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TRANSFER RIBONUCLEIC ACID FROM *SCENEDESMUS OBLIQUUS* D3: GENERAL PROPERTIES

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Key Word Index—*Scenedesmus obliquus* D3; Chlorophyceae; *t*RNA; isolation; properties.

Abstract—Transfer RNA has been isolated from the green alga *Scenedesmus obliquus* by phenol extraction at 60°. The proportions of major bases and some of the minor components in the mixed *t*RNA were determined. Optimum conditions for the charging of the *t*RNA with methionine are pH 7.8 and 4 mM Mg^{2+}. The *t*RNA is efficiently charged using aminoacyl *t*RNA synthetases from species of higher organisms, but less efficiently by synthetases from prokaryotes. Some species of *t*RNA have an unmodified uridine at position 23 from the 3′ end of the molecule.

INTRODUCTION

Many species of transfer ribonucleic acid (*t*RNA) from different classes of organism of the protista, plant and animal kingdoms have been isolated and studied. The primary sequence of about 95 of these *t*RNA species is now known [1]. However, algal *t*RNA has been largely neglected and except for *Euglena gracilis* *t*RNA [2] no sequences of algal *t*RNAs are known and little has been reported concerning their general properties.

The genetic code has been inert in the process of evolution but *t*RNA, the molecule involved in the interpretation of the code, has been subject to change. Variations both in nucleotide sequence and modification of the bases are seen in amino acid specific *t*RNAs from different sources [3]. In particular, differences have been noted between the *t*RNAs from eukaryotic and prokaryotic sources and in some instances these differences can be related to the differences in mechanism of protein biosynthesis in the eukaryote and prokaryote systems, in particular in the process of initiation [4].

We have commenced a study on the *t*RNA from the green alga, *Scenedesmus obliquus*, which is a simple unicellular eukaryote, having a defined nucleus, mitochondria, and a chloroplast which is large and in some stages of growth occupies most of the cell. The aims of our study are to compare this *t*RNA with *t*RNAs from other sources and in particular to compare the *t*RNAs of chloroplastic and cytoplastic origin with those of prokaryotes and other eukaryotes respectively. Here we report our initial studies on the isolation and general properties of *S. obliquus* D3 *t*RNA.

RESULTS AND DISCUSSION

Isolation of tRNA

S. obliquus has a remarkably tough cell wall which means that the milder methods used for the isolation of *t*RNA from other sources, e.g. *Escherichia coli* [5] or yeast [6] are unsuitable. *t*RNA can be extracted from *S. obliquus* either by treatment of a cell homogenate with PhOH at room temp. or by prolonged treatment of whole cells at 60°. Table 1 gives the results obtained when the *t*RNA was extracted by these methods. The amount of *t*RNA and its purity are estimated by measurement of the methionine-accepting *t*RNA per g of cells and per A_{260} unit of isolated nucleic acid respectively. It will be seen that the amount of *t*RNA obtained by treatment of whole cells harvested in the mid-exponential stage of growth with PhOH at 60° for 1.5 hr is just slightly less than that obtained by PhOH treatment of the cell homogenate, but the former method yields purer material (with respect to $tRNA^{Met}$). This is presumably because the homogenization requires drastic mechanical forces to break the tough cell wall, and this causes high MW nuclear material to be sheared and isolated in the crude *t*RNA fraction. Neither increasing the time of treatment of whole cells with PhOH at 60° beyond 1.5 hr nor incorporation of detergent (SDS) into the extraction buffer improved the yield of *t*RNA. In order to reduce oxidation of PhOH during the long extraction at 60°, 0.2% 8-hydroxyquinoline was included [7, 8]. Treatment of whole cells harvested at the mid-exponential stage of growth with PhOH under milder conditions (1 hr at 22°) did not release the *t*RNA.

*t*RNA could not be extracted from whole cells which were harvested in the late exponential or stationary phase with the harsher PhOH treatment (60°, 1.5 hr). Normal amounts of *t*RNA are certainly present in cells harvested in the late-exponential or stationary phase since a similar amount of *t*RNA to that obtained from mid-exponential phase can be obtained by PhOH treatment of the cell homogenate of stationary phase cells. Presumably the cell wall toughens as the cell ages and becomes more resistant to PhOH treatment.

