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RNA Editing in Plant Mitochondria: α-Phosphate Is Retained during C-to-U Conversion in mRNAs

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RNA editing in higher plant mitochondria frequently results in the post-transcriptional conversion of specific cytidine residues to uridine residues and infrequently results in the reverse conversion. The mechanisms by which this transition could occur are deamination or transamination of the amide at C-4 of cytosine, transglycosylation of the ribosyl residue, or deletion of a CMP residue and insertion of a UMP residue. Intact maize or petunia mitochondria were supplied with α-^{32}P-CTP to radiolabel CMP residues in the nascent transcripts, and the fate of the α-phosphate was examined by digestion of the RNA to nucleotide monophosphates and analysis by two-dimensional chromatography. A small fraction of radioactivity comigrated with UMP on two different two-dimensional thin-layer chromatography systems, and the amount of radiolabeled UMP increased between 10-min and 2-hr incubations. The conversion of cytidine-to-uridine residues was detected in the highly edited mRNA fraction but was not detected in the rRNA fraction. Recovery of radiolabeled UMP residues suggests that the α-phosphate is retained during the editing reaction. These results are consistent with either deamination or transamination, or transglycosylation mechanisms for RNA editing.

INTRODUCTION

RNA editing results in changes in the nucleotide sequence of a transcript such that the RNA sequence differs from the DNA template from which it was transcribed. RNA sequence modification by nucleotide conversion changes the identity of a nucleotide at a specific position, but not the number of nucleotides. Cytosine-to-uridine editing occurs frequently in higher plant mitochondrial transcripts (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989), in some maize chloroplast transcripts (Hoch et al., 1991), and at a single nucleotide in the coding sequence of apolipoprotein-B transcripts (Bostrom et al., 1990; Hodges et al., 1991; Lau et al., 1991). Adenosine deamination occurs in Xenopus oocytes by the double-stranded RNA unwinding deaminase (Bass and Weintraub, 1988; Polson et al., 1991). Transcripts for the glutamate-gated ion channel in mammalian brain tissue undergo an apparent "A-to-G" modification that may also result from adenosine deamination (Sommer et al., 1991). Editing has recently been reported in mitochondrial tRNAs of Acanthoamoeba that consist of single nucleotide conversions including U-to-A, U-to-G, and A-to-G (Lonergan and Gray, 1993).

RNA editing in plant mitochondria is ubiquitous among all the gymnosperms and angiosperms that have been examined (Gray and Covello, 1993) but probably does not occur in Marchantia mitochondria. Transcripts of almost every protein-encoding gene are edited, but the magnitude varies widely among transcripts for different genes. For example, only four nucleotides are edited in the 1533-nucleotide coding sequence of subunit 1 of ATP synthase (*atp1*) in Oenothera mitochondria, and editing results in a 0.4% change of the deduced amino acid sequence (Schuster et al., 1991). At the other extreme, 21 nucleotides are edited in the 357-nucleotide coding sequence of subunit 3 of NADH-ubiquinone dehydrogenase (*nad3*) in maize mitochondria, and editing results in a change of 15% of the amino acid residues (D. Grosskopf and R.M. Mulligan, unpublished results). In contrast to the high frequency of editing of many protein-encoding transcripts, the editing of rRNAs is extremely infrequent. Neither the 18S nor the 5S rRNAs are edited in Oenothera mitochondria, and editing of the 26S rRNA was observed at only two nucleotides and in only one of five cDNAs examined (Schuster et al., 1991). Thus, editing is prevalent among the protein-encoding transcripts but may be less important in expression of structural RNAs.

Most editing results in changes in the amino acid specified by a codon, and edited transcripts encode the evolutionary, conserved amino acid sequence of a polypeptide (Gualberto et al., 1989). RNA editing causes important changes in the amino acid sequence encoded by a transcript. Editing of subunit 2 of cytochrome oxidase (*cox2*) transcripts results in conversion of a CGU^{Arg} codon to a UGU^{Cys} at a position where cysteine is strictly conserved and required to coordinate the Cu_A site of cytochrome oxidase (Covello and Gray, 1990). RNA editing may occur in any position of the codon, but "silent" edits that do not affect the amino acid specified by the codon occur at a substantially reduced frequency (Gray and Covello, 1993). Thus, the primary role of editing appears to be in the expression of the correct amino acid sequence of

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a polypeptide; consequently, editing plays a genetic role in gene expression.

The mechanism of RNA editing in plant mitochondria has not been well characterized. Direct RNA and cloned cDNA sequence analysis has demonstrated that only specific C residues are subject to editing (Covello and Gray, 1990; Gualberto et al., 1991, Yang and Mulligan, 1991). Sequence analysis of unspliced pre-mRNAs and spliced mature mRNAs of *cox2* in maize and petunia mitochondria demonstrated that the unspliced pre-mRNAs are heterogeneous and incompletely edited relative to the nearly homogeneously edited, spliced transcripts (Sutton et al., 1991; Yang and Mulligan, 1991). These results indicated that RNA editing is not completed simultaneously with transcription, but that editing is a post-transcriptional process. Nuclear genes are known to influence the degree of editing of *nad3* transcripts in petunia, which demonstrates that RNA editing is at least partially under nuclear control (Lu and Hanson, 1992).

Cytidines could be converted to uridines by any of several mechanisms that are illustrated in Figure 1: deamination or transamination of the C-4 amide of cytosine would result in the direct conversion of cytosine to uracil; a transglycosylation reaction would remove the cytosine and replace a new base; or a deletion and insertion mechanism would remove the entire CMP residue and a new nucleotide would be inserted.

