Synthesis of Transfer Ribonucleic Acids with Uridine or 2'-O-Methylribothymidine at Position 54 in Developing Dictyostelium discoideum

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In amoebae of Dictyostelium discoideum the ribothymidine (rT) content of tRNA is 0.9 mol %, but decreases progressively during development into spores. To elucidate which nucleosides replace rT at position 54 in developmental tRNA we have characterized ‘vegetative’ and ‘developmental’ tRNAs from the slime mould. Specific tRNAs were separated by two-dimensional gel electrophoresis. During early developmental stages, all tRNA species that could be separated by this method were newly synthesized. A new tRNA with uridine in place of rT and having an electrophoretic mobility similar to ‘vegetative’ tRNA was detected during the early preaggregation stage. This ‘developmental’ tRNA was also extracted from purified polysomes. When development proceeds from preaggregation to postaggregation, tRNAs accumulate with 2'-O-methylribothymidine in place of rT. We suggest that these developmental tRNAs are important for the synthesis of specific developmental proteins.

INTRODUCTION

Eukaryotic tRNAs have been divided into four classes with respect to the nucleoside in loop IV at position 54 (numbering according to Gauss et al., 1979). Class A includes the initiator tRNAs that have an adenosine residue; class B tRNAs contain ribothymidine (rT), pseudouridine (Ψ) or 2'-O-methylribothymidine (Tm); in class C tRNAs uridine (U) is partially converted to rT; while in class D tRNAs position 54 is completely occupied by U (Roe et al., 1976). It has been suggested that the modifications of U54 in eukaryotic tRNAs are involved in regulatory mechanisms at the translational level (Roe & Tsen, 1977).

We have recently observed that in the lower plant Acetabularia mediterranea variations occur in the U54 modification of total tRNA during development (Schmidt et al., 1977b). In further experiments we have used the slime mould Dictyostelium discoideum to study the modification of tRNA in a simple developmental system. The slime mould can be grown vegetatively as single cells in a defined medium (Watts & Ashworth, 1970) and differentiation is induced by starvation (Loomis, 1975). After an early developmental preaggregation stage that involves signalling with pulses of cyclic AMP (Gerisch & Malchow, 1976), the cells aggregate into mounds each containing about $10^5$ cells. The mounds form slugs, which subsequently differentiate into mature fruiting bodies, containing spores at the top of a vacuolized stalk. As previously reported (Dingermann et al., 1977), the amount of rT in tRNA in D. discoideum decreases progressively from 0.9 mol % in vegetative cells to 0.5 mol % in the early preaggregation stage and 0.4 mol % in spores.

Dictyostelium discoideum has now been used in an attempt to elucidate the following:
tRNAs are formed that are used for protein synthesis during differentiation of the amoebae.

The results show a stage-specific formation of developmental tRNAs with U54 or Tm54 in place of rT. The specific ‘preaggregation tRNA U54’ is involved in protein synthesis.

**METHODS**

**Chemicals.** Chemicals were from the following sources: \(^{[\text{H}]}\)aspartic acid (15 Ci mmol\(^{-1}\)), \(^{[\text{H}]}\)threonine (2 Ci mmol\(^{-1}\)), \(^{[\text{H}]}\)phenylalanine (113 Ci mmol\(^{-1}\)) from New England Nuclear; \(^{[\text{H}]}\)asparagine (22 Ci mmol\(^{-1}\)) from CEA-France; potassium \(^{[\text{H}]}\)borohydride (18 Ci mmol\(^{-1}\)), \(^{[\text{H}]}\)S-adenosyl-L-[Me-\(^{3}\text{H}\)]methionine ([Me-\(^{3}\text{H}\)]SAM; (\(22 \text{ Ci mmol}^{-1}\)) and all other tritiated amino acids from Amersham Buchler, Braunschweig, Germany; alkaline phosphatase, snake-venom phosphodiesterase and RNAase T\(_1\) from Worthington Biochemical Corp.; RNAase T\(_4\) from Sankyo, Japan; RNAase A from Boehringer; acrylamide, bisacrylamide and \(N,N',N''\)tetramethylethylenediamine (TMED) from Serva, Heidelberg, Germany. X-ray films and Xr5 for fluorography were from Kodak. All other chemicals were of reagent grade. Streptomycin was a kind gift from Hormon-Chemie, München, Germany.

