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The nucleotide sequences of the initiator transfer RNAs from bean cytoplasm and chloroplasts

Jean Canaday, Pierre Guillemaut and Jacques-Henry Weil

Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue Descartes, 67084 Strasbourg Cedex, France

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ABSTRACT

The initiator tRNAs^{Met} from the cytoplasm and chloroplasts of Phaseolus vulgaris have been purified and sequenced.

The sequence of bean cytoplasmic initiator tRNA$_i^{Met}$ is : pA-U-C-A-G-A-G-U-m^{1}G-m^{2}G-C-G-C-A-G-C-G-G-A-A-G-C-G-U-m^{2}G-G-U-G-G-G-C-C-C-A-U-t^{6}A-C-C-C-A-C-A-G-m^{7}G-D-m^{5}C-C-C-A-G-G-A-ψ-C-G-m^{1}A-A-A-C-C-U-Gm-G-C-U-C-U-G-A-U-A-C-C-A$_{OH}$. The sequence of bean cytoplasmic tRNA$_i^{Met}$ is almost identical to that of wheat germ and shows a high degree of homology with other cytoplasmic initiator tRNAs.

The sequence of bean chloroplast initiator tRNA$_f^{Met}$ is : pC-G-C-G-G-A-G-U-A-G-A-G-C-A-A-C-U-U-Gm-G-D-A-G-C-U-C-G-C-A-A-G-G-C-U-C-A-U-A-A-C-C-U-U-G-A-A-m^{7}G-acp^{3}U-U-A-C-G-G-G-T-ψ-C-A-A-A-U-C-C-C-G-U-C-U-C-C-G-C-A-A-C-C-A$_{OH}$. Bean chloroplast initiator tRNA$_f^{Met}$ sequence shows procaryotic characteristics at the 5' end of the acceptor stem and in the TψC loop, but also contains some distinctive features.

INTRODUCTION

Chloroplasts and mitochondria contain their own DNA genome and a complete apparatus for protein synthesis, with ribosomes, tRNAs and aminoacyl-tRNA synthetases. Components of both organellar protein synthesis systems can be distinguished from their cytoplasmic counterparts (1,2). The mechanism of initiation of protein synthesis in organelles has been compared to that in procaryotes, since in both systems, formylated initiator tRNA$_f^{Met}$ is bound to the 30S ribosomal subunit, while in eucaryotic cytoplasm, unformylated initiator tRNA$_i^{Met}$ is bound to the 40S subunit. In all cases, the tRNA^{Met} used in initiation is distinguishable from the tRNA$_m^{Met}$ used in elongation. The elongating tRNAs^{Met} and the initiating species have been characterized in *Phaseolus vulgaris* chloroplasts, mitochondria and cytoplasm (3,4,5,6). The organelle initiator tRNAs, like most procaryotic initiators, were shown to be formylated, while the cytoplasmic initiator tRNA$_i^{Met}$

is not (3,6).

Nucleotide sequences of initiator tRNAs from a variety of procaryotic and eucaryotic sources have already been determined (7,8,9,10). Several distinctive features have emerged from comparison of these sequences : i) In loop IV the T-ψ-C-G sequence, found in most elongating tRNAs, is replaced by T-ψ-C-A in procaryotic initiator tRNAs and by A-U(or ψ)-C-G in eucaryotic cytoplasmic initiator tRNAs ; ii) Procaryotic initiator species lack a Watson-Crick base pair at the 5' end of the acceptor stem, while this pair is present in eucaryotic cytoplasmic initiator tRNAs ; iii) The pyrimidine-purine base pair (Y11-R24) found in the D stem in the standard cloverleaf is replaced by an A-U pair in prokaryotic initiator tRNAs.