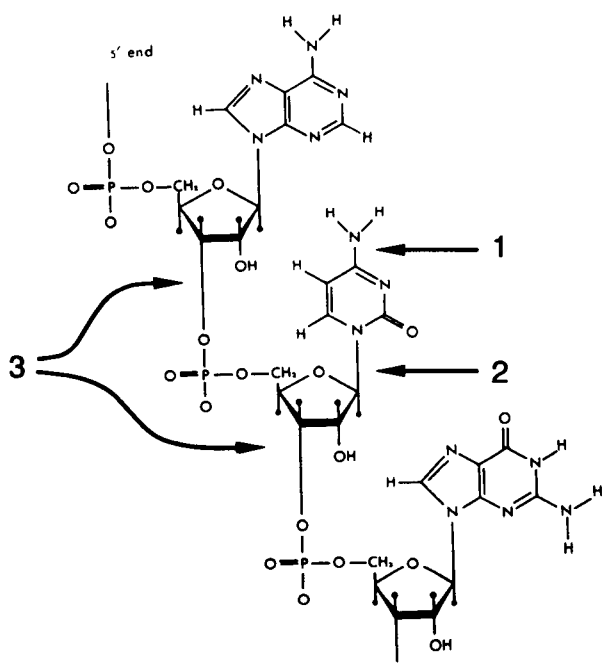

Figure 1. RNA Editing Mechanisms Potentially Responsible for C-to-U Conversion.

The bonds cleaved by various RNA editing mechanisms are indicated by the numbered arrows. Mechanism 1 involves deamination (or transamination) of the C-4 amide of cytosine to convert the base to uracil. Mechanism 2 involves transglycosylation of the ribosyl moiety to replace the base. Mechanism 3 involves deletion and insertion of a new nucleoside monophosphate.

Table 1. RNA Synthesis by Intact Maize Mitochondria

Time (min)	cpm/5-μL Aliquot	fmol Incorporated/ 100-μL Reaction
0	869	2.61
30	10,103	30.3
60	10,259	30.8

Aliquots (5 μL) were removed from a 100-μL transcription/editing reaction, and the reaction was terminated with 0.1% SDS. Unincorporated nucleotides were removed by centrifugation through a column packed with P-10 (Bio-Rad). Incorporated phosphorus-32 was determined by the Cerenkov method.

Nucleotide deletions at RNA editing sites have been reported in wheat mitochondrial cDNA clones (Gualberto et al., 1991). These cDNAs were interpreted to represent single-nucleotide-deleted intermediates of the editing process, and a deletion and insertion mechanism was proposed by these investigators. However, an in vitro RNA editing extract has been reported that apparently does not require exogenous nucleotides (Araya et al., 1992), and these data indicate that editing may proceed by a deamination mechanism. To directly evaluate the mechanism of RNA editing and discriminate among these possibilities, we have radiolabeled plant mitochondrial transcripts at CMP residues and observed that the α-phosphate is retained at UMP residues in the mRNA fraction. These results are consistent with deamination or transglycosylation mechanisms but are not expected for a deletion and insertion mechanism.

RESULTS

RNA Synthesis in Intact Mitochondria

To study nucleotide metabolism related to RNA editing, intact mitochondria were incubated with α-^{32}P-CTP to radiolabel CMP residues of the nascent transcripts. The total amount of label incorporated by intact maize mitochondria was constant over a 60-min incubation, as shown in Table 1. Thus, the transcription products were either stable, or transcript accumulation was maintained at steady state by synthesis and degradation. The relative amount of transcript accumulated for various maize mitochondrial genes was determined by hybridization of the radiolabeled transcription products to dot blots of cloned maize mitochondrial DNA sequences, as shown in Figure 2. The relative accumulation of each transcript in intact mitochondria after 1 hr was qualitatively similar to the relative rate of synthesis of the transcripts in run-on transcription assays with lysed maize mitochondria (Mulligan et al., 1991). Thus, after labeling

endogenous transcription products, the accumulation of transcripts is stable in isolated maize mitochondria. This system was used to study the subsequent processing of transcripts by RNA editing.

RNA Editing in Intact Mitochondria

Intact maize mitochondria were radiolabeled with α-^{32}P-CTP for 10 min or 2 hr. Nucleic acids were extracted and treated with DNase; high molecular weight RNA was purified by gel filtration chromatography. The RNA was digested to 5′-nucleotide monophosphates (NMPs) with nuclease P1 and analyzed by one-dimensional thin-layer chromatography (TLC), as shown in Figure 3, lanes 3 and 4. A small amount of nucleotide that comigrated with the UMP standard (lane 2) was observed after the 2-hr incubation (lane 4) but was not detectable as a discrete spot after the 10-min incubation (lane 3). The majority of the label was recovered as CMP, which comigrated with a CMP standard (lane 1). These results suggested that maize mitochondria convert CMP residues to UMP residues in a time-dependent fashion.

Comigration of the radiolabeled transcription/editing products with authentic CMP and UMP on two distinct two-dimensional TLC systems was utilized for diagnostic identification of CMP and UMP. Methods 1 and 2 utilize the same solvent system in the first dimension but fractionate CMP and UMP differently in the second dimension, as shown in Figures 4A and 4E. Control treatment of CTP-labeled synthetic RNAs yielded only CMP (Figures 4B and 4F) and demonstrated that the α-^{32}P-CTP had no detectable UTP contamination and that spontaneous or artifactual deamination did not occur during the preparation of the samples. Analysis of the transcripts extracted from maize mitochondria after a transcription/editing reaction indicated that a small amount of labeled nucleotide comigrated with UMP after 10 min of incubation (Figures 4C and 4G), but after 2 hr a distinct spot that comigrated with the UMP standard was present in each two-dimensional solvent system (Figures 4D and 4H).

