RNA Editing Is Required for Efficient Excision of tRNA\textsubscript{Phe} from Precursors in Plant Mitochondria\

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RNA editing corrects a \textit{C-A} mismatch to a conventional \textit{T-A} Watson-Crick base pair in the acceptor stem of the mitochondrially encoded tRNA\textsubscript{Phe} in plants. In vitro processing of edited and unedited Oenothera tRNA\textsubscript{Phe} precursor RNAs with pea mitochondrial protein extract shows a significant effect of this RNA-editing event on the efficiency of 5' and 3' processing. While mature tRNA molecules are rapidly generated by in vitro processing from edited precursors, the formation of mature tRNAs from unedited pre-tRNAs is considerably reduced. Primer extension analyses of in vitro processing products show that processing at both 5' and 3' termini is governed by the RNA-editing event. Investigation of edited and unedited precursor RNA by lead cleavage experiments reveals differences in the higher order structures of the pre-tRNAs. The differing conformations are most likely responsible for the altered processing efficiencies of edited and unedited precursor molecules. RNA editing of the tRNA\textsubscript{Phe} precursors is thus a prerequisite for efficient excision of the mature tRNA\textsubscript{Phe} in vitro. Hence RNA editing might be involved in regulating the amount of mature tRNA\textsubscript{Phe} in the steady state RNA pool of mitochondria in higher plants.

Plant mitochondrial RNAs have to undergo various posttranscriptional processing events to achieve their mature forms. These include splicing, 5' and 3' processing of mRNAs and rRNAs, excision of tRNAs from precursor molecules, and RNA editing (Gray et al., 1992). RNA editing has been described in different genetic systems and unlike all other processing steps alters the primary sequence of the affected RNA (for review see Bonnard et al., 1992; Wissinger et al., 1992; Gray et al., 1992). In plant mitochondria the editing process results in a conversion of genomically encoded cytidines (C) to uridines (U), however, in some rare cases reverse reactions have also been observed (Bonnard et al., 1992; Hiesel et al., 1994). Although specificity and mechanism of the RNA-editing reaction remain elusive, recent studies indicate that either deamination or transglycosylation of the ribosyl residue is involved in the conversion and that the sugar-phosphate backbone of the RNA remains intact during the editing process (Rajasekhar and Mulligan, 1993).

Most of the editing sites described in higher plant mitochondria are found in mRNA molecules and usually change the encoded amino acid identities. This frequently results in an evolutionary better conservation of the protein than predicted by the genomic sequence. The alteration of rRNA primary sequence by RNA editing remains unclear, since so far only a single edited cDNA has been found in Oenothera (Schuster et al., 1991). However, tRNA editing has unambiguously been identified in several instances and different plant species. One editing site located in the anticodon stem of tRNACys is only found in Oenothera, while a C to U conversion has been observed in the acceptor stem of tRNA\textsubscript{Phe} in several plants, i.e. potato, bean, and Oenothera. This nucleotide transition corrects a \textit{C-A} mismatch to a regular \textit{T-A} Watson and Crick base pair (Maréchal-Drouard et al., 1993; Binder et al., 1994). It has been speculated that this editing event might interfere with other maturation processes such as 5' and 3' end processing, in which endonucleases are implicated in both dicot and monocot plant species (Marchfelder et al., 1990, Hanicj oyce and Gray, 1990). Such a potential connection between RNA editing and the generation of mature 5' and 3' ends was indicated by the observation that all investigated mature tRNA\textsubscript{Phe} molecules are edited (Maréchal-Drouard et al., 1993), while in only 53.3% of the precursors is this nucleotide conversion detected (Binder et al., 1994).

In vitro processing assays with edited and unedited tRNA\textsubscript{Phe} precursor molecules and plant mitochondrial protein lysates now clearly show that the efficiency of 5' and 3' processing depends on RNA editing. Lead cleavage studies of the pre-tRNA molecules indicate that the single nucleotide transition caused by RNA editing leads to significant changes in higher order structures that are responsible for the differing processing efficiencies.

