato mitochondrial $tRNA^{Phe}_{GAA}$ can be observed with either a U_4-A_{69}, a C_4-A_{69}, or a U_4-G_{69} base pair. This confirms that the N_4-N_{69} base pair is not an identity element for phenylalanylation in plant mitochondria and does not play a role in aminoacylation, unless C_4-to-U_4 editing introduces a negative identity element which prevents mischarging of $tRNA^{Phe}$. Since we have been using a partially purified potato mitochondrion enzymatic extract, it is very unlikely that these results are biased by the occurrence of editing during the aminoacylation assay. This is also strongly supported by the fact that the same results were observed when an *E. coli* enzymatic extract was used, as no editing has been reported for *E. coli.*

Editing and processing. It is known that correct folding of the portion corresponding to the mature tRNA in larger precursors is important for recognition by processing enzymes. From the data presented here, it seems that indeed the nonedited precursor of potato mitochondrial $tRNA^{Phe}$ cannot be efficiently processed in vitro, and thus presumably in vivo, which is in accordance with the fact that only edited mature $tRNA^{Phe}$ can be found in mitochondria of potato (and bean) plants (25). That the nonedited in vitro-synthesized full-length $tRNA^{Phe}$ transcript is as stable as the edited one upon incubation with a potato mitochondrial lysate also makes it unlikely that this absence of nonedited mature $tRNA^{Phe}$ in vivo is simply due to fast turnover. In contrast to potato mitochondrial $tRNA^{Phe}$, $tRNA^{Asp}$ is present in both its edited and its nonedited form in marsupial mitochondria (15, 28), confirming that the role of editing is different in this case.

Although an effect on other processing activities cannot be excluded, our data are consistent with the possibility that editing of potato mitochondrial $tRNA^{Phe}$ affects cleavage of the 5′ leader rather than removal of the 3′ trailer from the precursor RNA. In all organisms studied so far, the 5′ leader of a tRNA precursor is removed by the site-specific RNase P, which cleaves precisely at the 5′ end of the mature tRNA (for a review, see e.g., reference 1). Structural studies have already indicated that (i) the conformation of the tRNA in the precursor is approximately the same as that in the final mature state and (ii) the correct cloverleaf folding and L-shaped three-dimensional structure of the tRNA moiety in the precursor are essential for recognition and cleavage by RNase P. Two domains, one near the cleavage site at the 5′ end of the tRNA and the other one in the T stem and loop, were shown to be important for the interaction between the eubacterial RNase P RNA and the tRNA precursor (36). The residue at position 4 was part of the 6 nt of the acceptor stem involved in this interaction. Detailed analyses of substrate recognition by RNase P and by the M1 RNA, the catalytic RNA subunit of *E. coli* RNase P, performed with different *Schizosaccharomyces pombe* pre-tRNAs led to similar results (17).

Considering all these data, it can be speculated that the U introduced at position 4 of potato mitochondrial $tRNA^{Phe}$ by editing is essential for maintaining the cloverleaf folding of the tRNA part in the precursor RNA and thereby obtaining efficient cleavage by RNase P. Figure 5 shows that if the first base pairs of the acceptor stem are indeed destabilized when the precursor is not edited, the 5′ end of the tRNA could be recruited to form an alternate stem-loop folding involving the upstream region in the precursor. According to the results