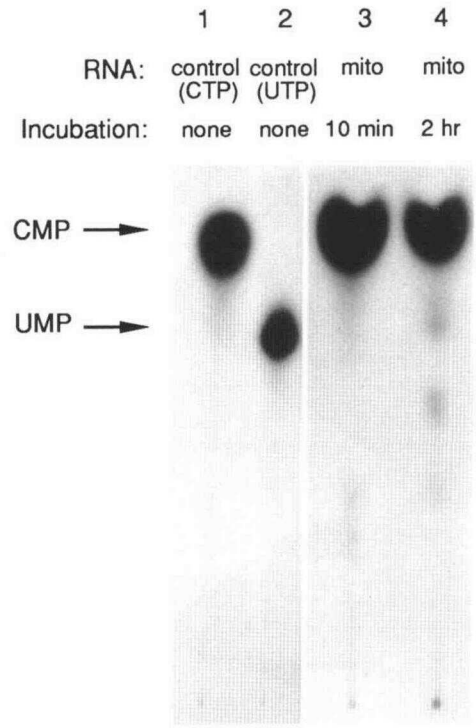

Figure 3. One-Dimensional Fractionation of NMPs from Maize Mitochondrial Transcripts.

Maize mitochondria were incubated with α-^{32}P-CTP and extracted after a 10-min or 2-hr incubation. Control RNAs were produced by transcription of pE203 with radiolabeled CTP or UTP. RNA was digested to NMPs with nuclease P1 and separated by one-dimensional TLC on polyethyleneimine-cellulose. Lanes 1 and 2 show digestion products of CTP- or UTP-labeled control RNAs, respectively. Lanes 3 and 4 show digestion products derived from mitochondrial (mito) transcripts after a 10-min or 2-hr incubation, respectively.

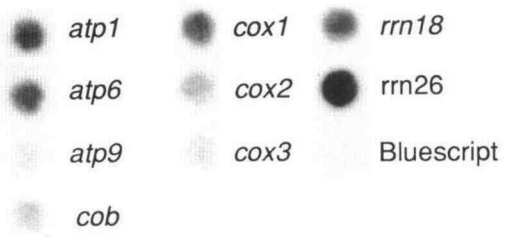

Figure 2. RNA Synthesis in Intact Maize Mitochondria.

RNA was isolated from the transcription reaction (500 μL) after 1 hr of incubation and hybridized to dot blots of maize mitochondrial genes. The clones represented are *atp1*, pBP1.7; *atp6*, pT25H; *atp9*, p117L; *cob*, p117C; *cox1*, pBN6601; *cox2*, pZmE1; *cox3*, pTL36; *rrn18*, p18B; *rrn26*, p26D; and Bluescript vector, pBluescript SK+.

Similar results were obtained with coupled transcription/editing reactions with isolated petunia mitochondria. A small amount of radiolabeled UMP was detected after 10 min of incubation, as shown in Figure 5A, and showed increased labeling of the UMP spot after a 2-hr incubation (Figures 5B and 5C). Additional radioactive products ("X" and an unmarked spot) were present in the TLC chromatograms of hydrolyzed petunia transcripts that do not comigrate with any known mononucleotides. The "X" spot was reported by Hodges et al. (1991) to have a similar migration to dinucleotide products (pCpA or pApC) that may result from incomplete digestion with nuclease P1. Thus, these unidentified products could result from dinucleotides that are present from incomplete digestion of the RNA.

