MODIFIED BASES IN tRNA OF *DICTYOSTELIUM DISCOIDEUM*: ALTERATIONS IN THE RIBOTHYMIDINE CONTENT DURING DEVELOPMENT

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1. Introduction

In essentially all procaryotic tRNAs ribothymidine (rT) is present in the TψC loop in molar amounts. However, several eucaryotic tRNAs from wheat germ and mammalian tissues have an incomplete modification or no modification of uridine into rT in this sequence [1–5]. In specific tRNAs from wheat germ the unmodified uridine residue can be converted to rT in vitro by the *E. coli* tRNA (uracil-5) methyltransferase and *S*-adenosylmethionine (SAM). The tRNAs function significantly less efficiently in cell free protein synthesis when the uridine residue was converted to ribothymidine [6]. We have previously shown, that tRNAs from undifferentiated *Acetabularia mediterranea* have low contents of rT, whereas tRNAs from developed cells have four times the amounts of rT [7]. All these observations suggest that in eucaryotic cells the modification of the uridine residue at position 23 from the 3′-terminus might have regulatory function on protein synthesis.

Here we present an overall analysis of modified bases in tRNA from the myxamoebae *Dictyostelium discoideum* an organism that allows to study the entire developmental sequence under well-defined experimental conditions. Bulk tRNA was isolated from the slime mould at three stages of the life cycle: the vegetative growth stage, the pre-aggregation stage, and after formation of spores. In bulk tRNA from cells of the vegetative growth stage 0.9 mol% rT is present. The amount of ribothymidine is significantly lower in tRNA from developing cells (0.5 mol%) and in tRNA from spores (0.4 mol%). The overall composition of other modified bases in bulk tRNA does not vary significantly during the whole life cycle.

2. Materials and methods

Chemicals were from the following sources: [^{3}H]-potassium-borohydride (18 Ci/mmol): from Amersham, Frankfurt/Main. Alkaline phosphatase and snake venom phosphodiesterase: Worthington, Freehold, USA. RNAase A: Boehringer, Mannheim. DEAE-Cellulose DE 32: Whatman, England. Sephadex G-200: Pharmacia, Upsala, Sweden. Acrylamide and Bisacrylamide: Serva, Heidelberg. X-ray film, XR-5 for fluorography: Kodak, Stuttgart. All other chemicals were of reagent grade and from commercial sources. Streptomycin was a kind gift from Fa. Hoechst, W. Germany.

Conditions for growth and differentiation: *D. discoideum*, strain AX-2, was obtained from Dr Gerisch, Basel. The myxamoebae were grown in axenic medium as described by Watts and Ashworth [8] containing in one litre: Oxoid bacteriological peptone (14.3 g), Oxoid yeast extract (7.15 g), maltose (Merck) (18 g), Na_2HPO_4 (0.64 g) and KH_2PO_4 (0.486 g), final pH 6.7. Maltose was autoclaved separately and added together with streptomycin (final concentration 200 μg/ml culture). Usually 200 ml AX-2 medium in a litre flask was inoculated with cells from a preculture to a final cell titer of $1-2 \times 10^5$ ml. The cultures were shaken (170 rev./min) in a New Brunswick 5 litre continuous culture apparatus and grow at 22–23°C exponentially at a doubling time of 8 h to a cell titer of 6×10^6. Thereafter the rate of growth decreases until the cells enter the stationary phase at a cell density of 1.5×10^7 cells/ml.

Vegetative growth stage: Cells were harvested from 3 litre culture during exponential growth at cell titers of $2-4 \times 10^6$/ml.

Pre-aggregation stage: Amoebae were harvested at

a cell density of 7–8 × 10^6/ml. Differentiation was induced by resuspending the cells from the vegetative growth stage in 16.7 mM phosphate buffer (pH 6.7), to a final titer of 3–5 × 10^7/ml. The cells were harvested after 4 h or after 8 hours of starvation.

Spores: Amoebae, grown to a titer of 8 × 10^6/ml were harvested and washed once with the phosphate buffer. The cells were suspended in the same buffer to a final titer of 1 × 10^8/ml and 0.5 ml plated on petri dishes containing 2% oxoid agar in 16.7 mM phosphate buffer. Spores develop at room temperature in about 3–4 days. Spores from 30 Petri dishes were harvested, for the isolation of tRNA.

Preparation of tRNA: 5 g (wet weight) of cells or of spores were homogenized with 15 g of Alcoa and 20 ml of buffer (0.4 M sucrose, 10 mM Tris–HCl (pH 7.5), 100 mM KCl, 6 mM $MgCl_2$, 30 mM NaCl). Alcoa and cell debris were sedimented and tRNA prepared from the cell extract as described by Yang and Novelli [9]. The tRNA preparation was further purified by gel-chromatography.