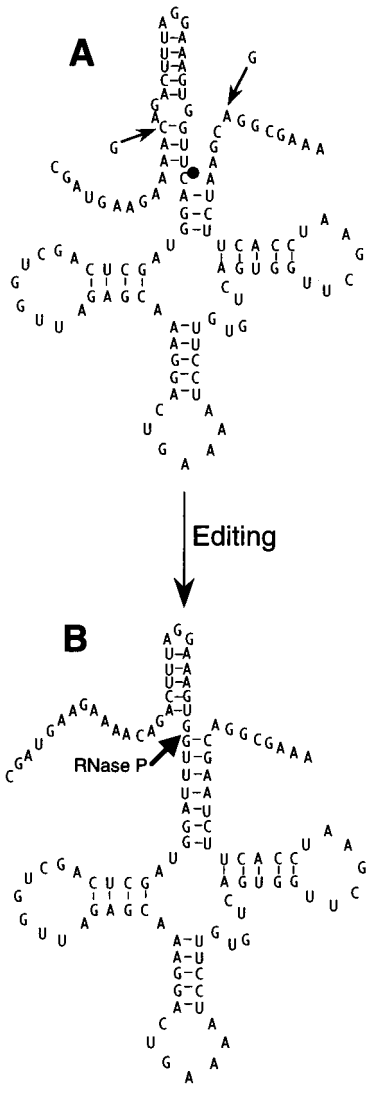


FIG. 5. Hypothetical effect of C_4-to-U_4 editing on the secondary structure of potato mitochondrial $tRNA^{Phe}$ in precursor RNAs and possible relation with 5′-end processing. Editing would enable complete base pairing of the amino acid acceptor stem of the tRNA in the precursor RNA (B) and therefore allow recognition and processing by RNase P. In the absence of editing, part of the acceptor stem of the tRNA would pair with an upstream sequence in the precursor, leading to another helical structure (A) and preventing RNase P recognition. Processing at the 5′ end would then be blocked. The editing site is indicated (dot). The two nucleotide differences found in the corresponding *Oenothera* mitochondrial $tRNA^{Phe}$ gene and its flanking regions (4) are indicated in panel A (arrows).

mentioned above obtained with other organisms, such an alternate folding is unlikely to be recognized by RNase P. One possible explanation for the low level of processing sometimes observed with the unedited precursor RNA is that occasionally a small proportion of the unedited molecules might fold correctly, either during in vitro synthesis or in the assay. In a parallel work, Marchfelder et al. recently pointed out that editing is also required for efficient excision of *Oenothera* mitochondrial $tRNA^{Phe}$ from precursor RNAs (23). A similar, although less stable, alternate folding can be proposed for the *Oenothera* $tRNA^{Phe}$ precursor (Fig. 5).

In the protozoan *Acanthamoeba castellanii*, 8 of the 10 mitochondrial tRNAs characterized are edited (20) or are predicted to undergo editing (21) at the 5′ end of the acceptor stem. These editing events are also likely to allow proper folding of the tRNA in the respective precursors and therefore might be required for recognition by RNase P and/or other processing enzymes.

The fact that replacing the unedited C_4-A_{69} mispairing in the potato mitochondrial $tRNA^{Phe}$ by a correct C_4-G_{69} base pair, instead of the normally edited U_4-A_{69} base pair, restored only 40% processing efficiency of the precursor RNA seems somehow contradictory with the previous view, as a C-G base pair is stronger than a U-A base pair and thus should even better stabilize the acceptor stem. This suggests that either introducing a C_4-G_{69} base pair makes the tRNA structure too tight or the U at position 4 by itself plays a role during interaction of the tRNA with plant mitochondrial RNase P. In contrast to the situation in dicotyledonous plants, such as potato plants, the native $tRNA^{Phe}$ gene has become a pseudogene in mitochondria of the monocotyledonous plants wheat and maize. When the sequences of these pseudogenes are folded into the cloverleaf structure, a correct C_4-G_{69} base pair is found (12, 37). It can be speculated that an A_{69}-to-G_{69} mutation appeared in the native $tRNA^{Phe}$ gene of monocotyledonous plants after their divergence with dicotyledonous plants, giving rise to a $tRNA^{Phe}$ transcript which could no longer be edited and was unefficiently processed. In another evolutionary step, the transfer of the chloroplast $tRNA^{Phe}$ gene into the mitochondrial genome of monocotyledons (16) might have enabled efficient expression and processing of an alternative $tRNA^{Phe}$, leading to further divergence of the native $tRNA^{Phe}$ gene into a pseudogene.