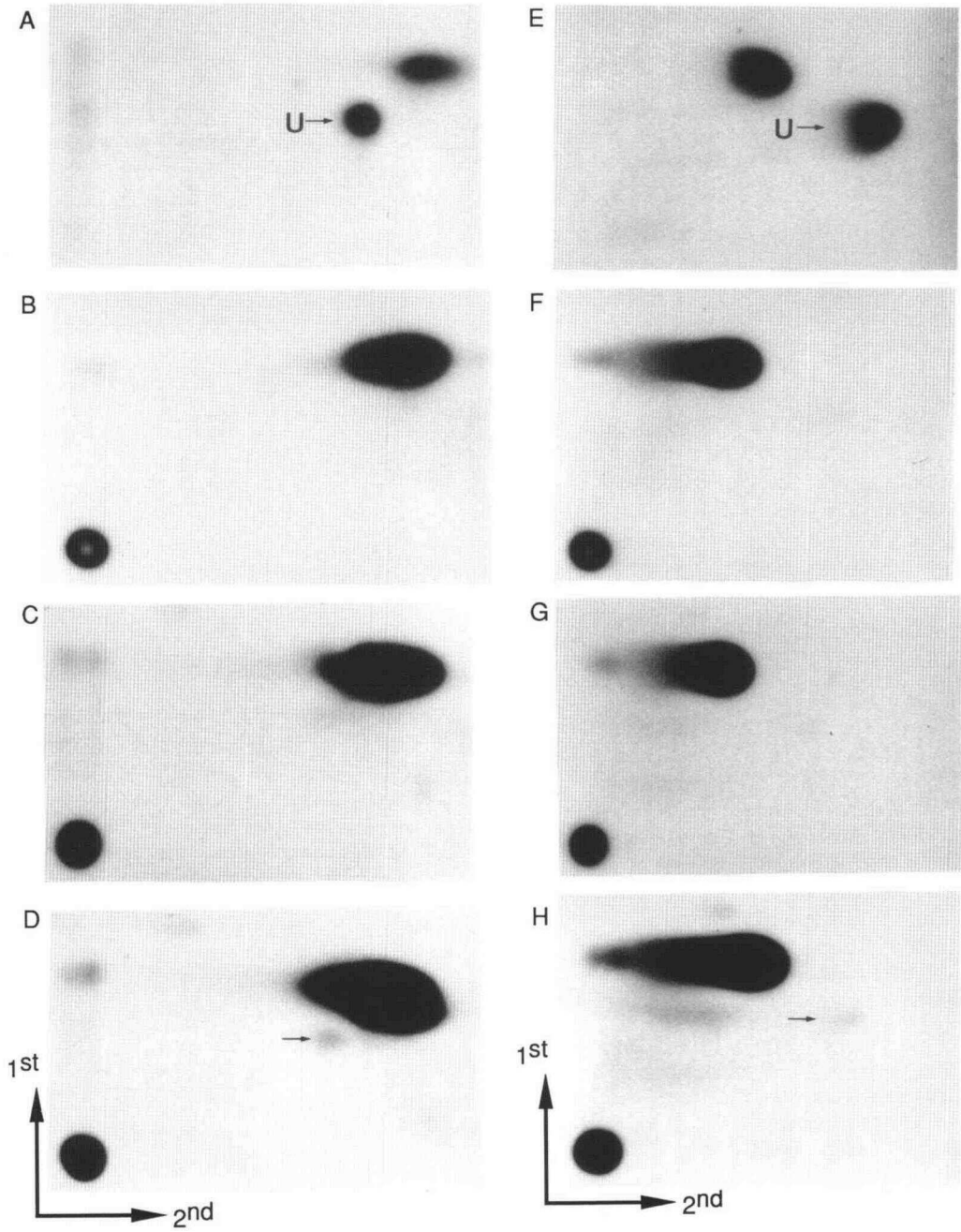

Figure 4. Two-Dimensional Fractionation of NMPs from Maize Mitochondrial Transcripts.

Maize mitochondrial transcripts were radiolabeled with CTP and extracted after 10 min or 2 hr of incubation. RNA was digested to NMPs and fractionated by two-dimensional chromatography on thin layer cellulose plates by method 1 (**[A]** to **[D]**) or method 2 (**[E]** to **[H]**). The migration of CMP and UMP standards was located as dark spots against a fluorescent background. The arrows at the bottom of the figure indicate the directions of the first and second dimensions of TLC.

(A) and **(E)** Control RNAs were prepared from transcription of clone pE203 with radiolabeled CTP and UTP. The migration of UMP is indicated by the arrows.

(B) and **(F)** Control RNAs were prepared from transcription of clone pE203 with radiolabeled CTP.

(C) and **(G)** CTP-labeled mitochondrial RNA was extracted after a 10-min incubation.

(D) and **(H)** CTP-labeled mitochondrial RNA was extracted after a 2-hr incubation. The radioactive spots indicated with the arrows were superimposable with the fluorescence quench of the UMP standard.

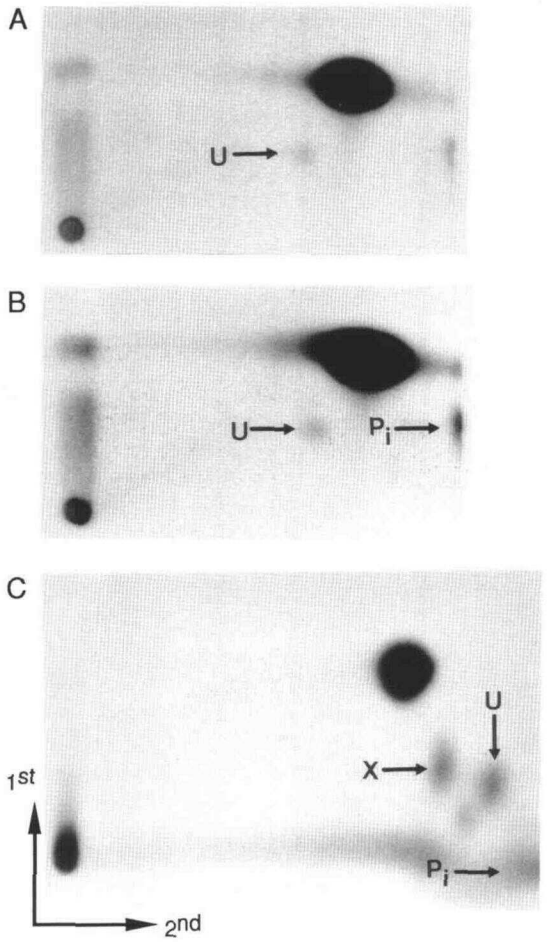

Figure 5. Two-Dimensional Fractionation of NMPs from *Petunia* Mitochondrial Transcripts.

Petunia mitochondrial transcripts were radiolabeled with CTP and extracted after 10 min or 2 hr of incubation. RNA was digested to NMPs and fractionated by two-dimensional chromatography on thin-layer cellulose plates by method 1 (**[A]** and **[B]**) or method 2 **[C]**. The arrows at lower left indicate the directions of the first and second dimensions of TLC. The arrows on the autoradiogram indicate the migration of uridine monophosphate (U), inorganic phosphate (Pi), and an unknown product (X).

(A) and **(B)** Mitochondria were labeled and incubated for 10 min or 2 hr, respectively, and NMPs were fractionated by TLC method 1.

(C) Mitochondria were labeled and incubated for 2 hr and NMPs were fractionated by TLC method 2.

