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Methylation of an adenosine in the D-loop of specific transfer RNAs from yeast by a procaryotic tRNA (adenine-1) methyltransferase

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ABSTRACT

tRNA (adenine-1) methyltransferase occurs in *Bacillus subtilis*. Eucaryotic $tRNA^{Thr}$ and $tRNA^{Tyr}$ from yeast in which 1-methyladenosine (m^1A) is already present in the TψC loop, can be methylated in vitro with S-adenosylmethionine and *B. subtilis* extracts. Each of the specific tRNAs accepts 1 mol of methyl groups per mol tRNA. The enzyme transforms into m^1A the 3'-terminal adenylic acid residue of the dihydrouridine loop, a new position for a modified adenosine residue in tRNA. Both tRNAs have the sequence Py-A-A-G-G-C-m_2^2G in the D-loop and D-stem region. Other tRNAs with the same sequence in this region also serve as substrates for the tRNA (adenine-1) methyltransferase.

INTRODUCTION

1-methyladenosine (m^1A) (1), a constituent of *B. subtilis* tRNA (2-4), has been shown to occur in $tRNA^{Tyr}$ of *B. subtilis* (5,6). The most abundant position of m^1A within the sequences of tRNAs is position 19 from the 3'-end. Exceptions were noted for $tRNA^{Phe}$ from rabbit liver and from calf liver in which a second m^1A is present in the dihydrouridine (D-loop) at position 14 (7). The tRNA (adenine-1) methyltransferase from chloroplasts and mitochondria of *Phaseolus vulgaris* methylates an adenylic acid residue in the acceptor stem of yeast $tRNA^{Asp}$ (8).

Agris et al. (9) reported recently that tRNA (adenine-1) methyltransferase from HeLa cells, which specifically methylates position 19 from the 3'-end transfers methyl groups to mature tRNA from *B. subtilis*. This result led us to suggest that the m^1A residue present in *B. subtilis* tRNA might be located at a site different from position 19. The tRNA (adenine-1) methyltransferase activity in extracts of *B. subtilis* has been measured with submethylated homologous tRNA (10) as

well as with heterologous tRNAs from *E. coli* (2) as substrates. We have now tested as to whether eucaryotic tRNAs in which m^1A is present in the TψC loop can serve as substrates for the *B. subtilis* enzyme.

Purified $tRNA^{Thr}$ and $tRNA^{Tyr}$ from yeast that have m^1A in molar amounts were found to accept one mol of methyl groups from S-adenosylmethionine with *B. subtilis* enzymes. The methyl moiety was found to be attached exclusively to the 1-position of adenosine. Since the sequences of both tRNAs are known (11,12) it was possible to determine the position in yeast $tRNA^{Thr}$ and in yeast $tRNA^{Tyr}$ that had become methylated. This was found to be the 3'-terminal residue of the D-loop.

MATERIALS AND METHODS

Materials: Bulk tRNA from yeast was from Boehringer, Mannheim, GFR. Sepharose 4B was from Pharmacia Uppsala, Sweden. Cellulose thin-layer plates (20x20 cm) were from Schleicher & Schüll, Dassel, GFR and DEAE-cellulose (n° 70, 0.9 meq/g) from Schleicher and Schüll, USA. DEAE paper (DE 81) for high voltage electrophoresis was from Whatman, Great Britain. Enzymes were from the following sources: RNAase T_2: Calbiochem; pancreatic RNAase, phosphomonoesterase (BAPF) and snake venom phosphodiesterase: Worthington. S-adenosyl-L-[Me-^{14}C] methionine was from the Radiochemical Centre Amersham, Great Britain, specific activity of 56 Ci/mol. Osray T4 films from Agfa-Gavaert were used for autoradiography.

Growth conditions: *B. subtilis W23* was grown in an enriched medium according to Doi (13). Cells were harvested during the logarithmic phase of growth.

