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Identification of Isoleucine-Accepting tRNA in Maize Mitochondria

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Abstract: Maize mitochondrial tRNAs for isoleucine have been isolated using a putative $tRNA^{Ile}$ gene probe which has been previously isolated and characterized. It contains the 5'-CAT anticodon which would normally recognize the AUG methionine codon. The nucleotide sequence of one of these tRNAs has been partially determined, and contains a modified nucleotide at the first position of the anticodon. This type of posttranscriptional modification event could change the specificity of amino acid acceptance of a tRNA, unlike that deduced from the corresponding gene. An aminoacylation experiment also demonstrated that these purified tRNAs have isoleucine acceptance activity but no methionine-accepting activity.
Key words: genetic code, mitochondria, plant, tRNA.

Determination of the DNA sequence of the maize mitochondrial tRNA genes provides useful information about tRNA structures and evolutionary relationships and history but it is inadequate as a sole approach to the understanding of tRNAs, the genetic code, and patterns of codon coverage in the organelle. In many cases it is necessary to demonstrate the specific amino acid acceptance of a tRNA (reacting with a homologous enzyme) in addition to determining its primary sequence. In particular, if a tRNA represents a departure from the "universal" genetic code, as observed in mammalian and fungal mitochondrial systems (Barrell *et al.*, 1980; Bonitz *et al.*, 1980; Breitenberger and RajBhandary, 1985), then it is essential to establish its amino acid acceptance activity as well as its anticodon sequence.

Moreover, the sequence of a tRNA gene will occasionally cause an incorrect deduction of the amino acid acceptance of the tRNA, even if it conforms to the universal code. This can happen if posttranscriptional modifications of anticodon nucleotides change their base pairing characteristics from those normally expected of the parent nucleotide. An important example of this phenomenon is provided by the AUA-specific isoleucine tRNAs of *E. coli* and bacteriophage T4 (Fukada and Abelson, 1980; Harada and Nishimura, 1974; Kuchino *et al.*, 1980). These tRNAs must decode AUA but avoid a "normal" wobble recognition of the AUG methionine codon. By RNA sequence analysis, the nucleotide in the anticodon wobble position in each case is a highly modified pyrimidine, yet to be fully characterized. However, when the T4 isoleucine tRNA gene sequence was determined, it showed that the nucleotide from which the highly modified pyrimidine is derived is a C residue. Thus the modified nucleotide derived from this C residue will base-pair with an A residue rather than with the usual G residue. However, in the absence of data about amino acid acceptance or posttranscriptional modification, it would be interpreted as a methionine tRNA gene to decode the AUG methionine codon.

We have isolated the putative isoleucine tRNA gene containing its anticodon sequence of 5'-CAT from maize mitochondria. Ordinarilly it may recognize the methionine codon, AUG. However, comparison of its structure resembles that of other $tRNA^{Ile}$ rather than that of $tRNA^{Met}$. But this tRNA has been previously reported as a $tRNA^{Met}$ by others (Parks *et al.*, 1984). Therefore isolation of $tRNA^{Ile}$ has been attempted using the putative $tRNA^{Ile}$ gene DNA probe. Two possible $tRNA^{Ile}$ have been isolated and one of these demonstrated that maize mt $tRNA^{Ile}$ contains the modified nucleotide at its wobble position of the anticodon by RNA sequence analysis. Also an aminoacylation experiment for these tRNAs showed that both accepted isoleucine rather than methionine.

Materials and Methods

Materials

The seeds of *Zea mays* strain FRB73 (male fertile)

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were purchased from Illinois Foundation Seeds, Inc. *E. coli* strains used as host strains for recombinant plasmid DNAs were DG75 (O'Farrell *et al.*, 1978) and JM 109 (Yanisch-Perron *et al.*, 1985).

Crude *E. coli* total tRNA was obtained from Boehringer Mannheim (Indianapolis, USA). The nucleoside 5'-monophosphates, pA, pC, pU and pG, were purchased from Sigma Chemical Co. (St. Louis, USA).