Higher Plant Mitochondria Lack Detectable CTP Deaminase

To ensure that the radiolabeled UMP observed by TLC analysis was not due to the production of α-^{32}P-UTP by a CTP deaminase activity, mitochondria from the transcription/editing reaction were assayed for radiolabeled UTP. Maize mitochondria were collected from the transcription/editing reaction by centrifugation after 10 min or 2 hr and washed by four cycles of resuspension and centrifugation. The mitochondria were extracted with phenol, phenol-chloroform, and diethyl ether, and the aqueous phase was digested with phosphodiesterase to convert unincorporated nucleotide triphosphates (NTPs) to NMPs. No UMP was detected by two-dimensional TLC from mitochondrial fractions obtained after 10-min or 2-hr incubations, as shown in Figures 6A and 6B, respectively. These data indicate that these organelles do not have detectable CTP deaminase activity and suggest that radiolabeled CTP was not converted to UTP in the mitochondria prior to incorporation into RNA.

C-to-U Conversion Is Specific for the mRNA Fraction

To establish whether the CMP-to-UMP conversion represented RNA editing, we fractionated and separately analyzed the highly edited mRNA fraction from the sparsely edited rRNA fraction. Protection of rRNA transcripts from digestion with nuclease P1 was utilized to fractionate the rRNAs from the mRNAs and tRNAs. Purified RNA from a transcription/editing reaction was hybridized with single-stranded DNAs for the

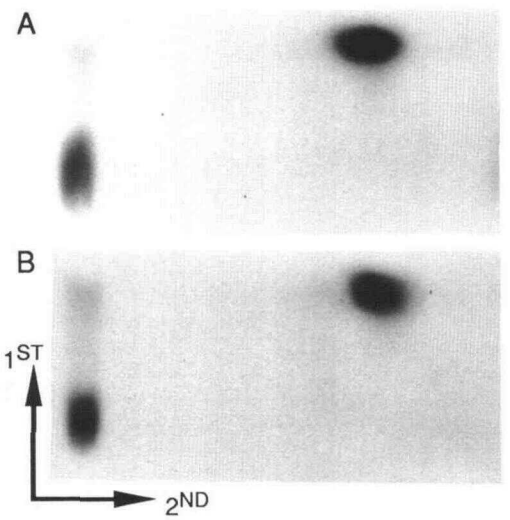

Figure 6. CTP Deaminase Assay from Maize Mitochondria.

The transcription/editing reaction was incubated for 10 min or 2 hr, and the mitochondria were collected by centrifugation. The mitochondria were washed by resuspension and centrifugation in nonradioactive reaction mixture and extracted with phenol and chloroform. The aqueous phase was treated with phosphodiesterase to convert unincorporated NTPs to NMPs and fractionated by TLC method 1. The arrows at lower left show the directions of the first and second dimensions.

(A) NMP products from mitochondria incubated in transcription/editing reaction for 10 min.

(B) NMP products from mitochondria incubated in transcription/editing reaction for 2 hr.

antisense strand of the 18S and the 26S rRNAs, and the hybridized nucleic acids were digested with nuclease P1. The protected rRNA transcripts were purified by gel filtration chromatography from the digested transcripts that included the mRNAs and tRNAs. Each fraction was subsequently digested to completion with nuclease P1, and ~2000 cpm of each digested RNA was analyzed by TLC with method 1. The rRNA fraction had no detectable radioactivity at the UMP position, as shown in Figure 7A, but the mRNA-enriched fraction exhibited a radioactive spot that comigrated with UMP (Figure 7B). Thus, the C-to-U conversion is specific for the highly edited mRNA-enriched fraction, and the rRNA fraction showed no detectable production of radioactive UMP. These results indicate that the C-to-U conversion observed in these studies is specific to the highly edited mRNA fraction.

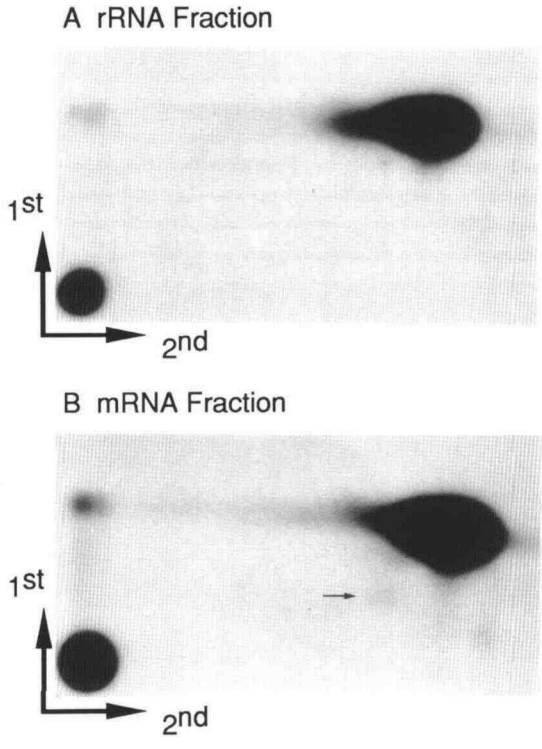

Figure 7. Two-Dimensional Fractionation of NMPs from rRNA and mRNA-Enriched Fractions from Maize Mitochondria.

Maize mitochondria were radiolabeled with CTP for 2 hr. High molecular weight RNA was prepared through the column chromatography step and hybridized with antisense single-stranded DNA for the 26S and 18S rRNAs. The duplexes were digested with nuclease P1, and protected rRNA fragments were separated from digested mRNAs and tRNAs. The rRNA and mRNA-enriched fractions were digested to completion with nuclease P1. Equivalent aliquots (~2000 cpm, determined by the Cerenkov method) were fractionated by TLC method 1 and autoradiographed for 7 days. The arrows at lower left show the directions of the first and second dimensions.

