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PRIMARY STRUCTURE OF THREE LEUCINE TRANSFER RNAs FROM BEAN CHLOROPLAST

M.L. OSORIO-ALMEIDA, P. GUILLEMAUT, G. KEITH, J. CANADAY and J.H. WEIL

Institut de Biologie Moléculaire et Cellulaire, Université Louis Pasteur,
15 rue Descartes, 67084 Strasbourg, France

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SUMMARY

*Three tRNAs^{Leu} have been purified from bean chloroplasts and their nucleotide sequence determined. tRNA$_1^{Leu}$ has 88 nucleotides and a U*AA anticodon, tRNA$_2^{Leu}$ has 85 nucleotides and a CmAA anticodon, and tRNA$_3^{Leu}$ has 83 nucleotides and a UAm^{7}G anticodon.*

INTRODUCTION

Chloroplasts contain their own DNA and have their own systems for replication, transcription and translation. As far as the translation machinery is concerned, chloroplasts contain tRNAs and aminoacyl-tRNA synthetases which are different from their cytoplasmic counterparts (1, 2). In *Phaseolus vulgaris* chloroplasts, three tRNAs^{Leu} have been found by reverse-phase chromatography (3) and by two-dimensional gel electrophoresis (4). In our laboratory, ribosome binding studies using the six leucine codewords showed that these three tRNAs preferentially recognize the U-U-G codon (5). To see whether these three isoaccepting tRNAs^{Leu} differ in their primary structures or only in post-transcriptional modification and to determine the anticodons, the nucleotide sequences were determined. It should be pointed out that only two sequences of chloroplast tRNAs have been published so far, namely Euglena chloroplast tRNA^{Phe} (6) and bean chloroplast tRNA^{Phe} (7) which differ by only five nucleotides, in addition to small differences in the post-transcriptional modifications.

MATERIAL AND METHODS

Bean leaf total tRNA was prepared as previously described (8). The three chloroplast tRNAs^{Leu} were first fractionated into three peaks on Sepharose 4B (Fig. 1). Then tRNA$_1^{Leu}$ (peak 1) and tRNA$_3^{Leu}$ (peak 3) were further purified using two reverse-phase chromatography (RPC-5) columns(10) : the first column run at room temperature and at pH 7.4 (NaCl gradient from 0.45 to 0.8 M), the second column run at 37° and pH 4.6, using the same NaCl gradient. Because of its extreme lability on RPC-5 columns, tRNA$_2^{Leu}$ was purified by two-dimensional polyacrylamide gel electrophoresis (4).

To determine the primary structures of these three tRNAs^{Leu}, the following procedures were used :

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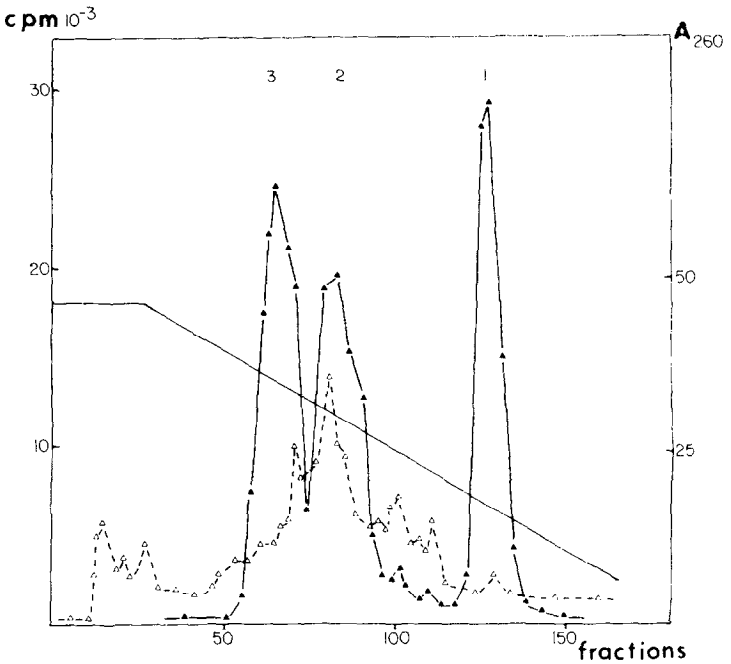