Preparation of extracts: Frozen cells (10g) were ground with 25g alumina in a prechilled mortar. The resulting paste was suspended in 25 ml of buffer (20 mM triethanolamine-HCl, pH 8.0; 10 mM $MgCl_2$; 1 mM 2-mercaptoethanol; 10% ethyleneglycol). The supernatant from a low speed centrifugation was centrifuged at 105.000 x g for 2 h. The upper two thirds of the supernatant were frozen at -20°C. These extracts (S-100) retain tRNA (adenine-1) methyltransferase activity for at least two months. The protein content was determined according

to ref. 14.

Transfer RNAs were removed from S-100 preparations by passing the crude enzyme extract through 1 ml DEAE-cellulose columns. The tRNA (adenine-1) methyltransferase was eluted with the following buffer: 20 mM triethanolamine, pH 8.0; 10 mM $MgCl_2$; 1 mM 2-mercaptoethanol; 10% ethyleneglycol; 0.2 M NaCl. The eluate was immediately used for methylation.

Transfer RNA: Bulk tRNA from yeast was fractionated by counter current distribution as described previously (15). $tRNA^{Thr}$ was purified further by chromatography on Sepharose 4B with a decreasing gradient of ammonium sulfate (16). Details of the purification procedure will be published elsewhere (J. Weissenbach et al., in preparation). The purification of $tRNA^{Tyr}$ was achieved by the same method.

Methylation of tRNA: Reaction mixtures always contained per ml: 30 µmol triethanolamine-HCl, pH 8.0; 6 µmol 2-mercaptoethanol; 120 nmol of S-adenosyl-L-[Me-^{14}C] methionine (10.5 Ci/mol) or an equal amount of unlabeled S-adenosylmethionine; 6 A_{260} units of purified $tRNA^{Thr}$ or $tRNA^{Tyr}$ or 11.2 A_{260} units of bulk tRNA; enzyme fraction corresponding to 600-700 µg protein. For kinetic measurements the total reaction volume was 340 µl. At intervals, indicated in Fig. 1, samples of 50 µl were withdrawn and acid precipitable radioactivity was measured as described by Mans and Novelli (17). Methylations of tRNA for subsequent analyses of nucleotides and nucleosides were carried out with 3 A_{260} units of $tRNA^{Thr}$ or 6 A_{260} units of $tRNA^{Tyr}$. The incubation time was 4 h. The tRNAs were recovered from the reaction mixtures by chromatography on DEAE-cellulose columns as described previously (2). Methylations of tRNA for the determination of modified sites were as follows: 7 A_{260} units of the purified tRNAs were methylated with [^{14}C]-labeled S-adenosylmethionine at a specific activity of 10.5 Ci/mol; 50 A_{260} units of $tRNA^{Thr}$, respectively of $tRNA^{Tyr}$, were methylated with unlabeled S-adenosylmethionine. Incubations were carried out at 37° for 4 h. The tRNAs were recovered from the reaction mixtures as described above. [^{14}C]labeled tRNAs and non-labeled tRNAs were mixed.

Analysis of nucleotides, nucleosides and bases: In vitro methylated tRNA was digested to nucleotides with one unit of

RNAase T_2 in 100 µl 0.05 M ammonium acetate (pH 4.5) for 24 h at 37°C. The digest was chromatographed on thin-layer plates with solvents A and B. Nucleosides were obtained from RNAase T_2 digests by incubation with alkaline phosphatase in 100 µl 0.02 M ammonium formate (pH 7.5) for 1 h at 37°C. The digests were chromatographed according to Rogg et al. (18) with solvents C and D. In addition nucleotides and nucleosides were treated with 0.05 M $(NH_4)_2CO_3/NH_4OH$, pH 9.8 for 20 h at 50°C and chromatographed as the non-treated samples. In vitro methylated tRNA was hydrolysed in 88% formic acid for 2 h at 100°C (19). The resulting bases were chromatographed with 1-methyladenine and 7-methyladenine-markers in solvent E or F.