MATERIALS AND METHODS

Plasmids containing the tRNA\textsubscript{Phe} gene were derived by polymerase chain reaction from cDNA reverse transcripted from edited and unedited precursor RNAs. cDNA synthesis used Superscript\textsuperscript{TM} reverse transcriptase (Life Technologies, Inc.) primed with oligonucleotide P7 5'ATAAGCTTGAGTCTTCTCCCAAATCCGG-3' complementary to the 3'-terminal part of the precursor. Using primer P6 5'-CAGGAGGACGGAAGACGGACG-3' (complementary to the 5'-terminal sequences of the precursor) and primer P7, single strand cDNA was amplified on a Biomed thermodenaturer under the following conditions: 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C. Polymerase chain reaction was performed using 2.5 units of Taq DNA polymerase in a buffer supplied by the manufacturer (Boehringer) with 50 nm dNTP and 500 ng of each primer. After 30 cycles and a final incubation at 72 °C for 5 min, DNA fragments were digested with HindIII and cloned into pBluescript II KS +, cut with Smal/HindIII. Edited (ptrnfbs300+) and unedited (ptrnfbs300-) tRNA\textsubscript{Phe} cDNA clones were confirmed by DNA sequencing. The tRNA precursor molecules transcribed with T7 RNA polymerase were used for in vitro transcription and translation.
polymerase from a HindIII-linearized template consist of 55-nt vector and 180-nt mitochondrial sequences, which correspond to sequences between nucleotide positions 313 and 492 in EMBL data bank entry X74449 (Binder and Brennicke, 1993).

Preparation of the Pea Mitochondrial Protein Lysate—The pea mitochondrial protein extract was prepared basically following a method established by Hanic-Joyce and Gray (1990) for wheat. Briefly, Percoll-purified mitochondria (5 g) were lysed in the presence of Triton X-100 and KCl. Membranes were removed by centrifugation at 100,000 × g, and the supernatant (S100) was fractionated by (NH₄)₂SO₄ precipitation into 20% (w/v) and 50% (w/v) fractions. After dialysis, the 50% fraction was further purified by anion exchange chromatography on a Mono-Q column (Pharmacia Biotech Inc.). Bound protein was eluted stepwise from the column at 100, 200, 250, 300, and 400 mM KCl. Fractions of each elution step were pooled, dialyzed, and concentrated by ultrafiltration using Centricon 10 (Amicon). The protein extracts were rapidly frozen in liquid nitrogen and stored at −80 °C. Protein concentrations were measured using a Bio-Rad (Bradford) protein assay.

In Vitro Processing Assays—Equal amounts of HindIII-linearized edited and unedited DNA templates were transcribed in vitro using T7 RNA polymerase in the presence of [α-³²P]UTP (3000 Ci/mmol) under standard conditions. Transcripts were purified on 4% polyacrylamide gels as described elsewhere (Marchfelder et al., 1990). Processing reactions were carried out at 26 °C in 50-mM Tris-HCl (pH 7.6), 40 mM KCl, 10 mM MgCl₂, 20 mM spermidine and 2 mM dithiothreitol. About 60 μg of protein eluted from the Mono-Q column at 200 mM KCl were used in each processing assay. After incubation, processing products were analyzed by electrophoresis on 6% polyacrylamide gels and autoradiography.

In Vitro Processing of Edited and Unedited tRNAPhe Precursors—To investigate the influence of the RNA-editing event in the acceptor stem of tRNA_Phe on 5′-end processing of this tRNA, edited and unedited Oenothera and 3′ end processing of the mature tRNA_Phe. Oligonucleotide P1 was used for the investigation of 5′ ends of the trailer and thus for the 3′ end of the tRNAs as well as for analysis of lead cleavage products.

Miscellaneous Methods—Cloning and labeling reactions were carried out following standard protocols (Sambrook et al., 1989). DNA sequence analysis was performed with a T7 sequencing kit (Pharmacia). Northern blot analysis was carried out as described previously (Binder et al., 1994).