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REFERENCES

1. **Altman, S., L. Kirsebom, and S. Talbot.** 1995. Recent studies of RNase P, p. 67–78. *In* D. Söll and U. L. RajBhandary (ed.), tRNA: structure, biosynthesis, and function. ASM Press, Washington, D.C.
2. **Beier, H., M. C. Lee, T. Sekiya, Y. Kuchino, and S. Nishimura.** 1992. Two nucleotides next to the anticodon of cytoplasmic rat $tRNA^{Asp}$ are likely generated by RNA editing. Nucleic Acids Res. **20:**2679–2683.
3. **Benne, R. (ed.).** 1993. RNA editing: the alteration of protein coding sequences of RNA. Ellis Horwood Series in Molecular Biology. Ellis Horwood, New York.
4. **Binder, S., and A. Brennicke.** 1993. A tRNA gene transcription initiation site is similar to mRNA and rRNA promoters in plant mitochondria. Nucleic Acids Res. **21:**5012–5019.
5. **Binder, S., A. Marchfelder, and A. Brennicke.** 1994. RNA editing of $tRNA^{Phe}$ and $tRNA^{Cys}$ in mitochondria of *Oenothera berteriana* is initiated in precursor molecules. Mol. Gen. Genet. **244:**67–74.
6. **Binder, S., A. Marchfelder, A. Brennicke, and B. Wissinger.** 1992. RNA editing in *trans*-splicing intron sequences of *nad*2 messenger RNAs in *Oenothera* mitochondria. J. Biol. Chem. **267:**7615–7623.
7. **Börner, G. V., M. Mörl, B. Wissinger, A. Brennicke, and C. Schmelzer.** 1995. RNA editing of a group II intron in *Oenothera* as a prerequisite for splicing. Mol. Gen. Genet. **246:**739–744.
8. **Carneiro, V. T. C., A. Dietrich, L. Maréchal-Drouard, A. Cosset, G. Pelletier, and I. Small.** 1994. Characterization of some major identity elements in plant alanine and phenylalanine transfer RNAs. Plant Mol. Biol. **26:**1843–1853.
9. **Cattaneo, R.** 1991. Different types of messenger RNA editing. Annu. Rev. Genet. **25:**71–88.
10. **Conklin, P. L., R. K. Wilson, and M. R. Hanson.** 1991. Multiple *trans*-splicing events are required to produce a mature *nad*1 transcript in a plant mitochondrion. Genes Dev. **5:**1407–1415.
11. **Del Sal, G., G. Manfioletti, and C. Schneider.** 1989. The CTAB-DNA precipitation method: a common mini-scale preparation of template DNA from phagemids, phages or plasmids suitable for sequencing. BioTechniques **7:**514–520.
12. **Gualberto, J. M., H. Wintz, J. H. Weil, and J. M. Grienenberger.** 1988. The