(A) Autoradiogram showing radiolabeled NMPs derived from the protected rRNA fraction.

(B) Autoradiogram showing radiolabeled NMPs derived from the mRNA and tRNA fraction that was not protected by the antisense ribosomal DNAs. The radioactive spot at the tip of the arrow comigrated with the UMP standard.

DISCUSSION

Intact Mitochondria Edit RNA by Conversion of CMP Residues to UMP Residues with Retention of the α-Phosphate

Intact mitochondria are a useful system for the study of RNA synthesis and processing. Intact plant mitochondria in this study rapidly incorporated radiolabeled nucleotides into transcription products (Table 1; Figure 2). Examination of the nucleotide products after a 10-min incubation indicated that CMP residues of the nascent transcripts had become labeled (Figures 4C, 4G, and 5A), with only a trace amount of UMP. After a 2-hr incubation, radiolabeled UMP was identified in the digestion products by comigration with a UMP standard in two diagnostic TLC systems (Figures 4D, 4H, 5B, and 5C). Thus, CMP residues of newly synthesized RNAs are converted to UMP residues in intact mitochondria.

The production of radiolabeled UMP residues from CTP-labeled transcripts apparently results from RNA editing. First, the amount of radiolabeled UMP present increased in a time-dependent manner (Figures 3, 4, and 5), which suggests that α-^{32}P-CTP was incorporated into the nascent transcripts as radiolabeled CMP residues and was processed to UMP residues. Second, the conversion of CMP residues to UMP residues was enriched in the mRNA and tRNA fraction relative to the rRNA fraction (Figure 7). This result indicated that radiolabeled UMP is produced specifically from the highly edited mRNA-enriched fraction rather than the sparsely edited rRNA fraction.

Two types of artifacts that could result in UMP formation have been eliminated: CTP deaminase and nonspecific deamination. First, direct assay of CTP deaminase activity demonstrated that maize mitochondria lack detectable CTP deaminase. CTP deaminase could convert labeled CTP to UTP and result in direct labeling of UMP residues. Second, several control reactions demonstrated that nonspecific deamination is not responsible for these results. Synthetic RNAs prepared with radiolabeled CTP yielded only radiolabeled CMP products (Figures 3 and 4), with no detectable UMP. Thus, the CTP was free of UTP contamination, and the digestion and chromatography procedure did not result in artifactual deamination. Finally, the observation that mRNAs and rRNAs are differentially edited in this assay indicated that the production of UMP is specific for mRNA-enriched fractions and was not observed nonspecifically in the rRNA fraction (Figure 7).

These results indicate that RNA editing occurs by conversion of radiolabeled CMP residues into radiolabeled UMP residues. Thus, the α-phosphate is retained during the editing reaction; these results are consistent with the editing mechanisms proceeding by either mechanism 1, transamination or

deamination, or mechanism 2, transglycosylation (Figure 1). Mechanism 3 involves a deletion and insertion of nucleotides and would be expected to exchange the α-phosphate.

Earlier investigators proposed that RNA editing in plant mitochondria proceeds by a nucleotide deletion mechanism (Gualberto et al., 1991). cDNA clones were observed with single nucleotide deletions at pyrimidine-rich RNA editing sites. These cDNA clones were thought to have arisen from spurious ligation of single nucleotide–deleted intermediates in the editing process, i.e., after nucleotide excision but before nucleotide insertion. Reverse transcriptase has recently been implicated in generating deletions in some oligouridine stretches of RNA (Y. Chapdelaine and L. Bonen, personal communication), and cDNA clones with deletions at uridine-rich editing sites might result from a reverse transcriptase artifact.

An in vitro editing extract has been reported that edits wheat *atp9* transcripts (Araya et al., 1992) and requires no exogenous nucleotide. These results seem to support a deamination mechanism (Gray and Covello, 1993), although potential carryover of NTPs from in vitro RNA synthesis makes the role of NTPs difficult to evaluate. The apparent absence of a requirement for exogenous nucleotides for in vitro editing reactions and the retention of the α-phosphate in the C-to-U conversion suggest that RNA editing may result from a deamination reaction.

RNA editing typically involves C-to-U transitions, but occasionally U-to-C conversions are also observed (Schuster et al., 1990). Deamination of cytosine would result in the direct conversion of cytidine residues to uridine residues, and the thermodynamics of deamination are very favorable (i.e., Gln + $H_2O \rightarrow$ Glu + NH_4^+; $\Delta G^{o\prime} = -3.4$ kcal/mol). However, the reverse reaction is consequently highly unfavorable, and a uridine-to-cytidine conversion would be expected to be an ATP-driven reaction (UMP + NH_3 + ATP → CMP + ADP + Pi) or to utilize a transamination reaction (i.e., UMP + aspartate → CMP + oxaloacetate). The stimulation of UMP production in the coupled transcription/editing reaction was tested by including α-ketoglutarate and oxaloacetate, common α-amino acceptors for a transamination reaction. This resulted in no apparent change in the production of UMP (data not shown). Thus, the possible role of a transaminase activity and the mechanism of U-to-C conversion in RNA editing will need further evaluation.