RESULTS

In Vitro Processing of Edited and Unedited tRNA_Phe Precursors—To investigate the influence of the RNA-editing event in the acceptor stem of tRNA_Phe on 5′ and 3′ end processing of this tRNA, edited and unedited Oenothera precursor RNAs were analyzed by in vitro processing assays with mitochondrial protein extracts from pea. The pre-tRNAs transcribed from cDNA clones are identical except for the editing site at nucleotide position 121, which is either U (edited) or C (unedited) in the respective transcript. The precursor molecule is 235 nt long and contains, besides the 73-nt-long tRNA_Phe, 117 nt of leader and 55 nt of trailer sequences, respectively (Fig. 1). To compare processing efficiencies, equal amounts of the two different precursor RNAs were incubated under identical conditions in the in vitro assays.

Distinct RNA molecules are observed after incubation of the edited precursor for 15 and 30 min, respectively (Fig. 2, top). Control reactions were carried out under identical conditions for 30 min without protein. Numbers on the right indicate specific processing products derived from the edited precursor. Sizes of coelectrophoresed DNA length markers (left) are given in nucleotides.

FIG. 1. Schematic structure of the tRNA_Phe precursor. Both edited and unedited pre-tRNA molecules are 235 nt long and consist of a 117-nt-long 5′ leader, the 73-nt-long tRNA_Phe* (indicated by the classic clover leaf structure) and a 45-nt 3′ trailer sequence. The indicated C4 (cytidine) to U4 (uridine) editing site is located at position 121 of the precursor molecule, corresponding to nucleotide 4 in the tRNA sequence.
pattern of different reaction products (Fig. 2, left side). The very weak signal in the size range of the mature tRNA\(^{\text{Phe}}\) indicates a very diminished correct and complete processing reaction. All stronger signals observed after 15 min of incubation represent RNAs also arising in control reactions and thus most likely derive from nonspecific cleavage. After 30 min the mitochondrial lysate has degraded the vast majority of the unedited precursor RNAs to nonspecific reaction products.

Analysis of 5' and 3' Ends of the Processing Products—To determine the exact cleavage sites in the precursor RNAs, primer extension analyses were performed with reaction products from in vitro processing assays. All assays were carried out under identical conditions with equal amounts of unlabeled edited or unedited pre-tRNAs.

Investigation of the 5' terminus of total mtRNA reveals a clear major signal at the precise 5' end of the tRNA predicted by the secondary structure model. The major signal obtained in the primer extension from the same primer P5 on in vitro processed edited RNAs coincides with the in vivo 5' end, thus confirming correct in vitro excision (Fig. 3, lanes 1 and 2). Only a minor signal is observed at the mature 5' end of the tRNA molecule in the analysis of processing products from the unedited precursor (Fig. 3, lane 3). These relations confirm the reduced 5'-processing efficiency of the unedited precursor RNA seen in the in vitro product analyses (Fig. 2). Accuracy of 5'

Fig. 3. Analysis of 5' end processing of tRNA\(^{\text{Phe}}\). Primer extension analysis of in vitro processing products from edited (lanes 2 and 5) and unedited (lanes 3 and 6) precursor RNAs and total in vivo mitochondrial RNA (lane 1). All in vitro processing assays and control reactions (lanes 2, 3, 5, and 6) were incubated for 30 min. Control reactions were performed with nucleic acids extracted from the mitochondrial protein isolated (lane 4) and with the degradation products of in vitro synthesized precursors after incubation without mitochondrial protein lysate (lanes 5 and 6). Extension products were coelectrophoresed with sequencing reactions identically primed (primer P5) on an edited cDNA clone (cDNA). For easier interpretation labeling of the sequence is inverted to show the sequence of the sense strand. The 5' end of the mature tRNA\(^{\text{Phe}}\) and the editing site are indicated on the left.

