OCCURRENCE AND BIOSYNTHESIS OF RIBOTHYMIDINE IN tRNAs OF *B. SUBTILIS*

H. H. ARNOLD, W. SCHMIDT and H. KERSTEN

Institut für Physiologische Chemie, der Universität Erlangen-Nürnberg, 852 Erlangen, Wasserturmstrasse 5, West Germany

Received 16 January 1975

1. Introduction

Ribothymidine is considered to be a common nucleoside in the sequence GTψC of most tRNAs from bacteria, yeast and mammalian cells involved in peptide bond formation at the ribosome. The biosynthesis of the methylated nucleosides in tRNAs was originally shown by Borek [1] to occur at the polynucleotide level and to involve methionine as precursor and *S*-adenosylmethionine (SAM) as coenzyme Therefore [^{14}C] methionine is used in several laboratories to label the methylgroups of tRNAs in growing cells.

In a previous communication [2] we have shown that labeled ribothymidine [m^{5}U) is not formed in tRNAs of *B. subtilis* to any considerable extent when the cells are grown in the presence of [^{14}C] methionine. These results are consistent with those reported by Klagsbrun [3]. Moreover we failed to detect a SAM dependent m^{5}U tRNA methyltransferase activity in extracts of *B. subtilis* [2,4]. On the basis of these results and the present state of knowledge on the transfer of methylgroups to tRNA we concluded that ribothymidine does not occur in most species of tRNAs of *B. subtilis*.

In this paper evidence is presented that tRNAs of *B. subtilis* contain one ribothymidine per molecule. Several independent methods, not involving labeling with methionine, have been applied to identify ribothymidine. Since methionine cannot serve as precursor for most tRNA species of *B. subtilis*, the cells were grown in the presence of [^{14}C] formate or L-[3^{14}C] serine in the presence of unlabeled methionine. The results indicate that a tetrahydrofolic acid dependent m^{5}U tRNA methyltransferase might be involved in the biosynthesis of ribothymidine in several tRNA species of *B. subtilis*.

2. Materials and methods

Chemicals were from the following sources: L-[1^{14}C] methionine (55 mCi/mmol); [^{14}C] formic acid (58 mCi/mmol); [^{14}C] uridine (40 mCi/mmol); L-[3^{14}C] serine (40 mCi/mmol); [^{3}H] KBH_4 (2 Ci/mmol): Radiochemical Centre Amersham. RNAase T_2: Sigma. Snake venom phosphodiesterase: Worthington. Plastic sheets, Polygram Cel 300 for thin-layer chromatography: Machery and Nagel. X-ray films for autoradiography Osray T_4: Agfa. RP X-Omat for fluorography: Kodak. All other reagents, solvents and cellulose thin layer plates: Merck AG, Darmstadt.

B. subtilis W 23 was grown in minimal medium as described [2]. For labeling experiments 2 ml overnight culture was diluted into 100 ml fresh medium containing either of the radioactive precursors: L-[1^{14}C] methionine, 7.0 μmol, 100 μCi; [^{14}C] uridine, 0.2 μmol, 10 μCi; [^{14}C] formic acid, 1.8 μmol, 100 μCi; L-[3^{14}C] serine, 0.8 μmol, 50 μCi. Purines and thymine were added at concentrations of 100 μg/ml, when the cells were labeled with formic acid or serine. The cells were grown up to the early stationary phase ($A_{578\ nm}$ = 1.8; d = 1 cm). Labeling with L-[3^{14}C] serine was carried out in the absence or presence of added non-labelled methionine 30 μg/ml as indicated in table 3.

tRNA analysis: The tRNA was isolated as described in the previous paper [2]. The bases of tRNAs from uridine/formic acid or serine labeled cells were analyzed after perchloric acid hydrolysis and purification

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of the hydrolysate [2] by thin layer chromatography according to Kahle [5].

The 3′-mononucleotides were analyzed after alkaline or T_2 digestion of tRNA exactly as described by Nishimura [6].

The nucleosides were determined with the technique of Randerath [7]. After digestion of 0.5 A_{260} units to the nucleoside level, the ribose moieties were oxidized with periodate, followed by reduction with [^{3}H] KBH_4 (2 Ci/mmol). About 6 X 10^5 cpm (counting efficiency 15%) of the labeled digest was applied to the thin-layer plate and resolved by two-dimensional chromatography according to Randerath [8].