An amount of 100–200 A_{260} of the tRNA fraction was layered on a Sephadex G-200 column (90 cm length; 7 cm diameter), equilibrated with buffer (200 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5) before use. The tRNA was eluted with the same buffer at a flow rate of 20 ml/h. Only the low molecular weight fraction (4 S material) was collected and dialysed three times against each 5 litre of distilled water. The tRNA was precipitated with two volumes of ethanol at –20°C. An amount of 15 A_{260} tRNA was further purified by preparative PAA-Gel electrophoresis as described recently [10]. The separation was carried out in a 7 × 15 × 0.8 cm gel plate of 10% acrylamide and 0.5% bisacrylamide and a buffer of 90 mM Tris/borate (pH 8.3), 2.5 mM Na_2EDTA. Later experiments showed that the tRNA obtained after Sephadex G-200 chromatography was pure and that the electrophoretic step can be omitted.

An amount of 1 A_{260} of purified tRNA was used for analysis of nucleosides by ^3H post-labeling as described by Randerath et al. [11,12].

3. Results and discussion

The myxamoebae, strain AX-2 of the cellular slime mould *Dictyostelium discoideum*, grow in axenic medium in the presence of adequate food supply as independent cells. Exhaustion or depletion of nutrients initiates a developmental sequence whereby the cells stream toward one another and form large aggregates. Further differentiation results in the creation of the fruiting body, which consists of two distinct cell types: stalk cells and spores. If cells from the vegetative growth stage are starved by suspending them in phosphate buffer they develop to an pre-aggregation stage during which early developmental changes can be observed. We have isolated and purified bulk tRNA from exponentially grown cells, from starved cells and from spores. The tRNA preparations were free from ribosomal RNA and from DNA. After enzymatic digestion of tRNAs the resulting nucleosides were analysed by ^3H post-labeling and two dimensional chromatography on cellulose thin-layer plates.

The pattern of the nucleoside derivatives from bulk tRNA obtained from exponentially grown cells is shown in fig.1. Since the patterns of ^3H-labeled con-

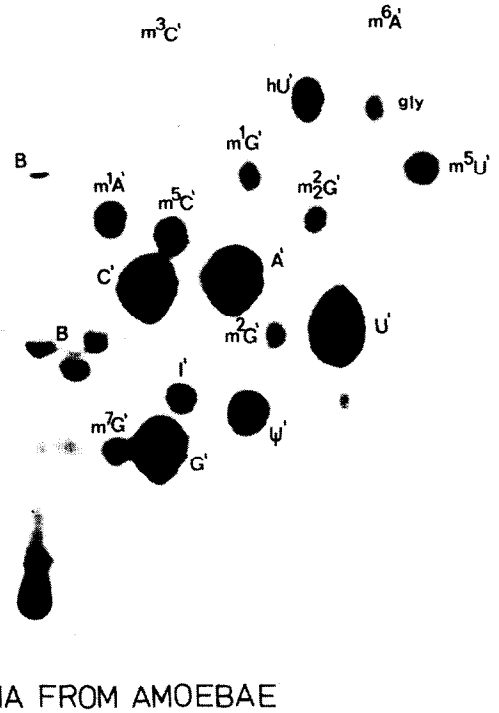

Fig.1. Reproduction of the autoradiographic map of ^3H-labeled digests of tRNA from *D. discoideum*, vegetative growth stage. About 8–9 × 10^5 cpm were applied on the plate. Fluorography at –70°C was performed for 3–6 days. Background spots are indicated as gly (glycerine) and B. (For further details see refs [11] and [12].)

stituents of tRNAs from the other developmental stages, did not show significant qualitative variations one pattern is shown as a representative only.

The following base – modifications of tRNA from *D. discoideum* were discovered: hU and ψ (2–2.5 mol%), m^5U, mA ($m^1A + m^6A$) and m^5C (0.8–1 mol%), m^7G, m^2G, m_2^2G and m^1G (about 0.5 mol%), table 1 first row. m^6A is probably derived from m^1A upon alkaline treatment during the analytical procedure.

The overall composition of modified bases in bulk tRNA from the slime mould agrees well with that described for eucaryotic tRNAs from other sources [13].

Minor changes were found in the overall base composition of bulk tRNAs from the three developmental stages with one exception: The amount of m^5U in tRNA decreases early during development. Already 4 h after starvation of the amoebae in phosphate buffer, the amount of rT in tRNA is reduced from 0.9 to 0.5 mol%. In bulk tRNA from spores only 0.4 mol% rT was found.

In tRNA preparations from eucaryotic cells small amounts of mitochondrial tRNA are present beside cytoplasmic tRNA. Mitochondrial tRNAs have only 0.2 mol% ribothymidine [13]. The decrease of rT in tRNA in developing *D. discoideum* can however not be explained by an increase in the amount of mitochondrial tRNA, relative to cytoplasmic tRNA because the appearance of mitochondria does not change during development and no evidence for transformation of mitochondria into prespore vesicles has been found [14].