genes coding for subunit 3 of NADH dehydrogenase and for ribosomal protein S12 are present in the wheat and maize mitochondrial genomes and are co-transcribed. Mol. Gen. Genet. **215:**118–127.
13. **Hanic-Joyce, P. J., and M. W. Gray.** 1990. Processing of transfer RNA precursors in a wheat mitochondrial extract. J. Biol. Chem. **265:**13782–13791.
14. **Hanson, M. R., C. A. Sutton, and B. Lu.** 1996. Plant organelle gene expression: altered by RNA editing. Trends Plant Sci. **1:**57–64.
15. **Janke, A., and S. Pääbo.** 1993. Editing of a tRNA anticodon in marsupial mitochondria changes its codon recognition. Nucleic Acids Res. **21:**1523–1525.
16. **Joyce, P. B. M., and M. W. Gray.** 1989. Chloroplast-like transfer RNA genes expressed in wheat mitochondria. Nucleic Acids Res. **17:**5461–5476.
17. **Kahle, D., U. Wehmeyer, and G. Krupp.** 1990. Substrate recognition by RNase P and by the catalytic M1 RNA: identification of possible contact points in pre-tRNAs. EMBO J. **9:**1929–1937.
18. **Knoop, V., W. Schuster, B. Wissinger, and A. Brennicke.** 1991. *trans*-splicing integrates an exon of 22 nucleotides into the *nad*5 messenger RNA in higher plant mitochondria. EMBO J. **10:**3483–3493.
19. **Lippok, B., A. Brennicke, and B. Wissinger.** 1994. Differential RNA editing in closely related introns in *Oenothera* mitochondria. Mol. Gen. Genet. **243:**39–46.
20. **Lonergan, K. M., and M. W. Gray.** 1993. Editing of transfer RNAs in *Acanthamoeba castellanii* mitochondria. Science **259:**812–815.
21. **Lonergan, K. M., and M. W. Gray.** 1993. Predicted editing of additional transfer RNAs in *Acanthamoeba castellanii* mitochondria. Nucleic Acids Res. **21:**4402.
22. **Marchfelder, A., and A. Brennicke.** 1994. Characterization and partial purification of tRNA processing activities from potato mitochondria. Plant Physiol. **105:**1247–1254.
23. **Marchfelder, A., A. Brennicke, and S. Binder.** 1996. RNA editing is required for efficient excision of $tRNA^{Phe}$ from precursors in plant mitochondria. J. Biol. Chem. **271:**1898–1903.
24. **Maréchal-Drouard, L., P. Guillemaut, A. Cosset, M. Arbogast, F. Weber, J. H. Weil, and A. Dietrich.** 1990. Transfer RNAs of potato (*Solanum tuberosum*) mitochondria have different genetic origins. Nucleic Acids Res. **18:**3689–3696.
25. **Maréchal-Drouard, L., D. Ramamonjisoa, A. Cosset, J. H. Weil, and A. Dietrich.** 1993. Editing corrects mispairing in the acceptor stem of bean and potato mitochondrial phenylalanine transfer RNAs. Nucleic Acids Res. **21:**4909–4914.
26. **Maréchal-Drouard, L., I. Small, J. H. Weil, and A. Dietrich.** 1995. Transfer RNA import into plant mitochondria. Methods Enzymol. **260A:**310–327.
27. **Maréchal-Drouard, L., J. H. Weil, and A. Dietrich.** 1993. Transfer RNAs and transfer RNA genes in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44:**13–32.
28. **Mörl, M., M. Dörner, and S. Pääbo.** 1995. C to U editing and modifications during the maturation of the mitochondrial $tRNA^{Asp}$ in marsupials. Nucleic Acids Res. **23:**3380–3384.
29. **Neuburger, M., E. P. Journet, R. Bligny, J. P. Carde, and R. Douce.** 1982. Purification of plant mitochondria by isopycnic centrifugation in density gradients of Percoll. Arch. Biochem. Biophys. **217:**312–323.
30. **Pallanck, L., M. Pak, and L. D. H. Schulman.** 1995. tRNA discrimination in aminoacylation, p. 371–394. *In* D. Söll and U. L. RajBhandary (ed.), tRNA: structure, biosynthesis, and function. ASM Press, Washington, D.C.
31. **Perret, V., A. Garcia, H. Grosjean, J. P. Ebel, C. Florentz, and R. Giegé.** 1990. Relaxation of a transfer RNA specificity by removal of modified nucleotides. Nature (London) **344:**787–789.
32. **Remacle, C., and L. Maréchal-Drouard.** 1996. Characterization of the potato mitochondrial transcription unit containing "native" *trnS* (GCU), *trnF* (GAA), and *trnP* (UGG). Plant Mol. Biol. **30:**553–563.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **Schuster, W., R. Ternes, V. Knoop, R. Hiesel, B. Wissinger, and A. Brennicke.** 1991. Distribution of RNA editing sites in *Oenothera* mitochondrial mRNAs and rRNAs. Curr. Genet. **20:**397–404.
35. **Steinberg, S., A. Misch, and M. Sprinzl.** 1993. Compilation of transfer RNA sequences and sequences of transfer RNA genes. Nucleic Acids Res. **21:**3011–3015.
36. **Thurlow, D. L., D. Shilowski, and T. L. Marsh.** 1991. Nucleotides in precursor tRNAs that are required intact for catalysis by RNase P RNAs. Nucleic Acids Res. **19:**885–891.
37. **Wintz, H., J. M. Grienenberger, J. H. Weil, and D. M. Lonsdale.** 1988. Location and nucleotide sequence of two tRNA genes and a tRNA pseudogene in the maize mitochondrial genome: evidence for the transcription of a chloroplast gene in mitochondria. Curr. Genet. **13:**247–254.
38. **Wissinger, B., W. Schuster, and A. Brennicke.** 1991. *trans*-splicing in *Oenothera* mitochondria: *nad*1 mRNAs are edited in exon and *trans*-splicing group II intron sequences. Cell **65:**473–482.
39. **Yokobori, S., and S. Pääbo.** 1995. Transfer RNA editing in land snail mitochondria. Proc. Natl. Acad. Sci. USA **92:**10432–10435.
40. **Yokobori, S., and S. Pääbo.** 1995. tRNA editing in metazoans. Nature (London) **377:**490.
41. **Zanlungo, S., V. Quinones, A. Moenne, L. Holuigue, and X. Jordana.** 1995. Splicing and editing of *rps*10 transcripts in potato mitochondria. Curr. Genet. **27:**565–571.