**Organisms, growth and development.** Dictyostelium discoideum, strain AX-2, was grown in axenic medium (Watts & Ashworth, 1970) (per litre): Oxoid bacteriological peptone, 14.3 g; Oxoid yeast extract, 7-15 g; maltose (Merck), 18-0 g; \(\text{Na}_2\text{HPO}_4\), 0-64 g; \(\text{KH}_2\text{PO}_4\), 0-486 g. The pH was adjusted to 6.7. Maltose was autoclaved separately and streptomycin was added at a final concentration of 200 \(\mu\)g ml\(^{-1}\).

Vegetative growth. Cells were grown at 22 to 23 °C in a 201 fermenter (Eschweiler, Kiel, Germany) under aeration and harvested at a cell density of \(1 \times 10^8\) to \(2 \times 10^8\) in a Cepa continuous flow centrifuge at 4 °C.

Preaggregation stage. Cells from the vegetative growth (1 \(\times 10^8\) to 3 \(\times 10^8\) cells ml\(^{-1}\)) were harvested, washed three times with cold phosphate buffer (2 mM-\(\text{Na}_2\text{HPO}_4\), 14.7 mM-KH\(_2\text{PO}_4\), pH 6.7, containing 200 \(\mu\)g streptomycin ml\(^{-1}\); subsequently referred to as 16-7 mm-phosphate buffer) and resuspended in the same buffer at 1 \(\times 10^7\) cells ml\(^{-1}\). After 12 to 16 h further incubation the cells were harvested.

Postaggregation stage. Cells (1-8 \(\times 10^9\)) from the vegetative growth (1 \(\times 10^8\) to 3 \(\times 10^8\) cells ml\(^{-1}\)) were suspended in 0-2 ml of 16-7 mm-phosphate buffer and spread on top of three filters (Schleicher & Schüll, 576, diam. 9-6 cm) saturated with the buffer in a Petri dish. After 10 to 12 h the cells formed ‘Mexican Hats’ and after 13 to 15 h they reached the culmination stage. These stages are referred to as postaggregation stages.

*Escherichia coli* MRE 600, which was used as source for the rT-forming enzyme, was grown in a medium containing (per litre): Bactopeptone, 10 g; yeast extract, 5 g; \(\text{NaCl}\), 10 g; glucose, 1 g. The cells were harvested during exponential growth.

**Enzyme preparations.** Aminoacyl-tRNA synthetases from exponentially growing cells of *D. discoideum* were prepared according to Palatnik et al. (1977) including DEAE-cellulose chromatography and ammonium sulphate precipitation. The dialysed extract was stored at -80 °C and was stable for at least 2 months. The protein concentration was 15 mg ml\(^{-1}\).

The crude rT-forming methyltransferase was prepared from *E. coli* MRE 600 as described by Reszelbach et al. (1977). The protein concentration of this extract was 14 mg ml\(^{-1}\).

**Preparation and analysis of tRNA.** Cells were washed twice with TMS buffer (10 mM-Tris/HCl, pH 7-5, containing 10 mM-Mg\(_2\)Cl\(_2\) and 2 mM-Na\(_2\)S\(_2\)O\(_3\)) and suspended in this buffer at 0-5 g wet wt ml\(^{-1}\). An equal volume of phenol saturated with TMS buffer was added and the mixture was stirred for 1 h at 4 °C. After centrifugation at 20000 g the aqueous phase was washed and 2.5 vol. absolute ethanol containing 2% (w/v) potassium acetate was added. The tRNA was stored in ethanol at -20 °C. Several tRNA preparations were combined and further purified according to Rogg et al. (1969). For nucleoside analysis, about 50 \(A_{260}\) units of this tRNA were further purified on a Sephadex G-200 column (90 cm x 1 cm diam.). The tRNA was eluted from the column with 10 mM-Tris/HCl, pH 7-5, containing 200 mM-NaCl and 10 mM-MgCl\(_2\). The column was equilibrated with the same buffer before use. The tRNA was collected, dialysed three times for 2 h against 51 distilled water and precipitated with 2.5 vol. ethanol at -20 °C. A sample (1 \(A_{260}\) unit) of purified tRNA was digested to nucleosides and analysed by \(^{3}H\)-postlabelling (Randerath et al., 1972; Randerath et al., 1974; Chia et al., 1976).