Table 1
Analysis of bulk tRNA from *Dictyostelium discoideum* by ^3H post-labeling

Compound	Percentage of total radioactivity recovered			
	Vegetative growth	Pre-aggregation		Spores
		4 h Starv.	8 h Starv.	
U	20.48	20.36	20.24	20.27
A + I	19.31	19.74	20.79	20.06
C	25.03	25.11	23.70	24.87
G	25.20	25.85	25.94	26.77
m^5U	0.87	0.52	0.55	0.41
hU	2.04	2.31	2.15	1.68
ψ	2.55	2.42	2.42	2.10
$m^1A + m^6A$	1.09	1.12	1.03	1.01
m^3C	0.14	0.20	0.17	0.16
m^5C	1.17	0.83	0.99	0.88
m_2^2G	0.48	0.41	0.54	0.40
m^2G	0.56	0.40	0.44	0.41
m^1G	0.52	0.42	0.43	0.40
m^7G	0.75	0.62	0.53	0.56
Total	100.19	100.31	99.92	99.98

A reduction in the amount of ribothymidine in cytoplasmic tRNA during differentiation of the slime mould can be caused by two alternative mechanisms: (i) The synthesis of new tRNA species in which uridine at position 23 from the 3′-terminus is not or only partially converted to ribothymidine; in addition tRNAs synthesized during vegetative growth might be subject to degradation. (ii) A hyper-modification of the ribothymidine residue in tRNA that cannot be detected by ^3H post-labeling of the nucleosides according to the method of Randerath.

Recently alterations in the spectrum of tRNA isoacceptors have been observed during morphogenesis of *D. discoideum* [15]. Whether these alterations reflect differences in the rate of synthesis of tRNA iso-accepting species or differences in the degree of modification of specific tRNAs is not yet understood.

Extracts obtained from differentiating *D. discoideum* have lower capacity to methylate submodified tRNA from *E. coli* than extracts from vegetative amoebae [16]. This decrease in methylating capacity is probably caused by the formation of inhibitors of tRNA methyltransferases [17]. Thus the accumulation of rT deficient tRNA during the life cycle of *D. discoideum* may well be a consequence of a stage specific synthesis of an inhibitor of the tRNA (uracil-5) methyltransferase.

On the other hand a hypermodification of ribothymidine to 2-*O*-methyl ribothymidine has been found in eucaryotic tRNAs [18]. Therefore we cannot exclude the possibility that the decrease in the amount of rT during development is caused by an additional modification at this specific position of tRNA. The mechanism that leads to a decrease in rT content of tRNA of the slime mould and it's biological significance during differentiation is currently being investigated in our laboratory.

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References

[1] Marcu, K., Mignery, R., Reszelbach, R., Roe, B., Sirover, M. and Dudock, B. (1973) Biochem. Biophys. Res. Commun. 55, 477–483.
[2] Keith, G., Picaud, F., Weissenbach, J., Ebel, J. P., Petrissant, G. and Dirheimer, G. (1973) FEBS Lett. 31, 345–347.
[3] Kimball, M. E., Szeto, K. S. and Söll, D. (1974) Nucl. Acids. Res. 1, 1721–1732.
[4] Raba, M., Limburg, K., Katze, J. R., RajBhandary, U. L. and Gross, H. J. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 301–302.
[5] Roe, B. A., Chen, E. Y. and Tsen, H. Y. (1976) Biochem. Biophys. Res. Commun. 68, 1339–1347.
[6] Marcu, K. and Dudock, B. (1976) Nature 261, 159–162.
[7] Schmidt, W., Kersten, H. and Schweiger, H. G. International Symposium on Cell Biology Boston-Massachusetts, Woodcock, C. L. (1977) Acetabularia 1976, Academic Press New York, in press.
[8] Watts, D. J. and Ashworth, J. M. (1970) Biochem. J. 119, 171–174.
[9] Yang, W.-K. and Novelli, G. D. (1971) Meth. Enzymol. 20, 44–55.
[10] Böcker, R. (1975) Thesis University Erlangen-Nürnberg
[11] Randerath, E., Yu, C. T. and Randerath, K. (1972) Anal. Biochem. 48, 172–198.
[12] Randerath, K., Randerath, E., Chia, L.-L. S. Y. and Nowak, B. J. (1974) Anal. Biochem. 59, 263–271.
[13] Chia, L.-L. S. Y., Morris, P., Randerath, K. and Randerath, E. (1976) Biochim. Biophys. Acta 425, 49–62.
[14] Loomis, W. F. (1975) Dictyostelium discoideum, p. 23, Academic Press, New York.
[15] Palatnik, C. M. and Katz, E. R. (1977) J. Biol. Chem. 252, 694–703.
[16] Pillinger, D. and Borek, E. (1969) Proc. Natl. Acad. Sci. USA 62, 1145–1150.
[17] Sharma, O. K. and Borek, E. (1970) J. Bacteriol. 101, 705–708.
[18] Gross, H. J., Simsek, M., Raba, M., Limburg, K., Heckman, J. and RajBhandary, U. L. (1974) Nucl. Acids. Res. 1, 35–43.