Solvent systems for thin-layer chromatography: A: isobutyric acid/NH_4OH (25 % NH_3)/H_2O (66:1:33) (20) B: HCl/propanol(2)/H_2O (17.6:68:14.4) (21) C: butanol(1)/isobutyric acid/NH_4OH (25 % NH_3)/H_2O (75:37.5:2.5:25) D: saturated ammonium sulfate/0.1 M sodium acetate, pH 6/propanol(2) (79:19:2) E: butanol(1)/H_2O (86:14) (22) F: acetonitril/ethylacetate/propanol(2)/butanol(1)/H_2O/NH_4OH(30 % NH_3) (40:30:20:10:5:22) G: NH_4OH (25 % NH_3)/propanol(1)/H_2O (30:60:10) (23).

Analysis of labeled nucleotide sequences: Hydrolysis of [Me-^{14}C] $tRNA^{Thr}$ or [Me-^{14}C] $tRNA^{Tyr}$ by pancreatic ribonuclease was performed according to Kuntzel et al. (24). Fractionation of the resulting oligonucleotides was achieved by DEAE-cellulose chromatography in the presence of 7 M urea. The radioactivity in each fraction was determined by counting 0.1 ml samples in 10 ml of dioxane scintillation fluid. The oligonucleotides were fractionated by high voltage electrophoresis on Whatman DE 81 paper. The radioactive oligonucleotides were further digested with phosphomonoesterase and with snake venom phosphodiesterase after removal of phosphomonoesterase (25). The non-radioactive oligonucleotides were hydrolysed by the combined action of phosphomonoesterase and phosphodiesterase. The resulting nucleotides and / or nucleosides were separated by two-dimensional thin-layer chromatography with solvent system A,B or G instead of A in the first dimension. The nucleotides and / or nucleosides were detected under UV light. The areas corresponding to UV-absorbing spots were scraped from

the tlc plates and eluted with 0.5 ml of water. Spectra were drawn from the eluates, and the amount of nucleotides respectively nucleosides were calculated from the absorbancy. Radioactivity was determined by counting samples of these eluates.

RESULTS

1. In vitro methylation of yeast tRNAs.

Bulk tRNA from yeast accepted considerable amounts of methyl groups from S-adenosyl-L-methionine upon incubation with *B. subtilis* extracts (Fig. 1). In order to find out which of the specific yeast tRNAs were acceptor molecules for methyl groups bulk yeast tRNA was fractionated by counter current distribution. Each of the 34 pools obtained was tested for methyl group acceptance (Fig. 2).

From the known distribution of amino acid accepting activities (15) the peaks that represent methyl group acceptance of tRNA could correspond to following specific tRNAs: peak A: $tRNA^{Thr}$, peak B: $tRNA_1^{Leu}$, peak C: $tRNA^{Ser}$ and $tRNA_3^{Leu}$, peak D: $tRNA^{Tyr}$. Purified $tRNA^{Thr}$ and $tRNA^{Tyr}$ accept methyl groups from S-adenosyl-L-methionine upon incubation with *B. subtilis* extracts

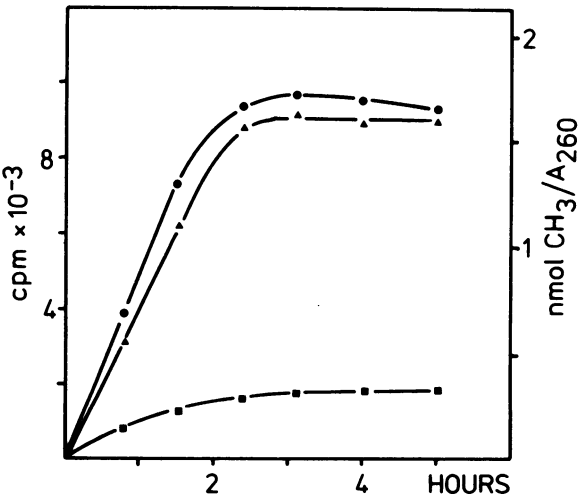

Fig. 1: Kinetics of methylation of tRNAs from yeast by *B. subtilis* extracts and S-adenosyl-L-[Me-^{14}C] methionine. Reactions were carried out as described under methods. Bulk tRNA: ■——■; $tRNA^{Thr}$: ▲——▲; $tRNA^{Tyr}$: ●——●.