In view of the similarities found between protein synthesis in chloroplasts and procaryotes, we thought it would be interesting to determine whether chloroplast $tRNA_f^{Met}$ has the same distinctive features as procaryotic initiator tRNAs. No chloroplast initiator tRNA sequence has yet been published and it should be noted that only very few chloroplast tRNAs have been sequenced so far (11,12,13). We could expect that bean cytoplasmic $tRNA_i^{Met}$ would resemble eucaryotic cytoplasmic initiator tRNAs, since a high degree of homology (73-100%) is observed between the cytoplasmic $tRNA_i^{Met}$ sequences determined so far. Among the $tRNA_i^{Met}$ sequences compared, there is only one plant cytoplasmic initiator, that of wheat germ (7). While animal cytoplasmic $tRNAs_i^{Met}$ can be formylated *in vitro* using the *E.coli* formyl transferase, this is not the case for plant cytoplasmic initiators, which might therefore have some special structural features. We have determined the sequence of bean chloroplast initiator tRNA (chl $tRNA_f^{Met}$) and cytoplasmic initiator tRNA (cyt $tRNA_i^{Met}$) to gain an insight into the relationship between structure and the specialized functions of initiator tRNAs.

MATERIAL AND METHODS

Purification of bean chloroplast $tRNA_f^{Met}$ and cytoplasmic $tRNA_i^{Met}$

Crude tRNA was extracted from frozen bean leaves using the procedure of Roe (14). Fractionation on a Sepharose 4B column (15) separates cyt $tRNA_i^{Met}$ and chl $tRNA_f^{Met}$. Cyt $tRNA_i^{Met}$,identified

by aminoacylation with *E.coli* and bean cytoplasm enzyme extracts, was further purified by BD-cellulose chromatography at pH 4.5 in 1 mM EDTA using a linear gradient from 0.3 M to 0.9 M NaCl. Chl $tRNA_f^{Met}$, detected by aminoacylation and formylation of Sepharose 4B fractions was purified by two successive BD-cellulose chromatographies at pH 4.5 using a linear gradient 0.45 M NaCl 0% ethanol to 1.5 M NaCl 20% ethanol. After the first BD-cellulose column, chl $tRNA_f^{Met}$,detected by aminoacylation,was charged and formylated preparatively as described (6,16) using a crude *E.coli* enzyme extract, methionine and [^{14}C]-formyltetrahydrofolate prepared as described (17) and provided by Dr. R. Martin. Since formylation of methionyl $tRNA_f^{Met}$ changes its chromatographic properties on BD-cellulose (18), chl fMet $tRNA_f^{Met}$ is separated from all other tRNA species by a second passage on the same BD-cellulose column. When necessary, two-dimensional polyacrylamide gel electrophoresis done according to Fradin *et al.* (19) was used as a final purification step.

Sequencing techniques

Analysis of RNAase T_1 and RNAase A digestion products : Cyt $tRNA_i^{Met}$ and chl $tRNA_f^{Met}$ were digested completely with RNAase T_1 or RNAase A. The resulting oligonucleotides were labeled with γ-[^{32}P]-ATP (Amersham) and T_4 polynucleotide kinase as described (20,21). T_4 polynucleotide kinase was donated by Dr. G. Keith or purchased from P.L. Biochemicals. The labeled oligonucleotides were fractionated on DEAE-paper or by two-dimensional homochromatography (22). Individual oligonucleotides were partially digested with nuclease P_1 and the [^{32}P]-labeled products were analyzed by two-dimensional homochromatography (23).

5' terminal [^{32}P]-labeling of intact tRNA : Intact tRNA was dephosphorylated by incubation in 20 mM Tris-HCl pH 8, 0.1 mM $ZnCl_2$ with 10^{-2} units calf intestine alkaline phosphatase (Boehringer) per µg tRNA for 20 min. Optimum reaction temperature was found to be 62°C for chl $tRNA_f^{Met}$ and 55°C for cyt $tRNA_i^{Met}$. After phosphatase inactivation, tRNA was labeled as described (23) using γ-[^{32}P]-ATP 3000 Ci/mmole (Amersham) and separated from degradation products by polyacrylamide gel electrophoresis.

Partial digestions of 5'-labeled tRNA : Partial hydrolysis, using RNAase T_1, RNAase U_2 or RNAase A, was done in buffer I (20 mM

sodium citrate pH 5, 1 mM EDTA, 7 M urea, 0.025% xylene cyanol and bromophenol blue (24). RNAase Phy I, donated by Dr. Bargetzi, was used in 10 µl buffer II (10 mM sodium acetate pH 5, 50 mM EDTA). Digestion at 50°C was stopped after 5' or 20' by addition of 10 µl buffer I. For each enzyme, two different digestions (10 µl each) were combined. Nuclease/RNA ratios are given in Fig. 2. Non-specific partial hydrolysis (see the "ladder" in Fig. 2) was done by incubating 5'-[^{32}P]-labeled tRNA and 5 µg yeast carrier tRNA (Boehringer) in 10 µl bidistilled water in a sealed capillary at 100°C for 10 or 20'. The two digests were combined and 20 µl buffer I added before loading on the polyacrylamide slab gel.