Fig. 4. Accuracy of 3' end processing. The position of the endonucleolytic processing event generating the 3' end of tRNA\(^{\text{Phe}}\) is indirectly investigated by primer extension analysis of the 5' end of the 3' trailer from primer P1. The extension products were coelectrophoresed with sequencing reactions performed with the same primer on a cDNA clone (cDNA, CTAG). For easier interpretation labeling of the sequence is inverted to represent the sense strand. Primer extension reactions were performed with isolated total in vivo mtRNA (lane 1) and with processing products from edited and unedited precursors (lanes 2 and 3). Control reactions were carried out both with precursors incubated without lysate (lanes 5 and 6) and with the lysate fraction without added pre-tRNA substrate (lane 4). The 3' end of the mature tRNA\(^{\text{Phe}}\) is indicated on the left. The 5'-terminal nucleotide of the 3' trailer is marked by an arrow.
processing, however, appears not to be affected by the editing event, since the weak signal at the mature 5' end is precise without scattering of the cleavage site to neighboring nucleotides. Mature 5' termini are not detected in the control reactions, confirming that the signals corresponding to the mature 5' tRNA ends result from genuine in vitro processing reactions of the mitochondrial protein lysate (Fig. 3, lanes 4–6).

The 3' ends of mitochondrial tRNAs from plants and other species are generated by endonucleolytic cleavage, allowing indirect investigation of this processing event by primer extension analysis of the 5' termini of 3' trailer fragments (Manam et al., 1987; Chen and Martin, 1988; Hanic-) oyce and Gray, 1990; Binder and Brennicke, 1993). Analysis of steady state Oenothera mtRNA identifies a guanosine as the 5'-terminal nucleotide of the trailer molecule, which coincides with the first nucleotide downstream of the mature 3' end of tRNAPhe (without the posttranscriptionally added CCA; Fig. 4, lane 1).

This 3' terminus is also detected with in vitro processed products derived from edited precursor molecules, suggesting correct cleavage at the 3' end in vitro (Fig. 4, lanes 1 and 2). One of the additional primer extension products also indicates incorrect processing, generating a tRNA molecule shortened by one nucleotide at the 3' end.

Only a very faint signal of correct 3' cleavage is observed with in vitro processed products derived from unedited precursor RNAs, indicating an almost completely reduced 3' tRNA processing activity (Fig. 4, lane 3). Slightly smaller extension products detected with both edited and unedited RNAs are also observed in the control reactions in the absence of protein lysate, identifying these signals as nonspecific breakage in the in vitro transcribed RNAs. However, no mature tRNA 3' termini were detected in these control reactions, confirming the genuine enzymatic origin of these 3' ends in the in vitro processing reactions (Fig. 4, lanes 4–6).

These primer extension analyses indicate that the single nucleotide transition by RNA editing is indispensable for efficient in vitro processing at both 5' and 3' ends of tRNAPhe.

Lead Cleavage Analysis of the Pre-tRNA Molecules—The investigation of tRNA maturation in various organisms identified correct secondary and tertiary structures of tRNA molecules as crucial features for efficient 5' processing (Thurlow et al., 1991; Kahle et al., 1990). The influence of the editing event on the conformation of the tRNA precursors was therefore tested by lead cleavage analysis. This method has been shown to be a powerful tool to detect the influence of single base substitutions on the tertiary structure of RNA molecules (Behlen et al., 1990; Zito and Pace, 1992). In vitro transcribed edited and nonedited precursor RNAs were incubated in the presence of 0.4 M lead acetate, and cleavage products were analyzed by primer extension experiments. Precursor RNAs incubated without lead but under otherwise identical conditions were used as controls (Fig. 5A). Comparison of the cleavage patterns identifies two main variations between the edited and unedited versions of the precursor molecules (Fig. 5A, marked by arrows). As indicated in Fig. 5B, these differences are located in the D-loop region and at the base of the acceptor stem. While the edited precursor is cleaved at C16/U17 and U17/G18, respectively (Fig. 5B, breakpoints 1a and 1b), the unedited pre-tRNA is more accessible to lead-induced cleavage between nucleotides G9 and U9 (Fig. 5B, breakpoint 2). These results confirm alterations in higher order structures due to the respective nucleotide identities present at the editing site. These distinct structures are most likely responsible for the altered in vitro processing efficiency of the edited and unedited versions of the tRNAPhe precursors.