(Fig. 1). The reaction was complete after 3 h. Each specific tRNA accepts one mol of methyl group per mol of tRNA.

2. Identification of the methylated nucleoside residue.

After the specific tRNAs had been methylated with *B. subtilis* extracts and S-adenosyl-L-[Me-^{14}C] methionine they were hydrolysed with RNAase T_2. The resulting 3'-mononucleotides were separated by two-dimensional thin-layer chromatography. Only one labeled product was detected after radioautography as shown for $tRNA^{Thr}$ as representative (Fig. 3A). The position of this product at the thin-layer chromatogram relative to the main nucleotides was found to be identical with that of 1-methyladenosine-3'-monophosphate. Since Agris et al. (26) have reported that 7-methyladenosine-3'-monophosphate migrates into a similar position, several experiments were done to confirm that position 1 of the adenylic acid residue had become methylated: (i) The nucleotides were hydrolysed to nucleosides and chromatographed in the system described by Rogg et al. (18). The labeled compound was found to migrate into an identical position as m^1A. (ii) Incubation at alkaline pH converted the methylated product into a nucleotide that comi-

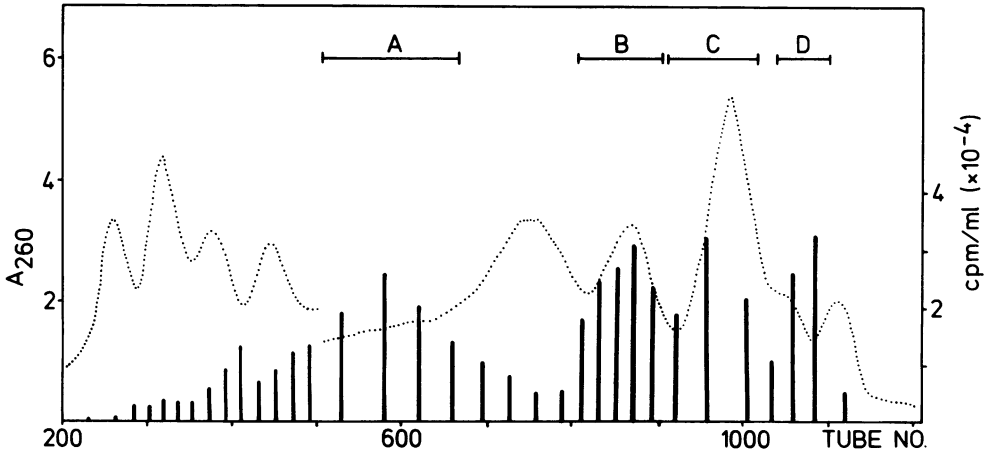

Fig. 2: Counter-current distribution of bulk yeast tRNA. The distribution was performed as described by Dirheimer and Ebel (15). The pools were tested for methyl group acceptance with *B. subtilis* extracts and S-adenosyl-L-[Me-^{14}C] methionine (bars). A_{260}:

grated with m^6Ap upon chromatography in solvent A and B (Fig. 3B). Correspondingly, the methylated nucleoside, after alkali treatment, comigrated with m^6A in solvent systems C and D. It has been shown (27) that m^1A (nucleotide or nucleoside) rearranges to m^6A under these conditions, whereas m^7A should undergo a ring opening reaction comparable to the ring cleavage of m^7G. (iii) The radioactive base released from [Me-^{14}C] $tRNA^{Thr}$ by formic acid hydrolysis cochromatographs with 1-methyladenine in solvents E and F, whereas 7-methyladenine is well separated from 1-methyladenine. We therefore conclude that the methyl group is attached to the position one of the adenylic acid residue. The analyses of the methylated nucleotide from $tRNA^{Tyr}$ gave the same results.