**Preparation of tRNA from polyosomes.** Polyosomes were prepared from a 600 ml culture (2 \(\times 10^9\) cells ml\(^{-1}\) of vegetative and preaggregation cells) according to Cocucci & Sussmann (1970) and precipitated with 2.5 vol. ethanol. The precipitate was dissolved in 3 ml TMS buffer containing 0.5% (w/v) SDS and stirred for 45 min with 3 ml buffer-saturated phenol. After DEAE-cellulose chromatography and Sephadex G-100 chromatography the tRNA was precipitated with ethanol.

**Electrophoretic separation of specific tRNAs.** Two-dimensional polyacrylamide gel electrophoresis was
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carried out according to Fradin et al. (1975) with the following modifications. Slab gels of 0.8 x 200 x 400 mm were used in the first dimension and 0.8 x 300 x 300 mm in the second dimension. Samples of 0.5 to 2.5 A_{260} units of labelled tRNAs were separated. To identify tRNA\textsuperscript{5}, 4 to 5 A_{260} units of bulk tRNA were separated on gels of 3 x 200 x 400 mm in the first dimension and 3 x 300 x 300 mm in the second dimension. For the first dimension gel 10 g acrylamide and 0.4 g bisacylamide were polymerized in 100 ml 80 mm-Tris buffer (adjusted to pH 8.3 with boric acid) containing 1 mm-EDTA and 4 mm-urea. For the spacer gel 1.9 g acrylamide and 0.1 g bisacylamide were polymerized in 35 ml 8 mm-Tris/HCl buffer, pH 6.7, containing 4 mm-urea. In both gels the concentrations of TMED and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} were 0.05 % (w/v). The second dimension gel contained (per 100 ml) 20 g acrylamide, 1.04 g bisacylamide, 80 mm-Tris/borate, pH 8.3, 1 mm-EDTA, 4 mm-urea and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} and TMED both at 0.075 % (w/v) final concentration. Electrophoresis was performed with 400 V (constant voltage) in the first dimension at room temperature for 30 h and in the second dimension at 4 °C for 75 h.

tRNA samples were dissolved in a mixture of 60 % (w/v) sucrose, 4 mm-urea, 0.1 mm-sodium acetate, pH 4.5, and 1 % (w/v) 'xylene-cyanol FF' and applied to the gel in 1.5 cm slots. Electrophoresis was terminated after the blue marker had reached the bottom and the gel was then cut into strips. Unlabelled tRNA served as a marker and was stained with 0.5 % ethidium bromide in running buffer for 30 min. For the second dimension, the gel strips were placed at the bottom of the chamber and embedded in 20 % (w/v) acrylamide. Electrophoresis was performed from the bottom to the top. To detect labelled tRNAs the gels were treated first with dimethyl sulfoxide/2,5-diphenyloxazole as described by Bonner & Lasky (1974), and then stained with 0.2 % methylene blue in 0.2 m-sodium acetate buffer, pH 4.5, for 3 to 4 h. The gels were destained under running water overnight and then dried and plated on Kodak XR-5 films for fluorography at -80 °C.