Fig. 1. Fractionation of bean leaf total tRNA on Sepharose 4B. 200 mg tRNA were loaded on the column (40 x 2 cm). Elution was performed with a 2 x 1000 ml decreasing gradient of $SO_4(NH_4)_2$ from 1.9 M to 0 M in sodium acetate buffer 0,01 M pH 4.5, $MgCl_2$ 0.01 M, EDTA 0.001 M, β-mercaptoethanol 0.006 M (9). Fractions of 10 ml were collected. (Δ—Δ) $A_{260\ nm}$; (▲—▲) $|^3H|$ leucine accepting activity revealed using a crude *E.coli* aminoacyl-tRNA synthetase preparation (3).

1) Characterization of modified nucleosides or nucleotides.

The nucleoside composition of $tRNA_1^{Leu}$ and $tRNA_3^{Leu}$ was determined according to Rogg *et al.* (11) whereas the nucleotide composition of $tRNA_2^{Leu}$ was determined according to Brown *et al.* (12).

2) Sequencing methods.

a) Most of the sequences were read off from sequencing gels done according to Donis-Keller *et al.* (13) and Simoncsits *et al.* (14) using 3'$|^{32}P|$ labeled $tRNAs^{Leu}$ (15) or 5'$|^{32}P|$ labeled fragments (7) obtained after partial T_1 RNAase hydrolysis of $tRNAs^{Leu}$.

b) Highly base-paired regions of tRNAs gave stacked bands even when thin gel slabs and high voltage electrophoresis were used (16). The sequence of these highly base-paired regions could be determined by analysis of the 5'$|^{32}P|$ labeled oligonucleotides obtained after complete T_1 or pancreatic RNAase digestion of $tRNAs^{Leu}$. These 5'$|^{32}P|$ labeled oligonucleotides were separated by two-dimensional homochromatography and their sequences were established after partial digestion by nuclease P_1 and analysis of products by two dimensional homochromatography according to the "wandering spot" technique (17, 18).

c) The position of the modified nucleotides in each $tRNA^{Leu}$ could be determined (19) by 5'$|^{32}P|$ labeling of the partial hydrolysis products of each tRNA, separation of the labeled fragments according to their size on a 20% polyacrylamide slab gel followed by extraction of each fragment from the gel and analysis of the 5'$|^{32}P|$ terminal mononucleotide by thin layer chromatography.