3. Sites of $tRNA^{Thr}$ and of $tRNA^{Tyr}$ methylated in vitro.

[Me-^{14}C] $tRNA^{Thr}$ and [Me-^{14}C] $tRNA^{Tyr}$ were hydrolysed with pancreatic ribonuclease and the resulting oligonucleotides fractionated according to their chain length by chromatography on DEAE-cellulose (Fig. 4 A,B). The elution profiles obtained for both tRNAs agree well with those described for $tRNA^{Thr}$ (J. Weissenbach et al., to be published) or $tRNA^{Tyr}$ (28) and

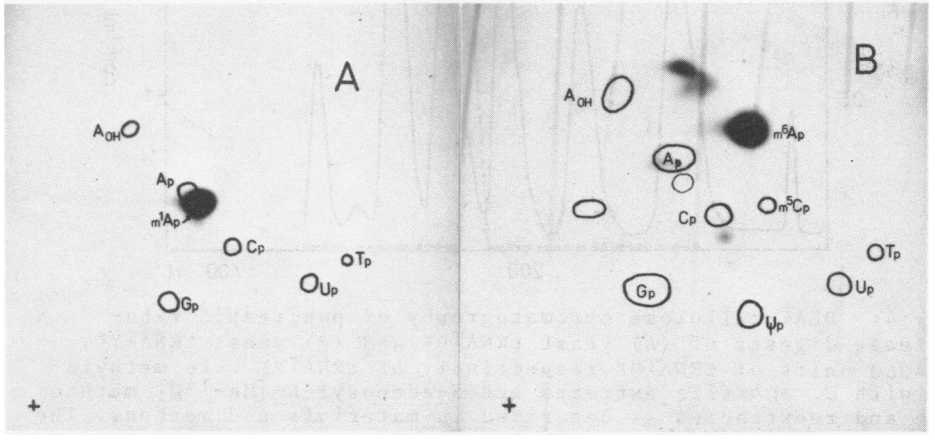

Fig. 3: Patterns of RNAase T_2 digests of yeast $tRNA^{Thr}$ after incubation with *B. subtilis* enzymes and S-adenosyl-L-[Me-^{14}C] methionine. The nucleotides were resolved by two-dimensional thin-layer chromatography in solvents A (bottom to top) and B (left to right). (A) Nucleotides untreated; (B) nucleotides incubated at pH 9.8 as described in methods.

allowed the assignment of the chain lengths of the individual peaks.

Only one peak of radioactivity was obtained in both cases that was eluted between the tri- and the tetranucleotide peak.