Base analysis

The base analysis of mixed *t*RNA isolated by the above procedure was determined according to the method described by Randerath *et al.* [9]. The method involves

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Table 1. Phenol extraction of *t*RNA from *Scenedesmus obliquus*

Material extracted	Conditions	$tRNA^{Met}$ extracted (pmol/g cell)	Methionine-accepting activity (pmol/A_{260})
Homogenate	23°, 10 min	187	2.6
Whole cells	22°, 1 hr	<10	—
(mid-exponential)	60°, 1.5 hr	148	14.0
	60°, 2.5 hr	138	11.6
Whole cells (late-exponential to stationary)	60°, 1.5 hr	18	0.8

The phenol extraction, isolation of *t*RNA and methionine-accepting assay were performed as described in the text.

enzymatic degradation of the *t*RNA to nucleosides, oxidation of the ribose rings to the dialdehyde with periodate and reduction with $NaBH_4$-[^{3}H] to give the [^{3}H]-labelled triols. The triols are then separated by 2D-TLC on cellulose and detected by fluorography. Quantitative estimation is made by cutting the triol spots from the cellulose sheets and measuring their radioactivity in the scintillation counter. The proportions of the major bases in *S. obliquus* mixed *t*RNA (A, 24.5%; C, 21.1%; G, 27.8% and U, 18.1%) and some of the minor components, identified by their position on the 2D chromatogram (m^1A, 0.3%; m^6A, 0.5%; m^5C, 0.6%; m^1G, 0.4%; m^7G, 0.4%; m_2^2G, 0.4%; D, 1.7%; m^5U, 0.5%; ψ, 1.4%), were determined alongside those for *E.coli* $tRNA_f^{Met}$. The experimental base analysis of *E. coli* $tRNA_f^{Met}$ agreed with the theoretical [10].

Similar to *t*RNA from other sources, *t*RNA from *S. obliquus* contains a number of methylated nucleosides in small amounts. Of particular interest is 5-methyluridine (m^5U), also called ribothymidine (T). This nucleoside occurs uniquely in the sequence GTψC of loop IV [2], a sequence common to most *t*RNAs. Exceptions to this are the eukaryote initiator *t*RNAs [11, 12] and the $tRNA^{Gly}$ involved in cell wall synthesis in *Staphylococci epidermides* [13]. Also it is reported [14] that certain species of wheat germ *t*RNA have an unmodified U in place of T in the GTψC sequence. Comparison of the compositions of m^5U in the mixed *t*RNAs from several sources shows that the value for *t*RNAs from higher organisms is lower than that found in yeast and several bacteria [15].

The value obtained here for *S. obliquus* *t*RNA is close to that of wheat germ and other plants. These results prompted further studies on the nature of the nucleosides occupying position 23 from the 3′ end of the *S. obliquus* *t*RNA, which represents the T position in the GTψC sequence.

Methylation of tRNA

Marcu *et al.* [14] have described a method for the specific methylation of an unmodified uridine residue at position 23 from the 3′ end of *t*RNA to form ribothymidine. The method is therefore a measure of the amount of unmodified uridine at this position in the mixed *t*RNA.

The method uses a crude *E. coli* supernatant as the source of methylase and *S*-adenosylmethionine as the Me donor. In our hands, saturating conditions for the methylation of *t*RNA were at an enzyme concentration of 0.12 mg protein/0.25 ml incubation mixture and for 6.5–6.6 hr incubation time. These conditions were used for the methylation of *t*RNA from wheat germ. *E. coli* and *S. obliquus*, where 0.1, 2.0 and 4.6 pmol of [^{14}C]-labelled Me group per pmol $tRNA^{Met}$ respectively was transferred to the respective crude *t*RNA preparations. Making the assumption that *ca* 5% of mixed *t*RNA is $tRNA^{Met}$, the results show that 0.6% of *E. coli* *t*RNA, 10% of *S. obliquus* *t*RNA and 20% of wheat germ *t*RNA is unmodified at position 23 from 3 OH end. The results found with *E. coli* and wheat germ *t*RNA agree with those of Marcu *et al.* [14] who state that *E. coli* and yeast *t*RNAs are not substrates for the *E. coli* uracil methylase, whereas in wheat germ there are at least 5 individual species of *t*RNA in which the uridine is unmodified. From their results Marcu *et al.* propose that this class of *t*RNA lacking ribothymidine and having uridine in its place may be limited to higher organisms. The results presented here with *S. obliquus* where there is an appreciable amount of unmodified uridine but less than in wheat germ, suggests that the algal *t*RNAs are to be classified with those of higher organisms.