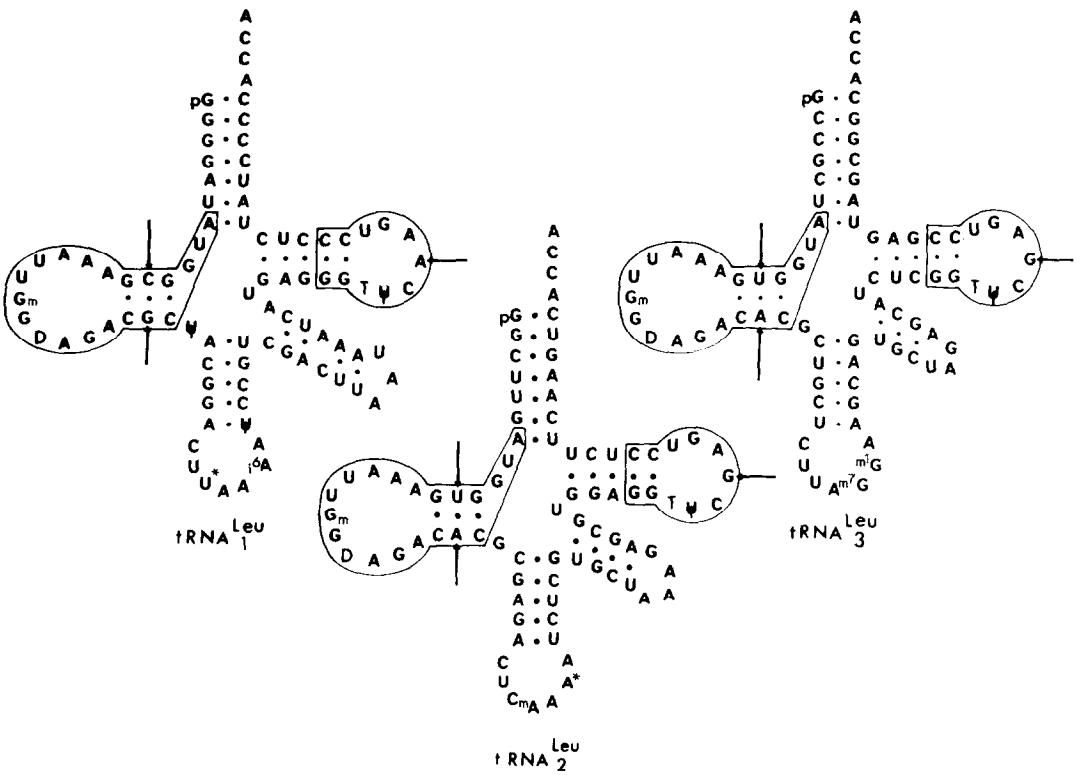

Fig. 2. Cloverleaf models of the three chloroplast tRNAs^{Leu}. Nucleotide sequences showing a large extent of homology are in boxes (arrows designate differences, within these sequences, between the three tRNAs^{Leu}).

RESULTS

The primary structures of the three chloroplast tRNAs^{Leu} are shown in cloverleaf form on figure 2. The anticodon regions of these three tRNAs^{Leu} deserve special attention.

a) tRNA$_1^{Leu}$

U* is an unknown uridylic acid derivative, resistant to pancreatic RNAase and to alkali. It could therefore correspond to a 2'-O-methyl derivative. Figure 3 shows the position of this 5' $|^{32}P|$ nucleotide (black spot) after analysis as described in "Material and Methods" (2c). The position of pU* on this chromatograph does not correspond to any known nucleotide. The modified A on the 3' side of the anticodon is either isopentenyl adenine (i^6A) or zeatin (io^6A).

b) tRNA$_2^{Leu}$

After analysis as described in "Material and Methods" (2c), the first nucleotide of the anticodon has a position upon thin layer chromatography (Fig. 3) which corresponds to a 2'-O-methyl cytidine-5'-monophosphate (pCm). Adjacent

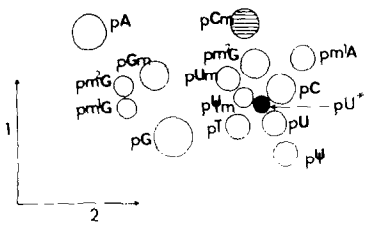

Fig. 3. Composite drawing of the two-dimensional chromatographs of 5'$|^{32}P|$ nucleotides present at the 5' end of the anticodon of $tRNA_1^{Leu}$ (black spot) and $tRNA_2^{Leu}$ (hatched spot). After partial hydrolysis of $tRNA_1^{Leu}$ or $tRNA_2^{Leu}$, the oligonucleotides were labeled at their 5' end with $|^{32}P|$, fractionated by electrophoresis on 20% polyacrylamide gel, extracted from the gel and hydrolyzed by P_1 nuclease. The unlabeled nucleoside 5'-monophosphate markers were obtained after P_1 nuclease digestion, followed by snake venom phosphodiesterase digestion, of mammalian $tRNA^{Phe}$ and $tRNA^{Trp}$ (20, 21). Solvent 1 was isobutyric acid - 25% ammonium - H_2O (66:1:33) and solvent 2 was 0.1 M sodium phosphate pH 6.8 : $(NH_3)_2SO_4$: n-propanol 100:60 g : 2.

to the 3' side of the anticodon, there is a modified A which probably corresponds to i^6A or ms^2i^6A (or their hydroxyl-derivatives : zeatin or ms^2 zeatin).