Fractionation of partial digests of 5'-[^{32}P]-labeled tRNA

Thin (0.5 mm) polyacrylamide slab gels (90 cm x 30 cm or 40 cm x 30 cm) described by Sanger and Coulson (25) were used to fractionate the digests produced by enzymatic and non-specific partial hydrolyses (read-off sequencing gels). In addition, the "ladder" hydrolysate was fractionated by two-dimensional polyacrylamide gel electrophoresis (2-dim. gel) (26,27), using thin (0.5 mm) slab gels in both dimensions.

Analysis of modified nucleotides

The technique described by Stanley and Vassilenko (28) was used with only a few minor modifications. Partial hydrolysis of tRNA (2-5 µg) was done in bidistilled water at 80°C for 3 min. After 5'-[^{32}P]-labeling, the fragments were fractionated on thin polyacrylamide gels (25). Each fragment was completely digested with nuclease P_1 and the 5' terminal nucleotide was identified by thin layer chromatography on cellulose plates.

RESULTS

Sequence analysis of bean chloroplast initiator $tRNA_f^{Met}$

The data obtained from read-off sequencing gels, from partial nuclease P_1 digestions and by the Stanley and Vassilenko technique, were used to determine the $tRNA_f^{Met}$ sequence. The oligonucleotides present in both total RNAase T_1 digests and RNAase A digests were aligned unambiguously by read-off sequencing gels (Results summarized in Fig. 1). Fig. 2 shows autoradiograms of partial hydrolysates of 5'-[^{32}P]-labeled chl $tRNA_f^{Met}$ separated on

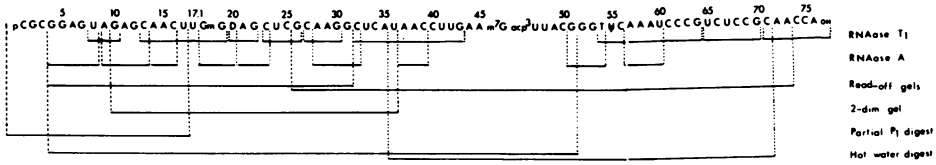

Fig. 1. Bean chloroplast $tRNA_f^{Met}$: Summary of sequence results obtained by different methods.

polyacrylamide gels. Numbers correspond to the positions in the final sequence indicated in Fig. 4. RNAase Phy I digestion (-C track in Fig. 2) was used to differentiate between pyrimidine residues since it cleaves more slowly after C than after U. Additional information was obtained from two-dimensional polyacrylamide gel separation (2 dim. gel in Fig. 1) of a "ladder" hydrolysate on which U (and G) can be distinguished from C (and A).

The technique of Stanley and Vassilenko was used to determine residues 4 to 71 (Hot water digest in Fig. 1). The results obtained confirmed data from read-off sequencing gels and from analysis of RNAase T_1 and RNAase A oligonucleotides and they provided a necessary complement by localizing modified nucleotides in the sequence. Modified nucleotides present in chl $tRNA_f^{Met}$ were determined by analysis of a complete RNAase T_1+T_2 digest according to (21). While both m^7G and acp^3U were poorly labeled by this technique, they were clearly present at the 5' ends of fragments corresponding to positions 46 and 47 obtained using the Stanley-Vassilenko method. The complete sequence of bean chl $tRNA_f^{Met}$ is shown in Fig. 4.

Sequence analysis of bean cytoplasmic initiator $tRNA_i^{Met}$

The sequence of cyt $tRNA_i^{Met}$ was determined using the same techniques described above for chl $tRNA_f^{Met}$ (results summarized in Fig. 3). Since the cytoplasmic initiator $tRNA_i^{Met}$ contains a number of other modified residues resistant to partial enzymatic cleavage, less information was obtained from sequencing gels than in the case of chl $tRNA_f^{Met}$. The data obtained from analysis of RNAase T_1 and RNAase A oligonucleotides were helpful in assigning positions to modified nucleotides, but most of the information was obtained using the technique of Stanley and Vassilenko (28).