Identification of tRNA\textsuperscript{5} after electrophoresis. Spot X (containing tRNA) and a reference spot (blank) were cut from the gel and eluted with 500 µl 0.3 M-NaCl containing 10 mm-MgCl\textsubscript{2}, 10 mm-sodium acetate buffer, pH 4.5, and 500 µl buffer-saturated phenol. After centrifugation the organic phase was extracted with 500 µl buffer and the combined aqueous phases were precipitated with 2 vol. ethanolic potassium acetate (2 %) in the presence of 2.2 A_{260} units of tRNA-free rRNA. tRNA together with added RNA was collected by centrifugation, dried \textit{in vacuo} and dissolved in 220 µl H\textsubscript{2}O. Each 10 µl of this solution was tested with one of 19 amino acids in 50 µl assay mixtures containing 100 mm-sodium cacydolate, pH 7-5, 10 mm-ATP (neutralized with NaOH), 20 mm-MgCl\textsubscript{2}, 2 µl \textsuperscript{3}H-labelled amino acid and about 100 µg protein of the purified extract containing tRNA synthetases. The reaction was started by adding the tRNA solution at 22 °C. After 25 min incubation each 40 µl was pipetted on to a Whatman 2MM filter (25 mm diam.) and the aminoacylated tRNA was precipitated in 10 % (w/v) cold trichloroacetic acid (TCA). After washing the filter [three times with 5 % TCA, three times with ethanol, once with ethanol/ether (1:1, v/v), and twice with ether], the tRNA was eluted from the filter with 1 h with 500 µl Soluene/water (9:1, v/v; Packard), dissolved in scintillant and the radioactivity was measured in a Packard liquid scintillation counter.

\textit{In vitro} methylation of tRNA with the \textit{E. coli} rT-forming enzyme extract. The methylation reaction mixture (final volume 90 µl) contained 33 mm-triethanolamine/H\textsubscript{2}O, pH 8-0, 5.5 mm 3-mercaptoethanol, 3.3 mm-MgCl\textsubscript{2}, 48 µM-[Me\textsuperscript{3}H]SAM (11.3 Ci mmol\textsuperscript{-1}), 0.5 A_{260} units of tRNA and enzyme extract corresponding to 280 µg protein. The mixture was incubated for 2.5 h at 37 °C. The reaction was stopped by adding 90 µl chloroform/phenol (1:1, v/v). After centrifugation the aqueous phase was re-extracted with 50 µl chloroform/phenol (1:1, v/v). After two back extractions of the combined organic phases, the pooled aqueous phases were layered on a Sephadex G-25 column (1 m x 1 cm diam.) to remove salt and [Me\textsuperscript{3}H]SAM. tRNA was eluted from the column with water, dried \textit{in vacuo} and then subjected to polyacrylamide gel electrophoresis.

\textit{In vivo} labelling of tRNA. During the preaggregation stage, 8 x 10\textsuperscript{7} cells in 4 ml 16-7 mm-phosphate buffer were labelled for 12 h with either 2 mCi [\textsuperscript{3}H]uridine (25 Ci mmol\textsuperscript{-1}) or 3 mCi [\textsuperscript{3}H]methionine (8-8 Ci mmol\textsuperscript{-1}). During the postaggregation phase, the cells were labelled for 3 h (from the 'Mexican Hat' stage to the culmination stage) by transferring the upper of the three filters to a Petri dish containing 1 mCi-L-[Me\textsuperscript{3}H]-methionine in 300 µl phosphate buffer.

During vegetative growth, a 20 ml culture (1 x 10\textsuperscript{8} cells ml\textsuperscript{-1}) was labelled with 2 mCi [\textsuperscript{3}H]methionine (8-8 Ci mmol\textsuperscript{-1}) for 18 h (4 x 10\textsuperscript{8} cells ml\textsuperscript{-1}). Me\textsuperscript{3}H-labelled tRNAs were digested to nucleosides and analysed as described by Rogg et al. (1976).
RESULTS

Separation of specific tRNAs

Two-dimensional gel electrophoresis was used in preference to RPC-5 column chromatography because it permitted the separation of tRNAs into several species in a single step. Bulk tRNAs were prepared from vegetative cells and from cells starved for 12 h in phosphate buffer (preaggregation stage). The nucleoside compositions of both tRNA preparations were determined by \(^3\)H-postlabelling analysis. In accordance with our previous findings (Dingermann et al., 1977), the rT content of vegetative tRNA was 0.9 ± 0.09 mol% and that of preaggregation tRNA was 0.55 ± 0.05 mol% (the standard deviation was calculated from six chromatographic analyses). Each preparation was separated by two-dimensional electrophoresis. The patterns of specific tRNAs were almost the same for 'vegetative' tRNA and 'preaggregation' tRNA. A typical electrophoretic pattern of tRNAs from vegetative cells is shown in Fig. 1. About 45 tRNAs were resolved as separate spots. Identical electrophoretic patterns were obtained in repeated experiments.