DISCUSSION

Application of Heterologous In Vitro Processing Reactions—Heterologous in vitro processing assays were used to show the requirement of RNA editing in the acceptor stem of plant mitochondrial tRNAPhe for efficient formation of the mature tRNA. The origin of precursor RNA and protein extracts from different plant species has no detectable influence on the results of these experiments since there is no difference in cleavage efficiency or in accuracy between homologous and heterologous processing assays (Marchfelder and Brennicke, 1993). The gene for tRNAPhe in pea mitochondria is also encoded in a nonedited version and consequently has to be edited to attain its mature form (Knoop et al., 1995). This assumption is substantiated by the fact that RNA editing of tRNAPhe has been confirmed in three different plant species as significant for
maturation of this tRNA species (Maréchal-Drouard et al., 1993; Binder et al., 1994).

Accuracy of in Vitro Processing Reactions—Although this tRNA editing has a strong effect on processing efficiencies, the accuracy of both 5' and 3' end processing is not affected. The 5' termini generated in vitro cleavage coincide precisely with the 5' ends determined for steady state RNA from Oenothera mitochondria. Primer extension products resulting from shifted specificities of the cleavage site are not detected in reactions with either edited or nonedited precursor RNAs.

Accuracy of the 3' reaction on the other hand differs between the two templates. The majority of edited precursor is correctly processed, with a minor adjacent primer extension product indicating cleavage products shortened by one nucleotide at the 3' tRNA terminus. In contrast, almost no correct 3' cleavage is observed upon processing of the unedited pre-tRNA. A weak primer extension signal indicates an incorrect cleavage at a cytidine residue, which generates a 3' terminus 2 nucleotides shorter than the correct 3' end determined with isolated in vivo mtRNA. This suggests that the altered conformation of the acceptor stem caused by the C-A mismatch influences the 3' cleavage specificity. It is also possible that the higher order structure of the unedited precursor molecule makes this RNA susceptible to cleavage by other nucleases not normally involved in 3' processing of tRNAs.

RNA Editing Changes the Higher Order Structures of RNAs—Changes of the amino acid identities introduced by RNA editing within coding regions of an mRNA generally improve the evolutionary conservation of the respective encoded protein, and the biological function of RNA editing within these regions is rather evident (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989). Much has been speculated, however, about the function of RNA-editing events located in non-coding regions of mRNAs or in untranslated functional RNAs such as tRNAs. In group I introns the function of these editing events is attributed to their influence on higher order structures by converting mispaired bases to regular Watson-Crick base pairs (Wissinger et al., 1991; Binder et al., 1992). Editing of domain 6 in intron II c/d of the Oenothera nad1 gene implanted in edited and nonedited versions into the yeast aSc intron was recently shown to be a prerequisite for splicing of this chimeric group II intron under in vitro self-splicing conditions (Börner et al., 1995). Since only the edited domain 6 can be folded into the typical secondary structure (Michel and Dujon, 1983; Michel et al., 1989), these results have been interpreted to show the influence of RNA editing on the splicing reaction via its alteration of the higher order structures. These conclusions are in the same vein as the lead cleavage data of tRNA precursors described here, which present evidence for the conformational alteration of the 235-nt-long pre-tRNA by the single nucleotide difference introduced by RNA editing. The altered structural assembly, on the other hand, changes the 5' and 3' processing efficiencies.