If the unmodified uridine-*t*RNA of *S. obliquus* represents specific *t*RNA species, as found in wheat germ *t*RNA, specific peaks of methylatable *t*RNA should be obtained on fractionation of the crude *t*RNA. On the other hand if the methylation observed represents general under-methylation owing to incomplete maturation of the *t*RNA at the time of harvesting of the cells, a diffuse pattern of methylation should be observed on fractionation of the *t*RNA. Fig. 1 shows the distribution of methylatable *t*RNA when crude *S. obliquus* *t*RNA was fractionated on a BD-cellulose column [16]. It will be seen that there are at least 3 and possibly 4 discrete peaks of methylatable *t*RNA. *Ca* 80% of the Me group acceptor activity was found in these peaks. Therefore it would appear that the *in vitro* methylation of *S. obliquus* *t*RNA is due to there being specific species of *t*RNA having an unmodified uridine residue at position 23 from the 3′ end.

S. obliquus *aminoacyl-tRNA synthetase*

The preparation was a crude post-ribosomal supernatant fraction which was treated with streptomycin sulphate or protamine sulphate to remove contaminating nucleic acids. Fig. 2 shows the extent of charging crude *S. obliquus* *t*RNA with methionine-[^{14}C] using increasing amounts of the *S. obliquus* synthetase preparation. It will be seen that preparations treated with prota-

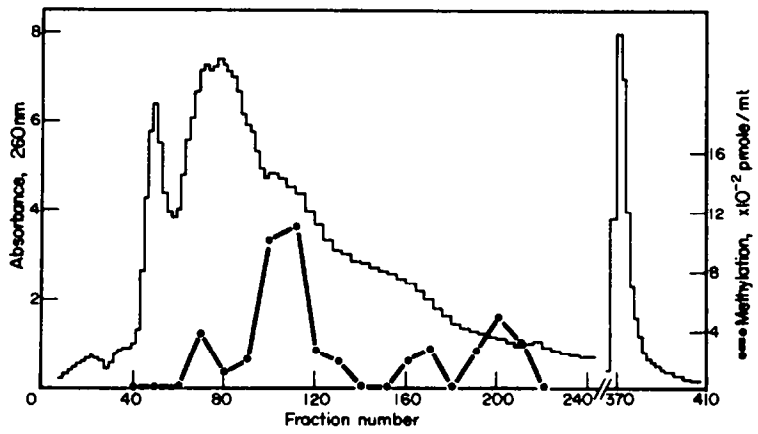

Fig. 1. Fractionation of methylated *S. obliquus* tRNA on BD-cellulose. Crude *S. obliquus* tRNA (4000 A_{260} units) was fractionated on a BD-cellulose column (1.5 × 80 cm). Elution was carried out with a 1.5 l. linear gradient of 0.45 M–1.0 M NaCl in 20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$. Fractions of 3.85 ml were collected at a flow rate of 26 ml/hr. Aliquots of the fractions were methylated with *S*-adenosyl-L-methionine-[methyl-^{14}C] and crude uracil methylase from *E. coli* as described in the Experimental.

mine sulphate or streptomycin sulphate have similar activity. The decrease in activity at higher enzyme concentration is presumably due to contaminating nuclease activity.

The stability of the synthetase is also illustrated in Fig. 2. When stored at −20° in 50% glycerol for 7 days the enzyme is only half as active. The loss in activity is not compensated by increasing the amount of protein added to the incubation mixture because of the introduction of more nuclease. When stored in liquid N_2, *ca* 10% of the synthetase activity is lost in the first 4 weeks after which there is negligible loss over the next 3 months.

The optimum pH for methionine-tRNA synthetase was 7.8. The synthetase has a requirement for magnesium

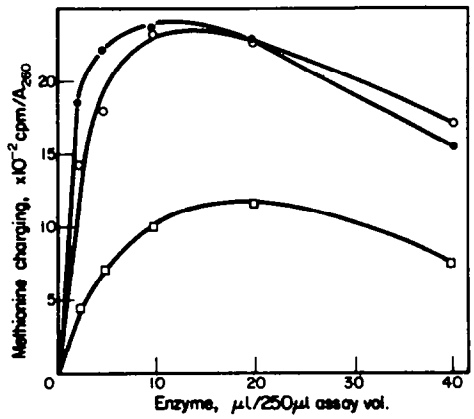

Fig. 2. Effect of crude *S. obliquus* synthetase concentration on the extent of *S. obliquus* tRNA charging with L-methionine-[methyl-^{14}C]. The assays were performed as described in the experimental section. O——O, enzyme preparation treated with streptomycin sulphate and stored in liquid N_2; ●——●, enzyme preparation treated with protamine sulphate and stored in liquid N_2; □——□, enzyme preparation treated with streptomycin sulphate and stored in 50% glycerol at −20° for 7 days.

ion which is optimal at 4.5 mM. However, up to 16.5 mM can be tolerated when 83% of enzyme activity is obtained. Beyond this concentration the enzyme activity decreases rapidly.