Isolation of maize mtDNA and mt tRNAs

Maize mtDNA and mt tRNA were isolated as described elsewhere (Park, 1991). Then the crude maize mt tRNA fraction was partially purified by RPC-5 column chromatography to remove contaminating DNA and larger RNA molecules (Kelmers and Heatherly, 1971). Separation of the pooled maize total mt tRNAs into a single species of tRNA was carried out by two-dimensional gel electrophoresis (Burkard *et al.*, 1982). The first and second dimensional gels were 10% and 20% in acrylamide respectively, and contained 4 M urea. Electrophoresis was carried out at 4℃ and 450 v. After staining the gel in the solution containing 0.2 M Na-acetate (pH 4.6) and 0.2% methylene blue for 2 h, it was destained under running tap water for approximately 3 h, until clear tRNA spots appeared on a translucent background. The tRNA spots were cut out from the gel and ground with a glass rod in a 4 ml glass tube. The tRNA was extracted with 0.5 ml of d-H_2O and 0.5 ml of phenol by vortexing for 1 min, followed by shaking for 3 h at 37℃. The phenol and urea were removed by passing the sample through a G-50 spin column in a 1 ml syringe. The tRNA was then lyophilized for future use.

Transfer RNA sequence analysis

Transfer RNA sequences were partially determined by the methods essentially described by Stanley and Vassilenko (1978), and Silberklang *et al.* (1979). For the radiolabelling, 2 μl of γ-^{32}P-ATP (2 nM, 400 Ci/mmol), 1 μl of 4X kinasing buffer containing 80 mM Tris-HCl, pH 8.0, 40 mM $MgCl_2$, 40 mM 2-mercaptoethanol and 12.8 mM spermidine, and 1 μl of T4 polynucleotide kinase (10 U) were added to the pellet of formamide-hydrolysate of tRNA and incubated at 37℃ for 30 min. The volume of the reaction mixture was brought up to 20 μl with d-H_2O and passed through a G-50 spin column to remove unreacted radioisotope. The radiolabelled hydrolysate of tRNA was then fractionated on a 15% polyacrylamide gel containing 8 M urea. After exposing it to X-ray film, bands corresponding to the fragments of tRNA were sliced out of the gel and the tRNA fragments were extracted in 400 μl of elution buffer containing 50 mM ammonium acetate (pH 5.3) and 1 mM Na_2EDTA at 37℃ overnight with vigorous shaking. The radiolabelled fragments of tRNA were recovered by ethanol precipitation and subsequently dried under vacuum. The dried sample was then redissolved in 10 μl of elution buffer containing 100 ng of nuclease P1 and incubated at 55℃ for 2 h to generate a ^{32}P-labelled nucleoside-5'-monophosphate. The nucleotide sequences of tRNA was then determined by thin-layer partition chromatography (TLC). One microliter of each sample was applied to cellulose plastic plates (Polygram CEL300, 0.1 mm, Brinkmann Instruments, Inc.) and chromatography was performed at room temperature in the following solvent systems: A) isobutyric acid/cNH_4OH/d-H_2O (66/1/33, v/v/v), B) 0.1 M sodium phosphate, pH 6.8/ammonium sulfate/n-propanol (100/60/2, v/w/v). The plates were then dried, covered with plastic wrap, and autoradiographed at −70℃.

Aminoacylation

Assays for a specific amino acid acceptance by tRNA were carried out in 0.1 ml reaction mixtures containing 50 mM sodium cacodylate (pH 7.4), 10 mM $MgCl_2$, 3 mM ATP (pH 7.0), ^{3}H-labelled amino acid (50~60 Ci/mmol) at a final concentration of 1 to 2 μM and 5 μl of synthetase for 1 A_{260} of the tRNA. Incubation was at 37℃ for 30 min and aliquots of 20 μl were applied to a quarter of a 2.4 cm Whatman 3 mm disc filter paper. The discs were washed by gentle swirling in three changes of 5% trichloroacetic acid (TCA) for 15 min each on ice to precipitate the aminoacylated tRNA. They were then washed with 95% ethanol once for 5 min and dried. The disc was placed in a scintillation vial containing 5 ml of scintillation medium which consisted of PPO (0.4 g/l) and dimethyl POPOP (1 g/l) in toluene. Samples were then counted in a Beckman LS-100C liquid scintillation counter.

CCA 3'-end labelling of mt tRNA

Transfer RNAs were first treated with snake venom phosphodiesterase (SVD) to remove the 3'-end terminal adenosine. Reaction mixtures containing 150 μg of tRNA, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 μl of SVD (1 mg/ml) in 100 μl of final volume were incubated at room temperature for 10 min. The mixtures were then extracted with phenol : chloroform : isoamylalcohol (25 : 24 : 1) 3 times followed by 3 ether extractions. After ethanol precipitation and lyophilization, tRNAs were labelled with α-^{32}P-ATP using *E. coli* tRNA nucleotidyl transferase (Silberklang *et al.*, 1977).