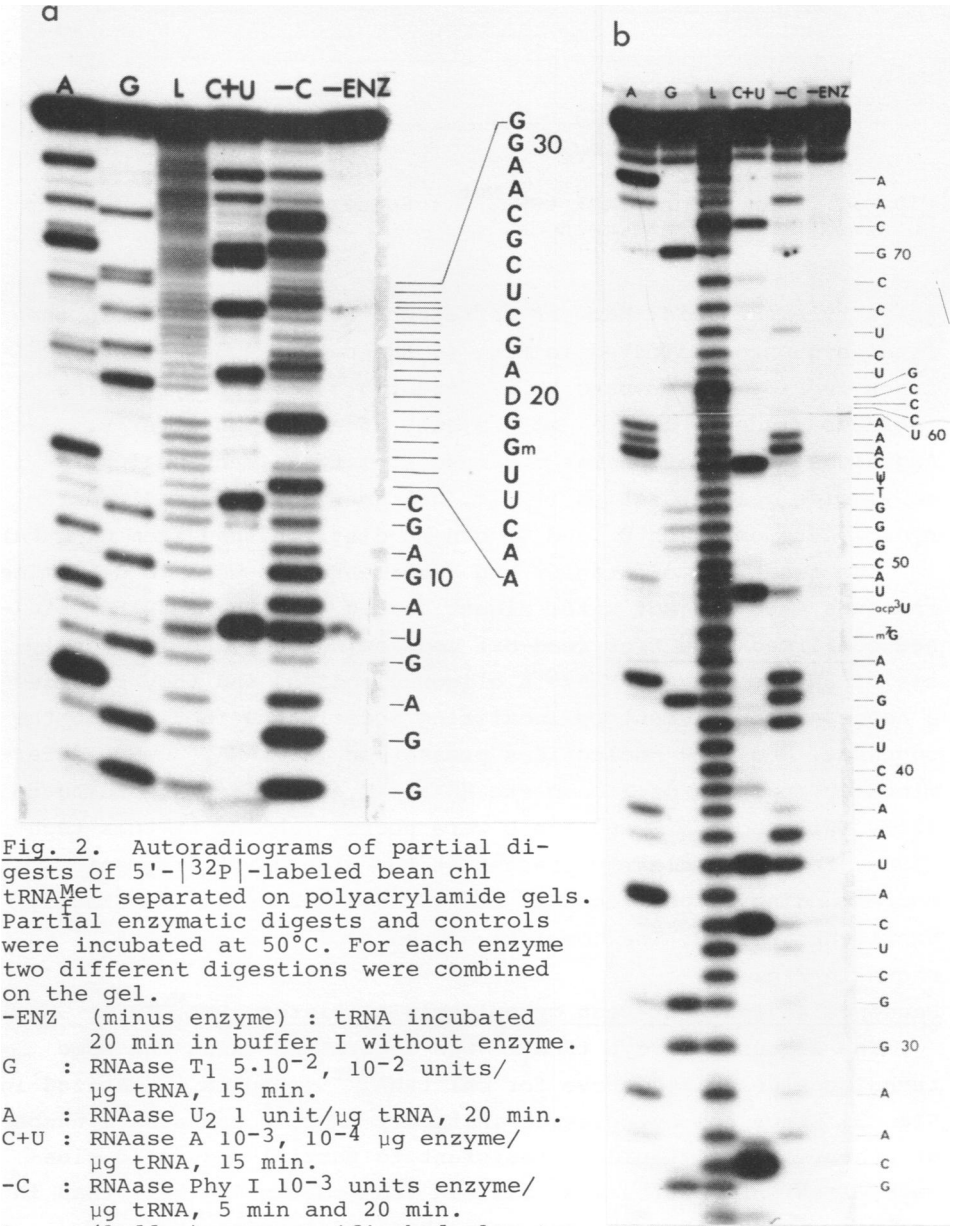

Fig. 2. Autoradiograms of partial digests of 5'-|^{32}P|-labeled bean chl $tRNA_f^{Met}$ separated on polyacrylamide gels. Partial enzymatic digests and controls were incubated at 50°C. For each enzyme two different digestions were combined on the gel.