Species specificity

The species specificity of the tRNA and synthetase preparations from *S. obliquus* was studied by comparing the extent of tRNA aminoacylation with methionine in homologous and heterologous systems obtained from *E. coli*, *Anacystis nidulans* (a blue–green alga), wheat germ and rat liver. The assays were carried out using optimal conditions and enzyme concentration for the homologous reaction except for the *A. nidulans* enzyme, where conditions chosen were those optimal for changing *E. coli* tRNA because *A. nidulans* tRNA was not available. The results are shown in Table 2. *S. obliquus* $tRNA^{Met}$ can be charged by *E. coli* and *A. nidulans* synthetase to only 70% and 74% respectively compared to the homologous enzyme. However, the rat liver enzyme can charge the *S. obliquus* tRNA with methionine up to 125%.

Table 2. Species specificity of tRNA aminoacylation reaction with methionine

Synthetase preparation	tRNA			
	Rat liver	Wheat germ	*S. obliquus*	*E. coli*
Rat liver	100	100	125	97
S. obliquus	84	92	100	87
A. nidulans	30	22	74	100
E. coli	29	17	70	100

Results are expressed as a percentage of the aminoacylation obtained using the homologous system, except for the *A. nidulans* enzyme, where charging of *E. coli* tRNA was given a 100% value and for wheat germ tRNA, where charging with rat liver enzyme was given a 100% value.

This high figure in comparison to the result with homologous charging may either be due to some mischarging of other *S. obliquus* tRNA species other than $tRNA^{Met}$, or to the inactivation or loss of a species of methionyl-tRNA synthetases from the *S. obliquus* enzyme preparation causing the homologous figure to be low. The *S. obliquus* synthetase preparation charges wheat germ tRNA more efficiently than it does rat liver or *E. coli* tRNA.

Examination of Table 2 as a whole shows there to be a decrease in species specificity of the synthetases of higher organisms and a decrease in the species specificity of the tRNAs of lower organisms. It will be seen *S. obliquus* fits in well with this pattern according to its position as a unicellular eukaryote in the phylogenetic tree [17].

EXPERIMENTAL

Materials. L-Methionine-[methyl-^{14}C] (54 mCi/mmol), *S*-adenosyl-L-methionine-[methyl-^{14}C] (55 mCi/mmol) and Na borohydride-[^{3}H] (11 Ci/mmol) were purchased from The Radiochemical Centre, Amersham. Streptomycin sulphate, protamine sulphate and micrococcal nuclease were from Sigma (London). Snake venom phosphodiesterase and *E. coli* alkaline phosphatase were from Worthington Biochemical Corporation (U.S.A.). Cellulose thin layers on a plastic backing and X-ray film were purchased from Eastman Kodak (U.S.A.). tRNA from *E. coli* was a gift from Dr. S. Nishimura, National Cancer Centre, Tokyo, Japan, and tRNA from wheat germ was a gift from Dr. A. B. Legocki, College of Agriculture, Poznan, Poland. Scintillation fluid was composed of 5 g 2,5-diphenyloxazole (PPO) and 0.3 g 1,4-*bis*-(5-phenyloxazol-2-yl)benzene (POPOP) per l. toluene. Benzoylated DEAE cellulose was prepared as described by Gillam *et al.* [17]. Rat liver tRNA was prepared as described previously [18].

Growth and harvesting of Scenedesmus obliquus *D3.* The organism was cultured heterotrophically on nitrate medium supplemented with glucose and yeast extract at 27° as described in ref. [19]. Cells were cultured in 13 l. batches with adequate stirring and aeration. Cells were harvested by centrifugation at 2500 rpm for 7 min and washed twice with H_2O. The yield was *ca* 10 g/l. The cell paste was stored at −20°.