The process of RNA editing in higher plant mitochondria is usually assumed to result from the conversion of a C residue to a U residue. Characterization of RNA editing usually involves reverse transcription and amplification with polymerase chain reaction or the specificity of an RNase (Covello and Gray, 1989). In principle, any modification of a C residue that causes it to base pair like a U residue or be recognized as a U residue by an enzyme would result in the apparent conversion to a "U" residue. For example, a modified cytosine, such as N^4-aminocytidine, is a potent mutagen in *Escherichia coli* that will base pair as cytidine or thymidine and consequently introduces A/T-to-G/C transitions (Bessho et al., 1989). N^4-acetylcytidine is known to exist as a minor base in tRNA (Stern and Shulman, 1978), and the derivatization of the C-4 amide could result in modified base pairing properties of the nucleotide. In addition, a novel pyrimidine, lysidine (4-amino-2-N^6-lysino-pyrimidinium), exists in the anticodon of $tRNA^{Ile}$ and base pairs with an A but not a G residue (Muramatsu et al., 1988). Thus, modification of an existing cytidine residue, or substitution with a novel base, could potentially result in the observation that cytidines are "edited to uridines." These experiments demonstrate that a radiolabeled product of CMP is produced that comigrates with UMP in both two-dimensional TLC systems and suggest that the base modification is probably a cytosine-to-uridine conversion rather than some other novel modification.

METHODS

Isolation of Mitochondria

Mitochondria were isolated from etiolated shoots of 8-day-old maize seedlings (*Zea mays*; B37N; Pioneer Hybrid International Inc., Johnston, IA) or from the green leaves of 8-week-old petunia (*Petunia hybrida* var Super Cascade Red; Park Seed Co., Greenwood, SC). Intact mitochondria were prepared as previously described (Mulligan et al., 1991), except that the homogenization buffer (HB; 10 mM 3-[*N*-morpholino]propanesulfonic acid, pH 7.2, 0.4 M mannitol, 1 mM EGTA, 0.05% cysteine, 0.1% BSA, 1 mM DTT) was supplemented with 3% polyvinylpolypyrrolidone for the isolation of mitochondria from petunia leaves. Mitochondria were purified by centrifugation on discontinuous sucrose density gradients (20, 36, 40, and 52% for maize; 20, 36, 40, 55, and 69% for petunia) as described by Kemble et al. (1980). Intact mitochondria were collected from 40 to 52% or 55 to 69% sucrose gradient interface for maize or petunia, respectively. All the above steps were performed at 4°C and/or in an ice bath, unless stated otherwise.

Transcription/Editing Reaction in Intact Mitochondria

Transcription reactions were performed as previously described (Mulligan et al., 1991), except that the reaction mixture was prepared isoosmotically with 330 mM mannitol. Purified mitochondria were incubated at 25°C in a final volume of 500 μL containing 10 mM $MgCl_2$, 50 mM KCl, 1.25 mM EDTA, 200 μM each of UTP, GTP, and ATP, 75 μCi of α-^{32}P-CTP (3000 Ci/mmol), 100 μg BSA, 200 units of RNasin (Promega), 2 mM DTT, and 330 mM mannitol. After 10 min or 2 hr of incubation, total nucleic acids were recovered from the mitochondrial suspension by two extractions with phenol/chloroform (1:1) and precipitation with ethanol. DNA was removed by treatment with RNase-free DNase (50 units, Boehringer Mannheim) at 37°C for 1 hr. The mitochondrial RNA fraction was purified by two extractions with phenol/chloroform (1:1) and was followed by at least three extractions with diethyl ether and precipitation with ethanol. High molecular weight RNA was purified by centrifugation through Sephadex G-50 columns. The excluded material from the first column was rechromatographed on a second Sephadex G-50 column. The high molecular weight RNA fraction was precipitated with ethanol and digested to nucleotide 5′ monophosphates in a final volume of 10 μL (50 mM sodium acetate, pH 6.6) with 1 to 2 units of nuclease P1 (Pharmacia) at 37°C for at least

3 hr (Hodges et al., 1991). The radioactivity of the digested samples was determined by the Cerenkov method (Goulding, 1981). Chromatograms for a given experiment were loaded with the same amount of radioactivity, which was routinely between 500 to 2000 cpm in 1 to 3 μL of digested RNA. Carrier CMP and UMP (25 μg each; Sigma) were added to the digested RNA, and the mixture was fractionated by thin layer chromatography (TLC).

Fractionation of Nucleotide Monophosphates by TLC

One-dimensional TLC was performed with 0.1 mm polyethyleneimine-cellulose plates embedded with fluorescence indicator (Sigma). The solvent system was 1 M acetic acid–3 M LiCl (9:1 [v/v]) (Bass and Weintraub, 1988).

Two-dimensional chromatography systems utilized fluorescent 0.1 mm cellulose TLC plates (E. Merck, Darmstadt, Germany) and were developed in the first dimension with isobutyric acid–NH_4OH–water (66:1:33 [v/v/v]). Two different solvent systems in the second dimension were utilized that fractionate CMP and UMP distinctly. Method 1 utilized 0.1 M sodium phosphate (pH 6.8)–NH_4SO_4–*n*-propanol (100:60:2 [v/w/v]) (Silberklang et al., 1979) and fractionates CMP with greater mobility than UMP. Method 2 used isopropanol–HCl–water (70:15:15 [v/v/v]) (Nishimura, 1972) and fractionates CMP with less mobility than UMP. UV-absorbing material was detected under a short-wave UV light source, and radioactive material was detected by autoradiography at −80°C with intensifying screens. Typical exposures were 4 to 7 days.