Results and Discussion

The identification of tRNA of maize mitochondria

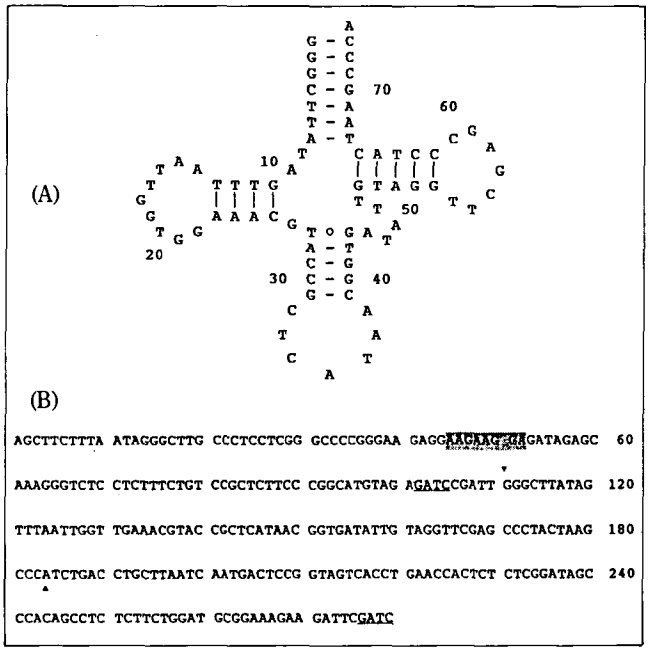

Fig. 1. (A) The secondary cloverleaf structure of the maize mt tRNA^{Ile} gene. (B) Nucleotide sequence of the maize mt gene encoding tRNA^{Ile} and its flanking regions. Arrows indicate the tRNA^{Ile} gene coding region. Shaded nucleotides indicate the possible promoter sequence. *Sau*3A sites are underlined.

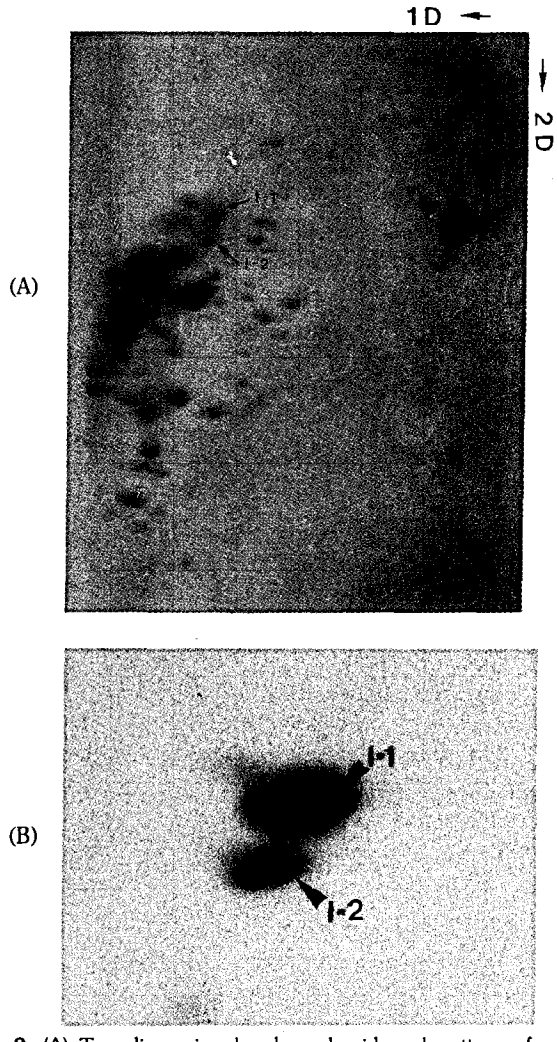

Fig. 2. (A) Two-dimensional polyacrylamide gel pattern of maize mt total tRNAs stained with methylene blue. First and second dimensional gels were 10% and 20% in acrylamide, respectively. The arrows show the directions of migration. Pure single species of tRNAs for isoleucine were extracted from tRNA spots on the 2-dimensional gel as indicated I-1 and I-2. (B) Autoradiogram for identifying tRNA^{Ile} containing spots on the 2-D polyacrylamide pattern of maize mt total tRNAs using hybrid-selected tRNA^{Ile} probe.