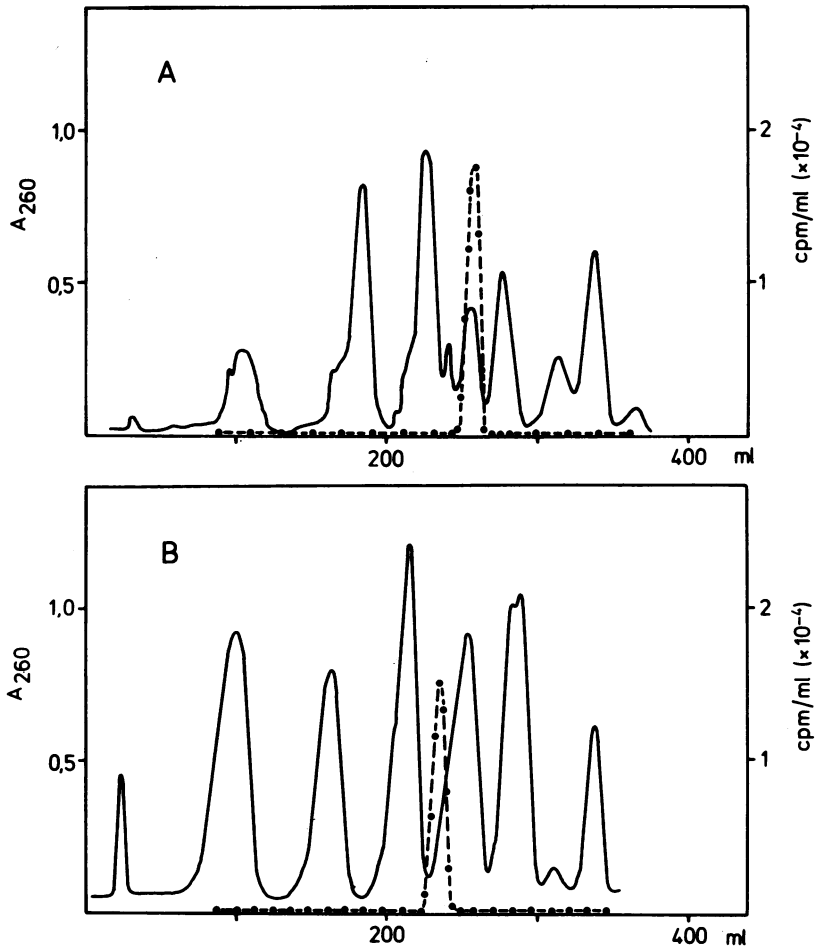

Fig. 4: DEAE-cellulose chromatography of pancreatic ribonuclease digests of (A) yeast $tRNA^{Thr}$ and (B) yeast $tRNA^{Tyr}$. 57 A_{260} units of $tRNA^{Thr}$ respectively of $tRNA^{Tyr}$ were methylated with *B. subtilis* extracts and S-adenosyl-L-[Me-^{14}C] methionine and reextracted as described in materials and methods. The tRNAs were then digested with 260 ug of pancreatic ribonuclease in 0.1 M Tris-HCl, pH 7.5, in a total volume of 1 ml for 3 h at 37°C. The digests were fractionated on DEAE-cellulose columns, 0.6x68 cm in (A), 0.6x60 cm in (B). Elution was performed with a linear gradient of NaCl (0-0.4M) in 7 M urea, 0.02 M Tris-HCl, pH 7.5 (total volume:500 ml). A_{260}: ——; radioactivity/ml: o--o.

The radioactive oligonucleotide could therefore probably be derived from a pentanucleotide that had acquired an extra positive charge by methylation. Oligonucleotides carrying a positively charged m^1A residue usually elute more than one chain-length earlier than oligonucleotides of the same chain-length without a modified base (29). The only pentanucleotide obtained upon digestion of $tRNA^{Thr}$ with pancreatic ribonuclease has the sequence A-A-G-G-C suggesting that this might be the sequence that had become methylated. This suggestion is supported further by the observation that the pentanucleotide peak is considerably smaller than the corresponding peak of unmodified $tRNA^{Thr}$. Digestion of $tRNA^{Tyr}$ by pancreatic tRNAase yields three pentanucleotides, A-A-G-A-C, G-A-G-A-D and A-A-G-G-C. Analysis of the pentanucleotide peak after chromatography of the hydrolysate of methylated $tRNA^{Tyr}$ showed a decrease in the content of A-A-G-G-C relative to the other pentanucleotides. This again suggests that the sequence A-A-G-G-C had become methylated.

The radioactive olignucleotides were separated from other oligonucleotides by high voltage electrophoresis (Fig. 5 A,B). The radioactive oligonucleotides from both tRNAs had practically the same mobilities relative to the marker B. The radioactive peak of $tRNA^{Thr}$ contained a molar amount of t^6A-A-ψ. The radioactive oligonucleotide of $tRNA^{Tyr}$ was slightly contaminated with several tetranucleotides which had eluted just behind in DEAE-cellulose chromatography. After elution the overall composition and the 5'-terminal residue of the labeled oligonucleotides were analysed.

The methylated oligonucleotides were reacted with phosphomonoesterase to remove the 3'-phosphate. After separation from the phosphomonoesterase, they were hydrolysed with snake venom phosphodiesterase yielding 5'-mononucleotides and a nucleoside from the 5'-end of the oligonucleotide. The products of phosphodiesterase digestion were separated by two-dimensional thin-layer chromatography. Quantitative analyses (Table 1) resulted in one A_{OH}, one $p[^{14}C]m^1A$, two pG and one pC.
We therefore conclude that the sequence A-A-G-G-C had become methylated and that the methyl group had exclusively been transferred to the internal adenylic acid residue of this sequence.