c) $tRNA_3^{Leu}$

The anticodon of $tRNA_3^{Leu}$ is U-A-m^7G, followed on the 3' side by m^1G. The nature of these minor nucleotides and their position in the $tRNA_3^{Leu}$ have been determined as follows : (i) the nucleotide analysis (12) of the T_1 oligonucleotide containing the anticodon showed upon thin-layer chromatography (see legend of figure 3) two minor nucleotides : pm^7G and pm^1G ; (ii) the "wandering spot technique" (see Material and Methods, 2b) applied to the above-mentioned 5'$|^{32}P|$ T_1 oligonucleotide gave the following sequence : $|^{32}P|$C-U-C-U-U-A-m^7G-m^1G-A-A-G (Fig. 4) ; (iii) the position of the above-mentioned T_1 oligonucleotide in $tRNA_3^{Leu}$ was confirmed using the technique described in reference 19 and the results are shown on figure 5. Oligonucleotides, generated by partial hydrolysis of $tRNA_3^{Leu}$, were labeled at their 5' end and fractionated by 20% polyacrylamide gel electrophoresis. The arrows indicate the fragments which are between 43 and 53 nucleotides long. P_1 nuclease digestion of these fragments, liberating the 5'$|^{32}P|$ nucleotide, has allowed the identification of the 5' terminal nucleotide of each fragment, as indicated on the figure. It thus became clear that these fragments contained the anticodon region and that the third nucleotide of $tRNA_3^{Leu}$ anticodon is m^7G which is followed, on the 3' side, by m^1G.

DISCUSSION

The bean chloroplast $tRNAs^{Leu}$ differ from each other in their length : $tRNA_1^{Leu}$ has 88 nucleotides, $tRNA_2^{Leu}$ 85 nucleotides and $tRNA_3^{Leu}$ 83 nucleotides. These differences are due to variations in the length of the extra-loop (Fig. 2).

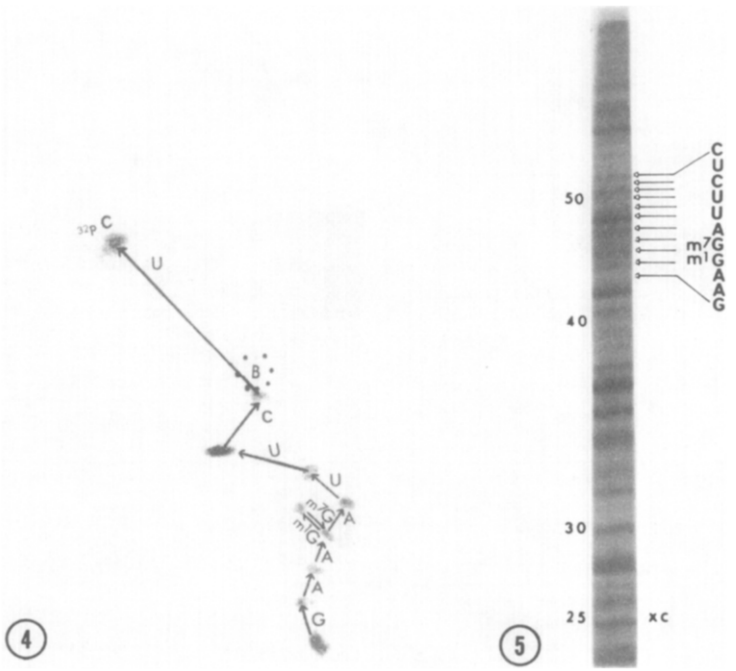

Fig. 4. Analysis by partial P_1 nuclease digestion of the 5'$|^{32}P|$ T_1 oligonucleotide containing the anticodon of $tRNA_3^{Leu}$ (see Material and Methods, 2b). Separation in the first dimension was achieved by electrophoresis on cellulose acetate at pH 3.5 and in the second dimension by homochromatography in 25 mM KOH-strenght homomix (17). Each radioactive spot corresponds to a 5'$|^{32}P|$ oligonucleotide different from the preceding one by the loss of its 3' nucleotide which is indicated on the figure. Spot B shows the position of the xylene cyanol blue dye marker.