Isolation of tRNA. Either whole cells were suspended in 2 vols of H_2O containing 0.1% bentonite and shaken with an equal vol. of 85% PhOH containing 0.2% 8-hydroxyquinoline at 60° for 90 min, or cells were homogenized in 1.5 vol. of H_2O containing 0.1% bentonite in a Dyno-Mill cell disintegrator cooled with circulating salt water at −15° for 5 × 2 min and the homogenate was shaken with 1 vol. of 85% PhOH at 23° for 10 min. In either case an equal vol. of *iso*amylalcohol–$CHCl_3$ (1:50) was added to the resulting mixture and after shaking at room temp. the phases were separated by centrifugation and the resulting upper phase was extracted twice with *iso*amylalcohol–$CHCl_3$. This upper phase was shaken with 85% PhOH at room temp. for 15 min. Again after addition of *iso*amylalcohol the phases were separated and the upper phase extracted 3× with *iso*amylalcohol–$CHCl_3$. NaCl was added to the final upper aq. phase to 0.3 M and nucleic acid pptd with 2 vols EtOH. The ppt. was collected by centrifugation and extracted twice with M NaCl at room temp. The extracted tRNA was pptd with 2 vols EtOH and after collection and drying *in vacuo* it was dissolved in 20 mM Tris–HCl pH 7.5, 150 mM NaCl. The soln was loaded onto a DEAE column (DE-23, Whatman) equilibrated in the same buffer. The column was washed with 20 mM Tris–HCl pH 7.5, 0.25 M NaCl before eluting the tRNA with 20 mM Tris–HCl pH 7.5, 0.7 M NaCl. The tRNA was recovered from the eluate by EtOH precipitation, washed once with 70% EtOH and dried *in vacuo.*

Aminoacyl-tRNA synthetases. E. coli [18] and rat liver [20] crude aminoacyl-tRNA synthetases were prepared as described previously. That from *A. nidulans* was prepared in a similar manner to the procedure described for the *E. coli* synthetase.

Crude aminoacyl-tRNA synthetase from S. obliquus. (All procedures were at 0–4° unless otherwise stated). Fresh *S. obliquus* cells (20 g) suspended in 20 ml 10 mM $Mg(OAc)_2$, 60 mM KCl, 6 mM 2-mercaptoethanol were homogenized with an equal vol. of 0.35 mm glass beads in a Dyno-Mill cell disintegrator, pre-cooled with circulating salt water at −15°. Homogenization was carried out for 5 × 2 min with 1 min intervals for cooling. After removing the glass beads by filtration through a sintered glass funnel (coarse) under suction, the cell homogenate was centrifuged for 30 min at 30000 *g* (at rmax in a Sorvall SS34 rotor) and the resultant green supernatant was centrifuged for 3 hr at 125000 *g* (at rmax in a Beckman 42.1 rotor). The clear orange–red supernatant was collected, avoiding the layer of lipid at the top and the material just above the pellet. A vol. of either 10% streptomycin sulphate or 10% protamine sulphate equal to one tenth of the supernatant was slowly added to the supernatant previously adjusted to pH 7 and the mixture left stirring for 30 min. The ppt. was removed by centrifugation and the supernatant was dialysed against 10 mM Tris–HCl pH 7.8, 10 mM $Mg(OAc)_2$ 1 mM 2-mercaptoethanol. The crude synthetase was stored in liquid N_2.

Assay of amino acid acceptor activity of tRNA. The aminoacylation assay of tRNA with *E. coli* synthetase was performed as described previously [21]. Assays for the aminoacylation of tRNA using crude synthetase from *A. nidulans* were performed similarly, and for synthetases from *S. obliquus* and rat liver the same except that the pH of the reaction mixture was 7.8.

Uracil methylase from E. coli *and methylation assay.* Crude uracil methylase preparation from *E. coli* was prepared according to the method of ref. [14].

The methylation reaction was performed in a 250 µl incubation mixture consisting of 50 mM Pi pH 8.2, 1 mM EDTA, 1 mM $MgCl_2$, 20 µM *S*-adenosyl-L-methionine-[Me-^{14}C], 0.05–0.5 A_{260} units of tRNA and a saturating amount of crude *E. coli* uracil methylase. (Before use the *S*-adenosyl-L-methionine-[Me-^{14}C] was diluted with non-radiolabelled material and H_2O to give a soln of 10 mCi/mmol and 5 µCi/ml). Incubation was at 30° for 6.5–7.5 hr and the reaction was terminated by dispensing 200 µl onto 2 filter paper discs (Whatman 3 MM; 24 mm) and immersing the discs in cold 10% TCA. The discs were washed, dried and counted as described for the assay of amino acid acceptor activity of tRNA.

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