CTP Deaminase Assay

CTP deaminase activity was assayed by analysis of maize mitochondria for the presence of radiolabeled UTP. Maize mitochondria were incubated in a 500-μL transcription/editing reaction mixture containing radioactive CTP. The mitochondria were collected by centrifugation after either 10 min or 2 hr of incubation and washed by four cycles of resuspension and centrifugation in 1.5 mL of a nonradioactive transcription/editing reaction mixture in which 50 nM CTP was substituted for the radioactive CTP. The mitochondria were resuspended in 100 μL of 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA and extracted sequentially with phenol, phenol/chloroform, and diethyl ether. An aliquot of the aqueous phase (2 μL) was treated with phosphodiesterase from *Crotalus durissus* venom (0.003 units; Boehringer Mannheim) in a final reaction volume of 10 μL (200 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$) at 37°C for 1 hr. Nucleotide monophosphates were fractionated by two-dimensional TLC with method 1.

Clones and Nucleic Acids

Clones for maize mitochondrial genes were described previously (Mulligan et al., 1991): subunit 1 of ATP synthase, *atp1*, pBP1.7, a 1.7-kb BamHI-PstI fragment with the *atp1* promoter; subunit 6 of ATP synthase, *atp6*, pT25H, a 2.7-kb HindIII fragment from the T genome (Dewey et al., 1985); subunit 9 of ATP synthase, *atp9*, p117L, a 1.7-kb BamHI-HindIII fragment (Mulligan et al., 1988b); apocytochrome *b*, *cob*, p117C, a 1.8-kb HindIII fragment; subunit 1 of cytochrome oxidase, *cox1*, pBN6601, a 3.9-kb EcoRI-BamHI fragment (Isaac et al., 1985); subunit 2 of cytochrome oxidase, *cox2*, pZmE1, a 2.4-kb EcoRI fragment (Fox and Leaver, 1981); subunit 3 of cytochrome oxidase, *cox3*, pTL36, a 1.3-kb BglII-XbaI fragment (Mulligan et al., 1988a); 18S rRNA, *rrn18*, p18B, a 0.7-kb BamHI-HpaII fragment (Mulligan et al., 1988b); 26S rRNA, *rrn26*, p26D, a 1.0-kb AvaI fragment (Mulligan et al., 1988a). Clones containing the maize mitochondrial rRNA genes were obtained from a mitochondrial genomic library of partially digested BamHI mitochondrial DNA (B37N) and cloned in pUC8 (gift of K. Newton, University of Missouri, Columbia). The *rrn18* gene was cloned into pBluescript SK+ as a 2.65-kb HindIII fragment (clone p206H2.6) that included 387 nucleotides upstream of the transcription initiation site for the 18S rRNA, all of the mature 18S rRNA sequence, and 49 nucleotides of the 18S/5S intergenic region (Mulligan et al., 1988a; Maloney and Walbot, 1990). The 26S rRNA sequences were cloned into pBluescript SK+ as a 5.2-kb HindIII fragment (p185H5.2) that included ~2500 nucleotides of 5′ untranslated sequence and ~2700 nucleotides of pre-26S and mature 26S rRNA sequences (Mulligan et al., 1988a; Maloney et al., 1989). The transcribed region of this clone represents ~85% of the 26S rRNA transcription unit. Single-stranded DNA was recovered from p185H5.2 and from p206H2.6 after transfection with bacteriophage M13KO7.

Synthetic RNAs were prepared from cloned DNA in the pBluescript vector. Clones pE203 and pE316 are unspliced cDNA clones containing intron and exon 2 sequences of *cox2*. Plasmids were linearized, and uniformly labeled RNAs were prepared by in vitro transcription according to manufacturer's instructions with T7 RNA polymerase (Promega). RNA was labeled with α-^{32}P-CTP or α-^{32}P-UTP for control reactions and analyses.

Nuclease Protection Assay

Protection of rRNA transcripts from digestion with nuclease P1 was utilized to fractionate the rRNAs from the mRNAs and tRNAs. Maize mitochondrial transcription/editing products were prepared through the second Sephadex G-50 column chromatography step. One microgram of α-^{32}P-labeled mitochondrial RNA was mixed with 25 μg of *Escherichia coli* tRNA and ~100 μg of antisense single-stranded DNA from each of the 26S and 18S rRNA clones (p185H2.6 and p206H5.2, respectively). The nucleic acids were resuspended in 20 μL of hybridization buffer (40 mM Pipes-NaOH, pH 6.4, 400 mM NaCl, 1 mM EDTA in 80% [v/v] formamide). The nucleic acids were denatured at 80°C for 10 min and immediately transferred to 42°C water bath for at least 4 hr. An aliquot (330 μL) of ice-cold nuclease P1 digestion buffer (50 mM sodium acetate, pH 6.0, 300 mM NaCl) containing 36 units of nuclease P1 was added directly to the hybridized nucleic acids in the 42°C water bath, and the samples were rapidly removed to an ice bath. The samples were digested with nuclease P1 at 30°C for 1 hr and extracted with phenol/chloroform (1:1), followed by diethyl ether, and precipitated with ethanol as described above.

The protected rRNA fragments were fractionated from the unprotected and digested nucleic acids by centrifugation on a 1-mL Sephadex G-50 column. The protected rRNA fragments voided on the column, and unprotected digestion products were eluted with successive application of 100-μL aliquots of sterile water and repeated centrifugation.

The chromatography of the protected rRNA fragments was repeated on a second column, and the protected rRNA fragments were collected by precipitation. The protected fragments were dissolved in water, denatured by heating for 10 min at 80°C, and quickly chilled in an ice bath. The denatured fragments were then digested with 6 units of nuclease P1 in a 10- to 15-μL volume at 40°C for 3 hr. Radioactivity of the digested RNAs was determined by the Cerenkov method. Aliquots (~2000 cpm) from the protected and unprotected fractions were analyzed by two-dimensional TLC and exposed for 7 days.

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