-ENZ (minus enzyme) : tRNA incubated 20 min in buffer I without enzyme.
G : RNAase T_1 5.10^{-2}, 10^{-2} units/µg tRNA, 15 min.
A : RNAase U_2 1 unit/µg tRNA, 20 min.
C+U : RNAase A 10^{-3}, 10^{-4} µg enzyme/µg tRNA, 15 min.
-C : RNAase Phy I 10^{-3} units enzyme/µg tRNA, 5 min and 20 min.
L : (ladder) non-specific hydrolysate, 5 µg tRNA done at 100°C in bidistilled water for 10 min and 20 min.

3 a : 20% gel (400 x 300 x 0.5 mm), 1500 Volts, 3 h.
3 b : 15% gel (900 x 300 x 0.5 mm), 1200 Volts, 72 h.

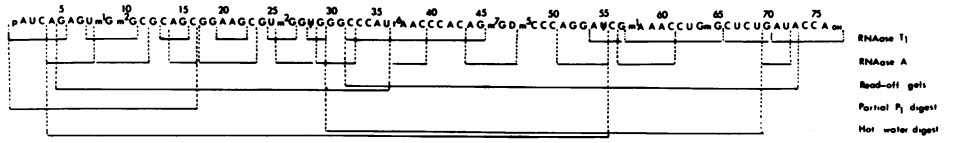

Fig. 3. Bean cytoplasmic $tRNA_i^{Met}$: Summary of sequence results obtained by different methods.

DISCUSSION

Bean chloroplast initiator $tRNA_f^{Met}$ contains the following modified nucleotides : Gm, D, m^7G, acp^3U, T and ψ. The only unusual modification is acp^3U 47 which has not been found previously in initiator tRNAs, but is found in position 47 in bean chl $tRNA^{Phe}$ (12) and in a number of *E.coli* tRNAs (29). Bean cyt $tRNA_i^{Met}$ contains m^1G, m^2G, t^6A, m^7G, m^5C, ψ, m^1A and Gm. Aside from ψ55 and Gm64, these modified nucleotides are found in the same positions in other eucaryotic initiator tRNAs. It should be

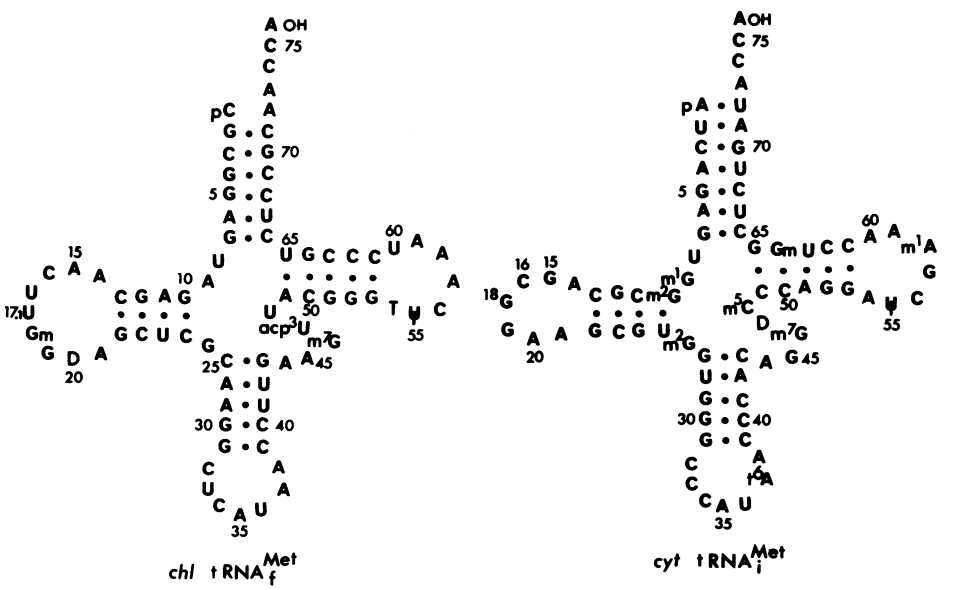

Fig. 4. Nucleotide sequences of bean chloroplast and cytoplasmic initiator tRNAs, numbered according to (7).