tRNA synthesis during development

When vegetative cells are starved by suspending them in phosphate buffer they stop growing and develop to the preaggregation stage at which the amoebae can be kept for about 12 to 18 h at 22 °C provided that aeration is adequate. To discover which tRNAs were synthesized and methylated during early development, vegetative cells were harvested, washed and incubated for 12 h in buffer containing \(^3\)H-uridine or \(^3\)H-methionine. The tRNAs were prepared from the labelled cells and separated by electrophoresis. It is evident that during preaggregation almost all tRNA species that can be separated by this method are newly synthesized (Fig. 2a, b). The radioactivity patterns were identical to the patterns obtained after staining the gel with methylene blue.

Detection of specific U54-containing tRNAs

In eukaryotic class C tRNAs, rT in position 54 has been partly replaced by U. The U54 residue can be converted to rT by the E. coli tRNA (uracil-5)-methyltransferase and S-adenosyl-L-methionine. tRNAs of class D in which rT has been fully replaced by U can also be methylated by this specific enzyme, but at a much lower rate than class C tRNAs (Roe & Tsen, 1977).

To detect the tRNA species with U54 we methylated bulk tRNA from both stages with \(^3\)H[SAM and the E. coli methyltransferase. Only a slight increase was observed in the overall U54 methylation of preaggregation tRNA as compared with that of vegetative tRNA (results not shown).

The tRNAs treated with the rT-forming enzyme were separated by electrophoresis. In vegetative tRNA, several species in which a U54 was methylated to rT were present. In preaggregation tRNA, a new tRNA with U54 methylated to rT was detected in repeated experiments (Fig. 3). Elution of the tRNA from this spot followed by enzymic digestion and nucleoside analysis revealed only rT (Fig. 4), indicating that the new 'developmental' tRNA had U in place of rT.

The developmental tRNA had the same electrophoretic mobility as vegetative tRNA\(^x\) in the schematic representation of Fig. 1(b). To identify the tRNA(s) present in this spot, 4 \(^A\) subunits of total tRNA were separated on a gel. Under optimal conditions the eluted tRNA from spot X contained on average 0.1 \(^A\) subunits. The eluate was divided into 20 portions to test the amino acid acceptance for each of 19 amino acids. Under these conditions an assay mixture contained less than 0.005 \(^A\) subunits of tRNA. The amino acids in the acceptor assay were therefore used undiluted at the highest available specific activity. From each gel, three reference spots were also eluted and treated in the same way as the sample. The experiment was repeated three times.
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Fig. 1. Pattern of *D. discoideum* tRNA after two-dimensional polyacrylamide gel electrophoresis. (a) One *A*$_{260}$ unit of bulk tRNA was applied to the gel and electrophoresis done as described in Methods. The gel was stained with 0.5% ethidium bromide in running buffer for 3 min. (b) Schematic representation of the electrophoretic separation of specific tRNAs. Electrophoresis was from right to left in the first dimension and from bottom to top in the second dimension. Spot X, indicated by the arrow, contains tRNA$^{^\text{AsU}}$ (see Table 1).

In all three cases the tRNA from spot X accepted only asparagine; the results for the other 18 amino acids were found to be identical with that of a corresponding blank within the calculated standard deviation (Table 1). We therefore suggest that the tRNA in question is tRNA$^{^\text{AsU}}$.

To elucidate whether the developmental tRNA U54 was used for protein synthesis, we prepared polysomes from vegetative and preaggregation cells. tRNAs were extracted from the polysomes, methylated with [Me-$^3$H]SAM and the rT-forming enzyme from *E. coli* and separated by electrophoresis. The specific developmental tRNA U54 was found to be present at polysomes, thus indicating that it is involved in protein synthesis (Fig. 5).
**Fig. 2.** Electrophoretic separation of newly synthesized tRNAs from cells in the preaggregation stage. Cells were labelled with (a) [3H]uridine (2 mCi) or (b) [Me-3H]methionine (3 mCi) as described in Methods; 2.5 A26O units of tRNA corresponding to \(1 \times 10^5\) c.p.m. (a) or 2.4 A26O units of tRNA with \(1 \times 10^6\) c.p.m. (b) were separated. The electropherograms were treated with scintillator, stained with methylene blue, dried and exposed for fluorography for 10 d at \(-80\) °C.