Fig. 5. Analysis of the anticodon region of $tRNA_3^{Leu}$. After partial hydrolysis (19) of $tRNA_3^{Leu}$, the oligonucleotides were labeled with $|^{32}P|$ at their 5' end, separated on a 20% polyacrylamide gel according to their size, and visualized by autoradiography. Each oligonucleotide was then extracted from the gel, hydrolyzed by P_1 nuclease, and its 5'$|^{32}P|$ nucleotide was identified by thin-layer chromatography. The xylene cyanol marker (XC) migrates with fragments about 25 nucleotides long.

In these three $tRNAs^{Leu}$ this loop is large (10, 12 and 15 nucleotides respectively), as in all $tRNAs^{Leu}$ sequenced so far (for a compilation see ref. 22). The three $tRNAs^{Leu}$ have quite different nucleotide sequences and as only two limited regions (see Fig. 2) of these three tRNAs show a high degree of homology, there must be (at least) three different $tRNA^{Leu}$ genes in bean chloroplast DNA.

The hU region of these three $tRNAs^{Leu}$ is almost identical and ressembles that of *E.coli* and phage T_4 $tRNAs^{Leu}$ (presence of a nucleotide at position 17 and lack of nucleotide at position 20:2), whereas it differs from that of yeast $tRNAs^{Leu}$ (see ref. 22), illustrating a similarity between chloroplast and procaryotic tRNAs already observed in the case of *Euglena* and bean chloroplast

$tRNAs^{Phe}$ (6, 7). This structural analogy between $tRNAs^{Leu}$ from *E.coli* and bean chloroplasts could account for the cross aminoacylation reactions observed between chloroplast $tRNAs^{Leu}$ and *E.coli* leucyl-tRNA synthetase and between *E.coli* $tRNAs^{Leu}$ and chloroplast leucyl-tRNA synthetase (3).

The anticodons are U^{*}-A-A and Cm-A-A for chloroplast $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ respectively. The $tRNA_3^{Leu}$ anticodon, U-A-m^7G, is rather unusual since it is the first case in which a m^7G is found in the third position of an anticodon. Furthermore, m^7G has so far been found only in the variable loop.

Of the six leucine codons, four can be recognized by these three chloroplast $tRNAs^{Leu}$, according to the wobble hypothesis (24) : U^{*}-A-A translates U-U-A and U-U-G ; Cm-A-A translates U-U-G;U-A-m^7G translates C-U-A and C-U-G. An additional $tRNA^{Leu}$ with a G-A-G anticodon would be necessary to translate C-U-U and C-U-C. Such a $tRNA^{Leu}_{GAG}$ has been found in *E.coli* K 12 (25) but has not been found in bean chloroplasts. Several hypotheses are possible : (i) a $tRNA^{Leu}_{GAG}$ exists in bean chloroplasts, but it is a minor species and has not yet been detected ; (ii) the C-U-C and C-U-U codewords are not used in chloroplast mRNAs ; (iii) U-A-m^7G can translate C-U-C and C-U-U. This third possibility cannot be ruled out, since a yeast $tRNA^{Leu}_{UAG}$ has been found to be able to translate all six leucine codons including C-U-C and C-U-U (25).

Both $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ contain cytokinin derivatives, i^6A or ms^2i^6A (or their hydroxyl derivatives zeatin and ms^2 zeatin) next to their anticodon. Chloroplast $tRNAs^{Phe}$ from *Euglena* (6) and *Phaseolus vulgaris* (7) also have ms^2i^6A at this position. These $tRNAs^{Leu}$ and $tRNAs^{Phe}$ read codons beginning with U, which is in agreement with McCloskey and Nishimura (26) who suggest that tRNAs which recognize codons starting with U almost always contain hydrophobic modified nucleotides adjacent to the anticodon (on the 3' side). Cytokinin activity has previously been shown to be associated with $tRNAs^{Leu}$ from peas (27) and wheat germ (28). Furthermore it was shown that of the six leucine isoaccepting tRNAs found in soybean cotyledons only two, which elute late on RPC-2 column, contain a cytokinin and recognize specifically codons beginning with U (29).

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