pointed out that m^1G, m^2G, t^6A, and m^5C are not found in procaryotic initiator tRNAs. Concerning Gm64, unidentified guanosine derivatives have been found previously in initiator tRNAs from wheat germ and *N.crassa* cytoplasm in position 65 (7) and in *Torulopsis utilis* (10) and *Scenedesmus obliquus* (30) in position 64.

Bean chloroplast $tRNA_f^{Met}$ shows 73-81% sequence homology with procaryotic initiator tRNAs (7), while cyt $tRNA_i^{Met}$ shows 73-100% homology with eucaryotic cytoplasmic initiator tRNAs (7,8,9,10). Aside from differences in post-transcription modifications at positions 49, 64, and 65, bean cyt $tRNA_i^{Met}$ is identical to wheat germ initiator $tRNA_i^{Met}$. Wheat is a monocotyledon, while bean is a dicotyledon. It has been reported recently that the sequence of lupin cytoplasmic initiator tRNA is also identical to that of the wheat initiator tRNA (31) suggesting that there may be a conservation of cytoplasmic initiator tRNA sequences among plants, analogous to that observed among vertebrate initiator tRNAs.

Concerning the distinctive features of procaryotic and eucaryotic initiator tRNAs noted in the introduction, bean chloroplast $tRNA_f^{Met}$ resembles procaryotic initiator tRNAs while $tRNA_i^{Met}$ shows eucaryotic characteristics. In chl $tRNA_f^{Met}$, C1 cannot form a Watson-Crick base pair and the Y11-R24 base pair (in the D stem) of the standard cloverleaf is replaced by A-U in chl $tRNA_f^{Met}$. The loop IV sequence, T-ψ-C-A-A-U is identical to that of three prokaryotic initiator tRNAs (The fourth one, *Thermus thermophilus* $tRNA_f^{Met}$ has two additional post-transcriptional modifications at position 54 and 58). While this sequence is found in other non-initiator tRNAs, it nonetheless differentiates procaryotic initiator tRNAs from eucaryotic initiator tRNAs. As in other eucaryotic initiator tRNAs, in bean cyt $tRNA_f^{Met}$ an A-U pair is found at the 5' end of the acceptor stem, there is a C-G pair in the D stem and the loop IV sequence is A-ψ-C-G-m^1A-A-A. While the modification of U to ψ55 is also found in wheat germ and starfish initiator tRNAs, it is absent in the others.

Several other features can be noted in the two bean initiator tRNA sequences. Initiator tRNAs (from both procaryotes and eucaryotes) which can be formylated by *E.coli* formyltransferase have a number of residues in common (32). Bean cyt $tRNA_i^{Met}$, which is

not formylated by the *E.coli* enzyme, contains all these residues except G2 and C71 which are replaced by A2 and U71. This change of G-C base pair to A-U in the acceptor stem might have an influence on the recognition by *E.coli* formyltransferase. In chl $tRNA_f^{Met}$, the presence of A15 is somewhat unusual since procaryotic as well as eucaryotic initiator tRNAs have G in this position. However, the tertiary interaction with U48 is possible. In the anticodon loop, bean chl $tRNA_f^{Met}$ has U in position 33, like most elongating and initiating tRNA species. Bean cyt $tRNA_i^{Met}$, however, has C in position 33 which is rather unusual, but is also found in cytoplasmic initiator tRNAs from wheat germ, *Drosophila*, and vertebrates (7,8). The anticodon stem of the bean chloroplast initiator tRNA differs from that of procaryotic initiator tRNAs. There are two G-C pairs next to the anticodon loop, while there are three G-C pairs in procaryotic as well as eucaryotic cytoplasmic initiator tRNAs (including bean cyt $tRNA_i^{Met}$). It has been suggested by Wrede *et al.* (33) that these three G-C pairs might contribute to an anticodon loop conformation specific for the initiator tRNAs and different from that of the elongator tRNAs.

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