**Fig. 3.** Electrophoretic patterns of tRNAs U54, detected by acceptance of methyl groups from [Me-3H]SAM with the rT-forming enzyme from *E. coli*: (a) vegetative stage; (b) preaggregation stage. One A26O unit of tRNA was methylated as described in Methods; 1.8 \(\times 10^5\) c.p.m. of ‘vegetative’ tRNA and 1.9 \(\times 10^5\) c.p.m. of ‘developmental’ tRNA were applied to the gels. The arrow in b indicates a newly synthesized developmental tRNA with U54 in place of rT.

**Formation of tRNA Tm54 during development**

In rat liver an enzyme is present that converts rT54 to 2'-O-methylribothymidine, Tm (Gross *et al.*, 1974). The previously observed decrease of rT in developmental tRNA (Dingermann *et al.*, 1977) might imply that in certain tRNAs rT is converted to Tm or that new tRNAs are formed with Tm in place of rT. To test this hypothesis, cells were labelled during vegetative growth, preaggregation and postaggregation with [3H]methionine and tRNA was isolated and hydrolysed to nucleosides. The nucleoside analysis of Rogg *et al.* (1976) permits the simultaneous detection of rT and Tm in a tRNA hydrolysate. The analysis of methyl-labelled nucleosides revealed that labelled Tm was absent from vegetative tRNA. Small amounts of Tm occurred in preaggregation tRNA. During postaggregation, between
Fig. 4. Product analysis of the specific ‘developmental’ tRNA after in vitro methylation. In vitro methylated tRNA from spot X was eluted, 1 $A_{600}$ unit of carrier tRNA was added and the tRNAs were digested to nucleosides. These were separated by thin-layer chromatography with the solvent systems described by Rogg et al. (1976).

Fig. 5. Electrophoretic pattern of ‘preaggregation’ polysomal tRNAs U54. Bulk tRNA was extracted from purified polyosomes and methylated in vitro with [Me-$^3$H]SAM and the rT-forming E. coli enzyme (see Methods).

Table 1. Identification of the specific tRNA$^X$ after electrophoresis

Three blanks and spot X (containing tRNA) were cut out from a preparative gel on which 4 $A_{600}$ units of bulk tRNA had been separated. The tRNA$^X$ and three blanks were eluted and each eluate divided into 20 portions. Each portion was tested for amino acid acceptance with 19 $^3$H-labelled amino acids. The experiment was repeated three times. Only Asn was accepted by the tRNA of spot X. The values for all other amino acids were in the range of the standard deviation of the mean value of the blank.

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<th>$^3$H-labelled amino acid</th>
<th>Specific activity (Ci mmol$^{-1}$)</th>
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</tr>
<tr>
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Fig. 6. Patterns of methyl-labelled nucleosides from developmental tRNAs after two-dimensional thin-layer chromatography. tRNA nucleosides from [3H]methionine-labelled cells in (a) preaggregation stage and (b) postaggregation stage. About 3 A_{260} units of tRNA with 1.2 × 10^6 c.p.m. (a) and 1.5 A_{260} units of tRNA with 1.6 × 10^6 c.p.m. (b) were digested with RNAase A, alkaline phosphatase, snake-venom phosphodiesterase and RNAase T_{1}. The nucleosides were separated according to Rogg et al. (1976).

formation of the 'Mexican Hat' and culmination, the amount of labelled Tm relative to labelled rT markedly increased (Fig. 6a, b).