The major breakpoint between nucleotides U27 and G18 detected in the analysis of the edited precursor (Fig. 5B, breakpoint 1a) coincides with cleavage sites observed in unmodified and modified mature yeast tRNA^Phe (Behlen et al., 1990). This specific sensitivity substantiates the significance of the cleavage sites and further supports the extrapolation that only edited pre-tRNAs are capable of forming correct secondary and tertiary structures.

The cleavage sites differing between edited and unedited precursor molecules are considered significant for the individual conformations, since these differences are consistently observed in the comparison of the results of different lead cleavage experiments of the same RNA well above the normal experimental variations (Fig. 5, data not shown). Primer extension signals occurring independently of lead cleavage are due to pauses of the reverse transcriptase on highly structured RNAs and have also been observed in the lead cleavage analysis of RNase P RNAs from different organisms (Zito and Pace, 1992).

tRNA Maturation Proceeds in a Defined Order—Our previous investigations have shown that the steady state RNA from plant mitochondria contains edited and nonedited tRNA^Phe precursors (Binder et al., 1994), which have to undergo a number of processing steps by different enzymatic activities. Based on the accumulated data available to date we suggest a defined order of the different processing steps; editing enzyme(s) bind to the unedited precursor, and the edited nucleotide moiety is introduced. The resulting conformational change of the pre-tRNA releases the editing enzyme(s). Now the 5'/3' processing enzymes recognize and bind to the precursor molecule and generate the 5' and 3' termini. Correct recognition of the precursor by the respective enzymes is thus based on the conformation of the pre-tRNA and requires unique specificities of the editing and processing enzymes, respectively. Simultaneous binding of both editing and 5'/3' processing enzymes with subsequent conformational change of the complete RNA-protein complex is rather unlikely, since in our experiments the 5'/3' processing enzymes recognize only edited precursor molecules.

Conformation of the precursor seems to be the crucial distinguishing feature for the binding and/or substrate specificities of the different enzymes including the RNA editing specificity, which may thus in part be guided by higher order RNA structure.

Regulation of tRNA Maturation by RNA Editing—The in vitro dependence of tRNA 5'/3' processing steps on the RNA
processing status of the precursor RNA and the in vivo observation of only edited nucleotides in mature tRNA\textsuperscript{Phe} molecules imply strongly that the amount of mature tRNA molecules present in the steady state RNA pool might be regulated by this nucleotide transition. The in vivo validity of these in vitro studies is supported by a Northern blot experiment with in vivo mRNA isolated from mitochondria of Oenothera (Fig. 6). Besides the mature tRNA\textsuperscript{Phe}, which is present in relatively low amounts, and an abundant larger RNA molecules are detected in this hybridization experiment. These RNAs include bona fide tRNA\textsuperscript{Phe} precursor molecules, but the large number of RNAs of so many different sizes indicates also the presence of degradation intermediates and products. These are most likely derived from turnover of the nonedited precursor RNA. Numerous different breakdown products are also observed in in vitro processing reactions with unedited precursors (Fig. 2, left part). This specific/unspecific breakdown is in vitro clearly enzymatically enhanced, since the degradation products progressively increase upon prolonged incubation in the lysate, whereas the correct (edited) products are rather stable in the mitochondrial extract.

Posttranscriptional regulation during maturation has also been suggested to control steady state abundance of tRNA\textsuperscript{Phe} in mitochondria of HeLa cells. Although a short primary transcript containing this tRNA is transcribed in a 25-fold higher rate than most other tRNAs, the steady state level of the mature tRNA\textsuperscript{Phe} is only 2–4 times higher than the average level of other less frequently transcribed tRNAs (King and Attardi, 1993). In Oenothera mitochondria a promoter has been identified just upstream of the tRNA\textsuperscript{Phe} gene, which could lead to a similarly selectively elevated transcription of this gene. The downstream-located tRNA\textsuperscript{Pro}, however, is cotranscribed and would thus be present in similar amounts and therefore likewise require extensive specific posttranscriptional regulation. Specific promoters located immediately upstream of other tRNA genes could analogously imply additional posttranscriptional regulation.

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