3. Results and discussion

The analysis of the methylated bases of tRNAs from [^{14}C] methionine labeled *B. subtilis* exhibits only one m^5U residue per 100 tRNA molecules [2]. Strikingly the nucleoside analysis by ^{3}H postlabeling of total tRNAs according to Randerath [7] showed a radioactive spot in a position identical with that of the derivative of m^5U (fig.1). A quantitative calculation revealed that this modified nucleoside occurs in *B. subtilis* tRNAs in nearly molar amounts (table 1).

Table 1
Relative amount (mol %) of m^5U in *B. subtilis* tRNAs compared with the relative amounts of purines and pyrimidines. Calculation from the Randerath analysis shown in fig.1.

m^5U'	0.99
U′	16.84
G′	31.07
C′	29.62
A′	17.48
M	4.02

Conditions as described in legend of fig.1. M = other modified bases.

This result agrees well with the analysis of ribothymidine in *B. subtilis* tRNAs reported by Randerath [9].

The failure to label m^5U of tRNAs with methionine as precursor may cast some doubt as to whether the compound in the position of the derivative of m^5U on the autoradiographic map is derived from ribothymidine (fig.1). Various methods were therefore applied to prove the identity of m^5U in *B. subtilis* tRNAs.

In a first series of experiments unfractionated non-labelled tRNAs were degraded by alkaline or

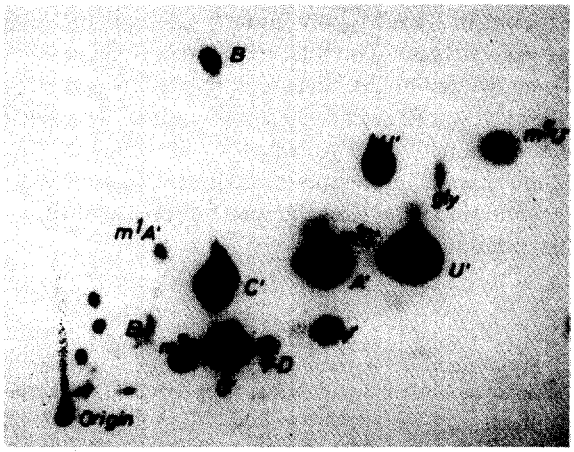

Fig.1. Reproduction of the autoradiographic map of a [^{3}H]labeled digest of *B. subtilis* tRNAs Film detection by fluorography at −35°C; exposure of the film 7 days. Other conditions and abbreviations as described in Materials and methods or [7] and [8].

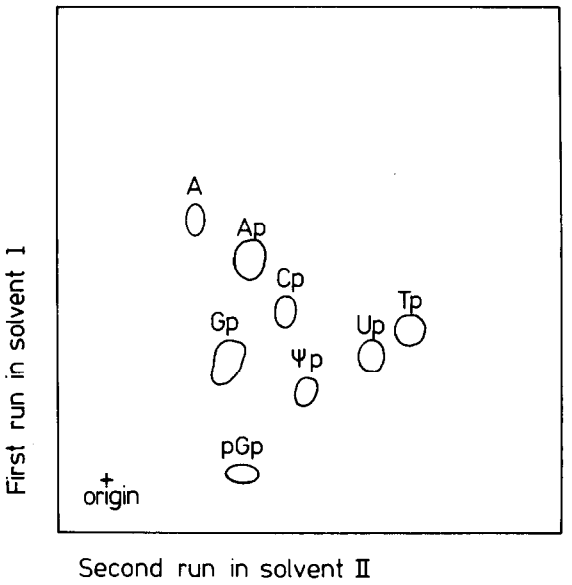

Fig.2. Separation of some minor base components of total tRNAs of *B. subtilis* and of $tRNA^{Phe}$ of *E. coli* by two-dimensional thin-layer chromatography. Conditions as described in Materials and methods and [6].

enzymatic hydrolysis with ribonuclease T_2. The resulting 3′ ribomononucleotides were separated by two-dimensional thin-layer chromatography [6]. Considerable UV absorption occurred in the position of 3′ ribothymidylic acid (fig.2). The UV absorption spectrum of the eluted spot was identical with that of 3′ ribothymidylic acid derived by the same procedure from $tRNA^{Phe}$ of *E. coli*.