DISCUSSION

Almost all prokaryotic and a great number of eukaryotic tRNAs that have been sequenced have the modified nucleoside rT at position 54 in a common sequence TUCG in loop IV of tRNA. The methylation of U54 to rT seems to play an important role in tRNA evolution because rT occurs only once in the tRNA molecule and always occupies the same position. In addition, rT is the unique methylated base for which two independent biosynthetic pathways have been evolved: the SAM-dependent pathway in Gram-negative bacteria and in eukaryotes (Mandel & Borek, 1963) and the tetrahydrofolate-dependent pathway in most Gram-positive micro-organisms (Schmidt et al., 1977a). As has been shown for E. coli, the methylation of U to rT in this position in tRNAs gives the cell a survival advantage (Björk & Neidhardt, 1974).

Acetabularia mediterranea and D. discoideum have their greatest amount of rT (0.9 mol %) in tRNA during vegetative growth stages when almost all proteins are synthesized at a high rate. During development the overall amount of rT in total tRNA is much lower (Schmidt et al., 1977b; Dingermann et al., 1977). Here we show that the observed decrease in the rT content of tRNA during the development of D. discoideum can be explained by an accumulation of tRNAs that have U or Tm in position 54. When vegetative amoebae stop growing and development ensues tRNAs are synthesized and methylated.

The new specific preaggregation tRNA U54 identified had almost the same electrophoretic mobility as vegetative tRNA^{Asn} and might be identical with one of the tRNA^{Asn} isoacceptors observed by Palatnik et al. (1977). These authors speculate that during early development an incompletely modified species of tRNA^{Asn} occurs, with G in place of the modified guanosine derivative Q in the first position of the anticodon. Rat liver tRNA^{Asn} has been sequenced recently and shown to contain the modified nucleoside Q (Chen & Roe, 1978).

In our experiments the developmental tRNA U54 was detected by the capacity to accept
methyl groups from S-adenosylmethionine by the rT-forming enzyme from *E. coli*. The preaggregation tRNA thus belongs either to class C or class D according to the classification of Roe et al. (1976) (see Introduction). Since class D tRNAs whose rT has been fully replaced by U54 are poor substrates for the *E. coli* rT-forming enzyme (Roe & Tsen, 1977) it is possible that further specific developmental tRNAs U54 are formed which cannot be detected by *in vitro* methylation.

The accumulation of tRNAs Tm during postaggregation might be the result of either (i) the rT residue in specific tRNAs that are present during vegetative growth and development becoming hypermethylated by a specific developmental tRNA (ribose-2'-O-methyltransferase) or (ii) synthesis of specific developmental tRNAs in which rT is methylated by a pre-existing enzyme. We conclude from our results that the overall distribution of tRNAs with U54 or rT54 or Tm54 (class B, C and D tRNAs) is changed when the slime mould starts to aggregate and finally forms spores.

The question arises as to whether developmental tRNAs with U54 or Tm54 are involved in protein synthesis or whether they serve other regulatory functions. The specific preaggregation tRNA U54 from *D. discoideum* was found to be present in purified polysomal fractions. This result strongly suggests that the developmental tRNA is used for protein synthesis. Our results therefore support an early proposal made by Sharma & Borek (1970) who analysed tRNA methylation in *D. discoideum*. They observed the occurrence of inhibitors of tRNA methyltransferases and suggested that tRNA methylation may serve a regulatory function in producing specific tRNA forms for new protein synthesis or by rendering some tRNA forms non-functional for old protein synthesis.

The nucleoside modification at position 54 of tRNA might have a regulatory function in protein synthesis. The following observations support this hypothesis: (i) prokaryotic and eukaryotic counterparts of specific tRNAs with U54 in place of rT function with an altered efficiency in *in vitro* protein synthesis (Albani et al., 1978; Marcu & Dudock, 1976; Roe & Tsen, 1977); (ii) during codon-anticodon recognition, aminoacyl tRNAs are bound to the ribosomal A-site, probably by interaction of the TUC or an analogous sequence with a complementary sequence of 5S rRNA in prokaryotes or 5-8S rRNA in eukaryotes (Erdmann, 1976, and references therein); (iii) mRNA seems to induce a conformational change in aminoacyl-tRNA exposing the trinucleotide sequence TUC for binding to the ribosome (Schwarz et al., 1974). Thus the nucleoside present in position 54 of a given tRNA species might influence codon-anticodon recognition and mRNA selection at the ribosome.

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