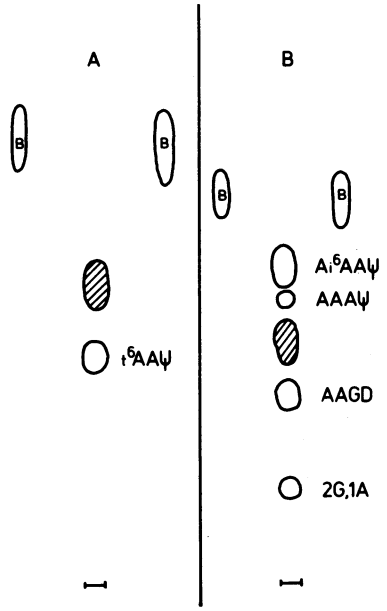

Fig. 5: Electrophoretic separation on DEAE-cellulose paper of the oligonucleotides recovered from the radioactive fractions of the DEAE-cellulose columns (Fig. 4 A,B). The oligonucleotides were submitted to electrophoresis on DE 81 paper in 7% formic acid. A: $tRNA^{Thr}$, 6 h at 1800 V, B: $tRNA^{Tyr}$, 12 h at 900 V. The ultraviolet absorbing spots were eluted from the paper with 1 M triethylammonium-bicarbonate buffer, pH 8.2. Samples from the eluates were counted in a liquid scintillation spectrometer. The shaded areas contained all the radioactivity. The other spots were assigned by analysing their nucleoside composition and compairing them with the oligonucleotide composition of $tRNA^{Thr}$ (11) and $tRNA^{Tyr}$ (28) respectively. B: blue marker xylene cyanol FF.

Table I: Analysis of dephosphorylated radioactive oligonucleotides by snake venom phosphodiesterase digestion and thin layer chromatography

	A	pm^1A (a)	pG	pC
$tRNA^{Thr}$	0.9	0.9	2.1	1
$tRNA^{Tyr}$	0.85	0.9	2.05	1

(a) In both cases 97% of the radioactivity was recovered in the pm^1A spot

The position of this residue within the sequences of $tRNA^{Thr}$ and $tRNA^{Tyr}$ is the 3'-terminal nucleotide of the dihydrouridine-loop (Fig. 6).

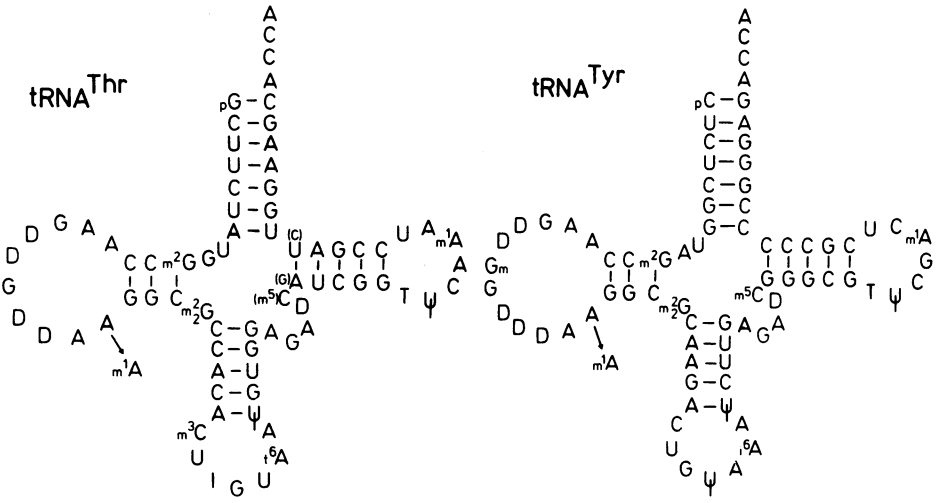

Fig. 6: Nucleotide sequence of yeast $tRNA^{Thr}$ (11) and yeast $tRNA^{Tyr}$ (12). The arrows denote the adenylic acid residue that becomes methylated by *B. subtilis* tRNA (adenine-1) methyltransferase.