has particular interest because the sequence of a tRNA^{Ile} gene contains an anticodon sequence 5′-CAT which has been identified earlier in this laboratory (Fig. 1A and 1B). Sequence comparison indicated that it is almost identical to those of potato or *Oenothera* mitochondrial tRNA^{Ile} genes, which also contain a 5′ -CAT anticodon (Weber *et al.*, 1990; Binder *et al.*, 1994). The anticodon 5′-CAT itself indicates that the tRNA may recognize the AUG codon and accepts methionine. But further characterization of potato mitochondrial tRNA^{Ile} showed that it contains a lysidine-like residue derived from the C residue at its wobble position of the anticodon. Also aminoacylation experiment for this potato mitochondrial tRNA^{Ile} demonstrated that it has isoleucine-accepting activity but no methionine-accepting activity.

However, the same gene from maize mitochondria has, in fact, been analyzed by another group and described as a tRNA^{Met} gene (Parks *et al.*, 1984). But this tRNA does not display features characteristic of either initiator or elongator methionine tRNAs in terms of its secondary cloverleaf structure and its primary nucleotide sequence (Sprinzl *et al.*, 1989). Therefore, we assigned it as a putative tRNA^{Ile}. It was suspected that it might be an AUA-specific isoleucine species containing a highly modified C derivative in the anticodon wobble position, analogous to the AUA-specific isoleucine tRNAs of *E. coli* and bacteriophage T4 (Fukada and Abelson, 1980; Harada and Nishimura, 1974; Kuchino *et al.*, 1980; Weber *et al.*, 1990; Binder *et al.*, 1994). In order to resolve this question, it was necessary to identify the product of this gene and determine whether the isolated tRNA has a modified nucleotide at the first position of its anticodon, and whether it could be aminoacylated with isoleucine or methionine.

Isolation of isoleucine accepting tRNA

*Sau*3A DNA fragment of 174 bp in size containing the tRNA^{Ile} gene, which has been identified earlier in the lab. (Fig. 1B) was used for hybrid-selection of the corresponding tRNA molecule from a population of 3′ -end labelled total maize mt tRNAs. The labelled

Table 1. Aminoacylation of maize mt $tRNA^{Ile}$ (spots I-1 and I-2) (U : cpm)

	Maize mt total tRNA	I-1	I-2	Control (no tRNA)
^{3}H-Ile	2,264	1,185	961	174
^{35}S-Met	45,247	1,254	1,203	1,225

Aminoacylation reaction were carried out in the presence of ^{3}H-Ile (specific activity, 45 Ci/mmol) or ^{35}S-Met (specific activity, 1095 Ci/mmol), and maize mt synthetase or *E. coli* synthetase, respectively.

tRNA, which hybridized to and could be eluted from the DNA of the cloned gene, was mixed with the unlabelled total maize mt tRNAs and fractionated on the 2-D preparative polyacrylamide gel electrophoresis shown in Fig. 2A. Autoradiography of the gel showed that most of the hybrid-selected tRNA comigrated with spot I-1 and the rest comigrated with spot I-2 (Fig. 2B).

Transfer RNA was eluted from each spot and subjected to aminoacylation with radiolabelled isoleucine or methionine. Results are shown in Table 1. Aminoacylation with isoleucine was carried out with maize mitochondrial enzyme and that with methionine was carried out with *E. coli* enzyme because, in each case, that enzyme gave the highest incorporation for total maize mitochondrial tRNA. The tRNAs from both I-1 and I-2 showed isoleucine acceptance activity well above background levels, but their methionine acceptance was at precisely the level of the minus-tRNA negative control. This suggests that both these spots contain isoleucine tRNAs, perhaps two isoacceptors sufficiently closely related to be hybrid-selected by the single cloned gene.