In a second series of experiments growing cultures of *B. subtilis* were labelled with [^{14}C] uridine, the tRNAs were isolated, hydrolyzed by perchloric acid to the bases and analyzed exactly as the tRNAs from methionine labeled cells. Another part of the tRNA was degraded by alkaline hydrolysis to the 3′ ribomononucleotides which were further separated and identified [6]. Radioactivity was found in uracil and in 3′ uridylic acid and also in thymine and in 3′ ribothymidylic acid (table 2). In both analyses about one m^5U residue was estimated per 20 uracil residues. This ratio is by 15% lower than the ratio of $m^5U/U = 1 : 17$ calculated from the Randerath analysis. The lower ratio of m^5U/U in tRNAs from the uridine labelled cells is caused by a contamination of the tRNA preparations with 5S tRNA which was not removed as in the Randerath analysis (table 1). We can therefore calculate from the U labeling experiment that about 1.0 m^5U residues occurs per tRNA molecule.

The labeling of m^5U with CH_3 from [^{14}C] methionine revealed 0.01 m^5U residues per tRNA molecule. This low value cannot be caused by a loss of m^5U during perchloric acid hydrolysis, since the tRNA labelled from uridine was hydrolyzed exactly as the tRNA from methionine labelled cells.

Taken together these results show that ribothymidine occurs in *B. subtilis* tRNAs and that it is present in nearly molar amounts, but that methionine is not the sole precursor of m^5U. The results are consistent with our previous findings that, crude extracts of *B. subtilis*, tested in an SAM dependent m^5U tRNA methyltransferase assay, fail to transfer significant amounts of methylgroups to methyldeficient tRNAs of *E. coli* or of *B. subtilis* [2,4].

To elucidate whether tetrahydrofolic acid might be involved in the biosynthesis of ribothymidine we have tested [^{14}C] formic acid and L-[3^{14}C] serine as precursors of m^5U. Cultures of *B. subtilis* were labelled with [^{14}C] formate or [^{14}C] serine. The tRNAs were hydrolyzed to the bases or 3′mononucleotides and analyzed with [^{14}C] formate, when the specific activities of U and T were calculated they were found in repeated experiments to be about 1 : 2. The labelled carbon atom(s) in the uracil ringsystem might be derived from formate via CO_2 and carbamoyl-phosphate or via the pyruvate pool. The differences in the specific activities found for U and T were significant, however considerably higher specific activities in m^5U were obtained when the cells were labelled with [^{14}C] serine in the absence (I) or presence (II) of added methionine. In experiment I and II the bases were analyzed after acid hydrolysis. In experiment III the tRNA from experiment I was hydrolyzed to the 3′ monophosphates with T_2 RNAase. The total radioactivity measured in U was divided by 20, because in this tRNA preparations one m^5U was found per 20 uracil-residues. The specific activity of m^5U after growing the cells with [^{14}C] serine in the absence or presence of added methionine was 20–30-fold higher than the specific activity of the uracil residues (table 3).

Table 2
Labeling of uracil and thymine respectively their 3′ribomononucleotides in tRNAs of *B. subtilis* grown in the presence of [^{14}C]uridine

Compound	cpm		Ratio
	I	II	U/T
U	12.170	7.440	
T	665	380	~ 1/20
rUMP	4.930	1.662	
rTMP	240	80	~ 1/20

Conditions of labeling, tRNA isolation hydrolysis and analysis of the bases respectively 3′ribomononucleotides as described in Materials and methods.

Table 3
Specific activity in uracil or thymine respectively their 3′ribomononucleotides of tRNAs from *B. subtilis* grown in the presence of L-[3 ^{14}C]serine

Compound	cpm			Ratio
	I	II	III	U/T
U (rUMP)	8	9	10	
T (rTMP)	200	190	270	~ 1/25

The results indicate that a tetrahydrofolic acid derivative might be involved in the transfer of methyl-groups during the biosynthesis of m^5U in tRNAs of *B. subtilis*. In this respect recent results of Delk and Rabinowitz are of importance [10]. When grown in the absence of folate $tRNA^{met}_f$ accumulates in *Streptococcus faecalis*, which contains instead of $GT\psi C$ the sequence $GU\psi C$. Thus the labeling of tRNA with $[^{14}CH_3]$ from methionine is not sufficient to elucidate the nature or the content of the methylated bases of tRNAs. Further experiments to clarify the biosynthetic pathway of m^5U in tRNAs of *B. subtilis* and of other grampositive micro-organisms are under way.

Acknowledgements

The authors are indebted to the Deutsche Forschungsgemeinschaft, Bad Godesberg/Bonn for a research grant.

We acknowledge with gratitude the skillful assistance of Ortrun Alter.

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