DISCUSSION

Eucaryotic tRNAs have considerably higher amounts of modified nucleosides than procaryotic tRNAs. With the exception of some ribothymidine lacking tRNAs from wheat germ and rat liver, that can be methylated by the *E. coli* tRNA (uracil-5) methyltransferase (30, 31), eucaryotic tRNAs do not serve as substrates for procaryotic tRNA methyltransferases.

We describe here another exception: The tRNA (adenine-1) methyltransferase from *B. subtilis* methylates an adenosine residue in yeast $tRNA^{Thr}$ and $tRNA^{Tyr}$. A modified adenosine residue has not been found in yeast tRNAs in that position suggesting that a corresponding enzyme does not occur in yeast.

The extent of methylation of the specific tRNAs from yeast by *B. subtilis* extracts is one mol of methyl group per mol of tRNA when reactions are carried out on an analytical scale (Fig. 1). However, for reasons not yet known, large scale methylations usually yield lower extents of methylation. Therefore in oligonucleotide preparations from digests of methylated tRNA

always the homologous unmodified oligonucleotide sequence is present (Fig. 4).

The identity with 1-methyladenosine of the product formed in tRNA during the transmethylation reaction was concluded from the following data: (i) The chromatographic behaviour of the methylated nucleotide or nucleoside; (ii) the rearrangement under alkaline conditions into a product that comigrates with 6-methyladenosine (m^1A is known to rearrange to m^6A under these conditions). Agris et al. (26) have recently described the presence of 7-methyladenosine in tRNA of *B. stearothermophilus*. 7-methyladenosine-3'-phosphate behaves like 1-methyladenosine-3'-phosphate in solvent systems A and B used in our experiments. The methylated product upon hydrolysis to the base was found to cochromatograph with 1-methyladenine and not with 7-methyladenine.

In yeast $tRNA^{Thr}$ and $tRNA^{Tyr}$ one 1-methyladenosine residue is present in the TψC-loop, therefore the enzyme must recognize another adenosine residue in both tRNAs. Sequence studies unequivocally showed that the 3'-terminal adenylic acid residue of the D-loop had been transformed into 1-methyladenosine. This is a new position of an adenosine residue methylated by a specific tRNA methyltransferase in vitro. Both $tRNA^{Thr}$ and $tRNA^{Tyr}$ have an identical sequence D-A-A^+-G-G-C-m_2^2G-C in the region of the adenosine residue that becomes methylated (denoted by (A^+). Several other tRNAs were also tested as substrates for the tRNA (adenine-1) methyltransferase from *B. subtilis*. Preliminary experiments revealed that $tRNA_1^{Leu}$, $tRNA_3^{Leu}$ and $tRNA^{Ser}$ from yeast accept methyl groups. These tRNAs have the common sequence Py-A-A-G-G-C-m_2^2G in the D-loop/D-stem region. If this specificity of the enzyme is general it would be the first case where a modification is associated with a specific long oligonucleotide sequence which could be considered as a specific enzyme recognition site. It must also be emphasized that our result is not in favour of the hypothesis of Hildesheim et al. (32) who stated that in order to be methylated a nucleotide must be adjacent to an U or a derivative of U.

Finally, yeast $tRNA^{Thr}$ and yeast $tRNA^{Tyr}$ are heterologous substrates for tRNA (adenine-1) methyltransferase from *B. subtilis*. In *B. subtilis* $tRNA^{Tyr}$ m^1A is present in molar

amounts (5,6). At the present time we do not know the position of m^1A in *B. subtilis* $tRNA^{Tyr}$. Sequence analysis of *B. subtilis* $tRNA^{Tyr}$ is underway to clarify as to whether sequence and site specificity of this procaryotic enzyme is maintained when heterologous eucaryotic tRNAs are used as substrates.

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