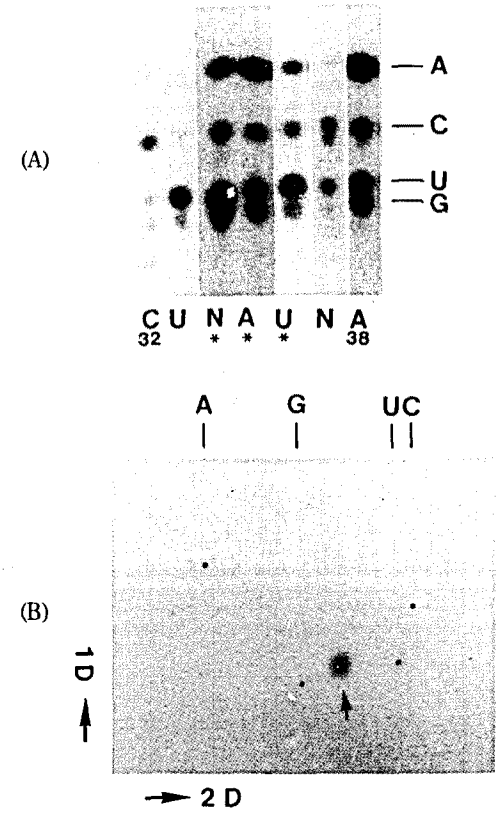

Fig. 3. Autoradiograms of nucleotide sequences of $tRNA^{Ile}$ from spot I-2, resolved by TLC (A) Nucleotide sequences from the anticodon loop region resolved with solvent. A The anticodon nucleotides are marked with *. The unknown nucleotide at position 34 migrated near pU but not exactly the same as the pU. (B) The nucleotide at position 34 was resolved by 2-D TLC with solvent A and B for the first and second dimensions, respectively. Arrows indicate the direction of solvent movement. The modified nucleotide is indicated by an arrow. Relative positions of pA, pC, pU and pG are marked with dots.

Sequence analysis of tRNA

Attempts were made to derive RNA sequence data from the tRNAs of spots I-1 and I-2. Those for I-1 were unsuccessful, but analysis of I-2 generated a sequence which throws some light on the question of isoleucine isoacceptors in this system. Although the partial sequence of the $tRNA^{Ile}$ from spot I-2 has been determined (data not shown), it is not the product of the putative $tRNA^{Ile}$ gene. But it was probably hybrid-selected by the gene because its high level of homology. Moreover, its anticodon, 5′-NAU, is consistent with its being an isoleucine tRNA (Fig. 3A). The first nucleotide of the anticodon, however, could not be identified because the spot resolved on TLC with solvent A migrated near pU but not exactly with it. The nucleotide at the wobble position was resolved by two-dimensional TLC with solvent A for the first dimension and solvent B for the second dimension. As seen in Fig. 3B, the spot appeared between pG and pU and it did not match any other known modified nucleotide spots (Silberklang *et al.*, 1979). Its exact identity remains unknown, but it is not identical to the modified C found in *E. coli* AUA-specific $tRNA^{Ile}$ (Kuchino *et al.*, 1980). The most likely resolution is that the wobble nucleotide in the spot I-2 $tRNA^{Ile}$ might be a modified G designed to enable the tRNA to decode the AUU and AUC isoleucine codons. Another posssibility is that the modified residue is a lysidine which is a cytidine with a 2-lysyl side chain. This changes anticodon CAU to LAU, then base-pairs with AUA isoleucine codon (Muramatsu *et al.*, 1988; Weber *et al.*, 1990; Jukes and Osawa, 1993).

Sequence analysis of the 5′-flanking region showed a purine-rich motif 58 nucleotides upstream of the putative maize mt $tRNA^{Ile}$ gene (Fig. 1B). Such a purine-rich motif has been proposed as a possible promoter

sequence at the region 50 to 65 nucleotides upstream of tRNA genes in higher plant mitochondria (Marechal-Drouard *et al.*, 1990). The AAGAAGGA motif found upstream of the putative maize mt $tRNA^{Ile}$ gene perfectly fits this consensus AAGAANRR sequence.

Further analysis will be necessary to resolve completely the questions of isoleucine codon coverage in the maize mitochondrion, but these studies show that the putative $tRNA^{Ile}$ gene is very likely an isoleucine tRNA gene. Namely, the recognition of AUA codon for isoleucine could be achieved by the existance of a $tRNA^{Ile}$ containing a highly modified C residue such as lysidine at its wobble position of the anticodon. Also it has been shown that the maize mitochondrial tRNAs contain an unusually rich and varied assortment of modified nucleotides, many of which may be previously unidentified. Thus studies on these tRNAs will benefit from a three-pronged approach of RNA sequence analysis, DNA sequence analysis, and aminoacylation.

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