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ANOMALIES IN 5'-END [^{32}P] LABELLING OF TRANSFER RNA AND PARTIAL SEQUENCE OF A tRNA^{Glu} FROM SCENEDESMUS OBLIQUUS

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SUMMARY

A chloroplast tRNA$^{Met}_{m}$ species from Scenedesmus obliquus is very poorly 5'-end [^{32}P] labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. In sequencing the tRNA using standard 5'-labelled methods a very minor contaminating tRNA is preferentially labelled. The partial tRNA sequence determined by this method has an anticodon (CUC) for tRNA^{Glu}.

INTRODUCTION

Rapid read-off gels [1,2] are being used to elucidate the sequence of DNA and RNA fragments. The method requires the radioactive labelling of the fragments at either the 5'-[1,2] or the 3'-[3,4,5]ends. 5'-labelling of DNA and RNA is achieved using T4 polynucleotide kinase and [γ-^{32}P] ATP and has been used successfully to label many DNA fragments and different types of RNA including tRNA.

We have been elucidating the nucleotide sequence of Scenedesmus obliquus (a green alga) chloroplast tRNA$^{Met}_{m}$. The material has been purified by several chromatographic steps [6] and aminoacylation assay with methionine shows it to be greater than 95% pure. The tRNA is [^{32}P] 5'-end labelled very poorly but a contaminating tRNA estimated to be present as an approximately 3% impurity is very efficiently labelled. Consequently the molecule whose sequence was obtained by the rapid read-off gel method was that of a very minor contaminating species. This serves to illustrate a hazard of this very sensitive method of nucleotide sequencing.

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MATERIALS AND METHODS

Uniformly labelled L-[^{14}C] methionine (52 mCi/mmol), L-[^{14}C] glutamic acid (285 mCi/mmol) and [γ-^{32}P] ATP (2000-3500 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, U.K. *Escherichia coli* $tRNA_f^{Met}$ was a gift from Dr. S. Nishimura (Tokyo). Calf intestinal alkaline phosphatase and T4 polynucleotide kinase were obtained from Boehringer Mannheim (BCL, London); the latter was also obtained from PL Biochemicals and bacterial alkaline phosphatase was from Worthington Enzymes.

Isolation of *S. obliquus* tRNA [7], purification of *S. obliquus* chloroplast $tRNA_m^{Met}$ [6], isolation of crude *E. coli* [8] and *S. obliquus* [7] aminoacyl-tRNA synthetases, and the aminoacylation assay [9] have been described previously. The [5'-^{32}P] labelling was performed as a two step reaction as described by Silberklang *et al.* [3,10]. Methods of sequential analysis were performed by slight modifications of literature methods [2,3,11,12,13] as described previously [14].

RESULTS

Purified *S. obliquus* chloroplast $tRNA_m^{Met}$ was [5'-^{32}P] labelled using polynucleotide kinase and [γ-^{32}P] ATP and the products were separated by electrophoresis on a 15% polyacrylamide gel. Fig. 1 [B] shows a typical autoradiogram of the gel. The conditions for labelling were varied considerably both in the alkaline phosphatase step, (e.g. both calf intestinal phosphatase and bacterial phosphatase were used,

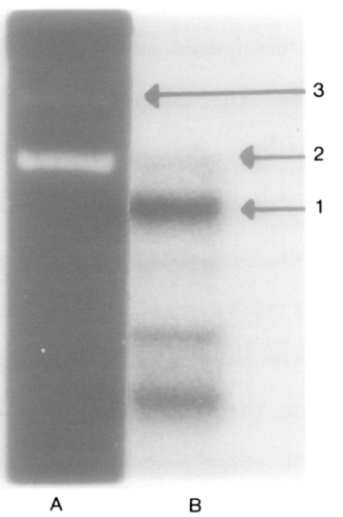

Fig. 1 Electrophoresis of [5'-^{32}P] labelled tRNA on a 15% polyacrylamide gel, 0.5 Kv for 16 hr. [A] Nucleic acid bands detected by fluorescence under UV light (254 nm) after soaking gel in ethidium bromide (10 µg/ml). [B] Autoradiogram of [^{32}P] labelled bands.

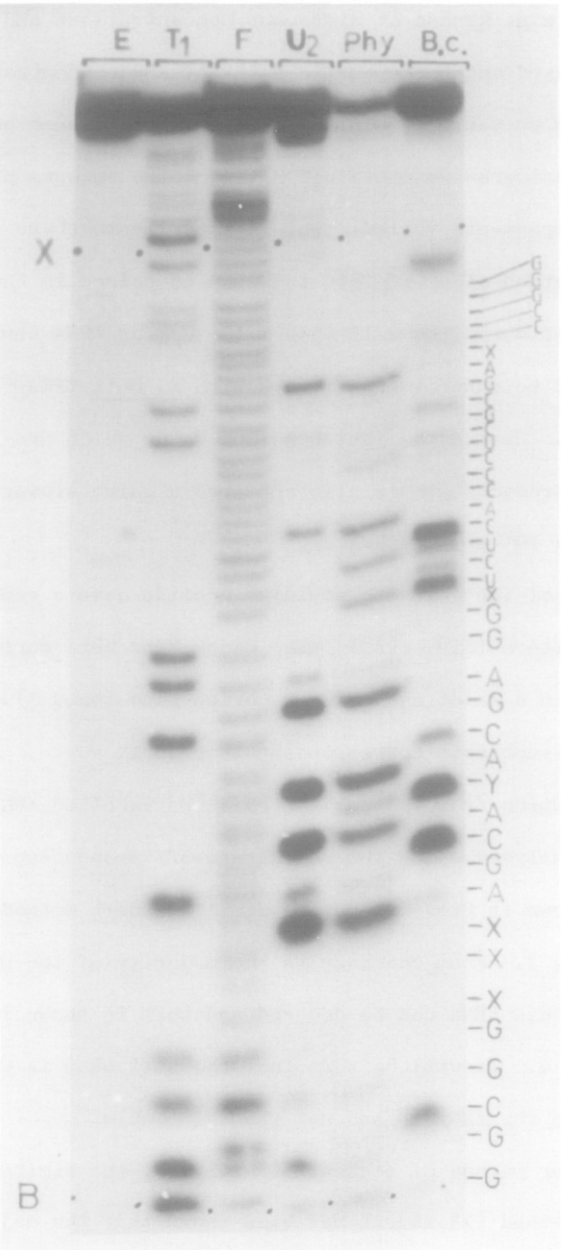

Fig. 2 Autoradiogram of partial digestions of [5'-^{32}P] labelled tRNA in band 1 (Fig. 1) fractionated by electrophoresis on a 10% polyacrylamide thin gel at 15 Kv for 2 hr. E, digestions in absence of enzymes; T_1, U_2, Phy, B.c.; digestions with RNase T_1, RNase U_2, Phy 1 RNase and B. cereus RNase respectively; F, hydrolysis with formamide. X, xylene cyanol blue dye; B, bromophenol blue dye.

and the temperature and length of incubation were varied) and the kinase step (e.g. kinase at different concentrations and from different sources was used and the specific activity and concentration of [γ-^{32}P] ATP were varied). In all experiments the same general pattern in the autoradiogram was obtained although the amounts of smaller degradation fragments varied. In all experiments there was only a low incorporation of [^{32}P] into the tRNA contained in the major band in the autoradiogram (band 1) (5.8×10^5 dpm/µg tRNA applied to the gel) compared to control experiments with E. coli $tRNA_t^{Met}$ (1.1×10^8 dpm/µg tRNA). Beside the faster moving bands which are likely to be degradation products, there also appeared a faint slower moving band (band 2) (6×10^4 dpm/µg tRNA).

Staining of the gel with ethidium bromide gave a remarkably different pattern (Fig. 1[A]). The major band here corresponds to band 2 with a faint even slower moving band (band 3). A band was hardly discernable corresponding to band 1.

After elution from the gel the [5'-^{32}P] labelled tRNA from band 1 was analysed using the rapid read-off sequencing gel method [2,11] as shown in Fig. 2, and the mobility shift method [3,12] as shown in Fig. 3. From the results the majority of the nucleotide sequence of this tRNA can be deduced and this is shown in cloverleaf form in Fig. 4. It will be seen that the anticodon is CUC, which is the anticodon for $tRNA^{Glu}$ and not $tRNA^{Met}$ (CAU).

By another method of sequential analysis, the limited hydrolysis sequencing method [13,14], it has been shown that the major species of tRNA in the purified sample has the anticodon CAU. The sequence obtained by this method is the same as that for the tRNA eluted from band 2, deduced from results using the direct read-off gel method and the mobility shift method (unpublished results, manuscript in preparation).

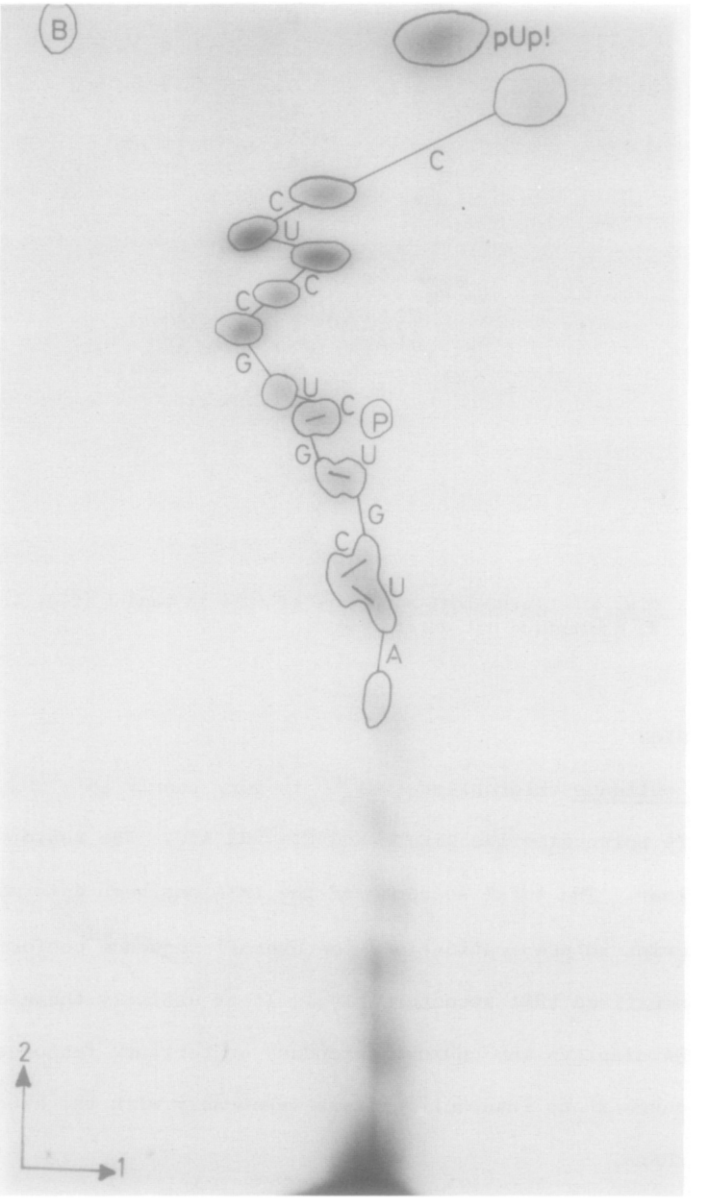

Fig. 3 Autoradiogram of [5'-^{32}P] labelled tRNA in band 1 (Fig. 1) partially digested with formamide and fractionated by two-dimensional homochromatography [3,12]. P and B; marker dyes.

Assays on the purified tRNA for methionine and glutamic acid charging, using E. coli and S. obliquus synthetase preparations, show that the tRNA is aminoacylated by methionine (1983 pmoles/E_{260} unit) only with the E. coli synthetase and by glutamic acid (63 pmoles/E_{260} unit) only with the S. obliquus synthetase.

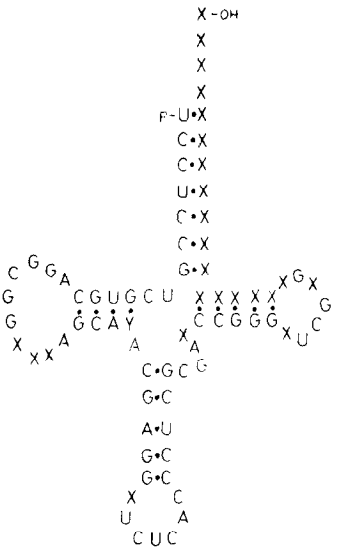

Fig. 4 Clover-leaf structure of tRNA in band 1 (Fig. 1). X, nucleotide not determined.

DISCUSSION

S. obliquus chloroplast $tRNA_m^{Met}$ is very poorly [5'-^{32}P] labelled using T4 polynucleotide kinase and [γ-^{32}P] ATP. The reason for this is unclear. The total sequence of the tRNA has been determined (manuscript in preparation) and the overall sequence conforms to the generalised tRNA structure [15]. It is unlikely therefore that the tRNA displays any unusual secondary or tertiary features which would cause it to interact any less favourably with the kinase than other tRNAs.

Because of the poor labelling of this tRNA a minor contaminating tRNA becomes the major [^{32}P] labelled species. From the amino-acylation assay and the fact that this contaminating tRNA is hardly visible on the gel with ethidium bromide staining, it is estimated that it is present as an approximately 3% impurity. Aminoacylation assays and sequential analysis of this tRNA (running as band 1 on the gel) indicate that it is a S. obliquus cytoplasmic $tRNA^{Glu}$ species.

Lillehaug and Kleppe [16] have examined the kinetic parameters for the phosphorylation of several tRNA species and show that these tRNAs (including E. coli $tRNA_f^{Met}$) can be completely phosphorylated. Even under conditions which they suggest are necessary for complete phosphorylation of tRNA (a large excess of ATP over the concentration of free 5'-OH groups) we have found that not all tRNA species are efficiently phosphorylated. This can lead to misleading results when tRNAs are [5'-^{32}P] labelled for detection and sequential analysis of very small quantities of material.

ACKNOWLEDGMENT

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