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Occurrence of 1-Methyladenosine and Absence of Ribothymidine in Transfer Ribonucleic Acid of *Mycobacterium smegmatis*

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The minor base composition of *Mycobacterium smegmatis* tRNA has been studied. Thin-layer chromatographic patterns of a ribonuclease T2 digest of mycobacterial tRNA indicated the presence of appreciable amounts of 1-methyladenosine (which is commonly present only in eucaryotic tRNA), dihydrouridine, and 7-methylguanosine. Ribothymidine was absent. The *S*-adenosylmethionine-dependent tRNA methylases of *M. smegmatis* catalyzed the formation of 1-methyladenosine when *Escherichia coli* tRNA was used as acceptor. Similarly, *E. coli* extracts methylated the tRNA of *M. smegmatis*, forming ribothymidine.

In the course of our work on tRNA methylase of *Mycobacterium* (11) we found that extracts of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* were able to methylate *Escherichia coli* tRNA. This finding led us to investigate the content of methylated nucleotides in tRNA of mycobacteria to find out whether these cells contain modified nucleosides in tRNA different from those present in *E. coli*. This paper reports a comparison of the methylated nucleosides and tRNA methylases in the two organisms.

The salient features of the minor base composition of *M. smegmatis* tRNA are (i) the absence of ribothymidine, which is present in most organisms, and (ii) the presence of 1-methyladenosine (m^1A), which is more prevalent in eucaryotes.

MATERIALS AND METHODS

Chemicals. DEAE-cellulose, RNase T2, and *E. coli* tRNA were obtained from Sigma Chemical Co., St. Louis, Mo. *S*-Adenosyl L-[^{14}C-CH_3]methionine (specific activity, 59 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. Thin-layer plates coated with cellulose powder were purchased from Funakoshi Co. Ltd., Tokyo, Japan. Other chemicals were of reagent grade. [^{32}P]orthophosphate (carrier free) was obtained from Bhabha Atomic Research Center, Bombay, India and the Japanese Atomic Energy Research Institute.

Bacterial strains and growth conditions. *M. smegmatis* SN_2 was grown at 37°C in Youmans and Karlson's minimal medium (14) with 0.1% Tween-80 to avoid cell clumping. *E. coli* D10 (*rel met arg*) was grown in nutrient broth for preparation of tRNA methylase and in the minimal medium of Youmans and Karlson, supplemented with L-arginine (30 mg/liter) and L-methionine (20 mg/liter), for preparation of methyl-deficient tRNA. *E. coli* B was grown in nutrient broth.

Isolation of tRNA. *M. smegmatis* tRNA was isolated from the cells as described by Deobagkar and Gopinathan (4). Cells were disrupted either by grinding with alumina (2 g/g of cells) or by toluenization (final concentration, 1% toluene in ethanol). The tRNA obtained was finally purified by DEAE-cellulose column chromatography as described previously (4, 8).

Isolation of tRNA methylases. The cells were ground with twice their weight of alumina and extracted with 10 mM Tris-hydrochloride (pH 7.8)-1 mM EDTA-2 mM β-mercaptoethanol-10% glycerol (2 ml of buffer per g of cells). The cell extract was centrifuged at 7,000 rpm for 15 min and then at 20,000 $\times g$ for 20 min. This S-20 preparation was centrifuged at 100,000 $\times g$ for 60 min in a Beckman model L2-50 ultracentrifuge, and the resulting S-100 preparation was used as the source of enzyme in the assays.

Assay of tRNA methylase activity. tRNA methylase activity was assayed as described by Taya and Nishimura (12). Incubation was carried out for 30 min.

Analysis of ^{14}C-methylated nucleotides. The methylation reaction was carried out in 200 μl of reaction mixture containing 2 absorbancy units at 260 nm of tRNA as specified and 800 μg of protein as enzyme source, as described previously (12). ^{14}C-methylated tRNA was isolated and digested with RNase T2, and the digest was subjected to two-dimensional thin-layer chromatography (9, 12). The solvent systems used were isobutyric acid-0.5 N NH_4OH (5:3, vol/vol) in the first dimension and isopropanol-concentrated HCl-water (70:15:15, vol/vol/vol) in the second dimension. The thin-layer plates were subjected to autoradiography.

Analysis of ^{32}P-labeled nucleotides. The ^{32}P-labeled tRNA was isolated from *M. smegmatis* cells

grown in phosphate-deficient medium containing 100 μCi of inorganic ^{32}P in 5 ml of culture medium. Phosphate was removed from the medium as follows. One liter of medium (1% tryptone [Difco], 1% yeast extract [Difco], 2% glucose, 0.5% NaCl) was stirred with 5 ml of 1 M $MgSO_4$ and 5 ml of concentrated NH_4OH for 30 min at room temperature. Insoluble material was removed by filtration through a membrane filter (Millipore Corp.), and the filtrate was adjusted to pH 6.8 to 7.0 with HCl.

RESULTS

The tRNA methylase activity of the S-100 preparation from *M. smegmatis* was measured with various tRNA's as substrates. The ability of *M. smegmatis* tRNA to act as a substrate for *E. coli* tRNA methylase was also tested. Significant amounts of methyl groups were incorporated into *M. smegmatis* tRNA by *E. coli* tRNA methylase and into normal *E. coli* tRNA by *M. smegmatis* tRNA methylase (Table 1).

For identification of the ^{14}C-methylated nucleotides formed in vitro, tRNA was isolated from the reaction mixture and hydrolyzed with RNase T2, and the digest was analyzed by two-dimensional thin-layer chromatography (9). tRNA methylase from *M. smegmatis* catalyzed the formation of m^1A when *E. coli* tRNA was used as acceptor (Fig. 1). On the contrary, *E. coli* tRNA methylase catalyzed the formation of ribothymidine into *M. smegmatis* tRNA. These results suggest that *M. smegmatis* tRNA contains m^1A but lacks ribothymidine.

For confirmation of these results, ^{32}P-labeled tRNA was isolated from cells of *M. smegmatis* grown in the presence of ^{32}P. ^{32}P-labeled *M. smegmatis* tRNA was hydrolyzed, and the digest was analyzed by two-dimensional thin-layer chromatography (Fig. 2). As expected from the previous in vitro methylation experiment, no ribothymidine 3′-phosphate was detected in the chromatogram of the digest of unfractionated *M. smegmatis* tRNA. Table 2 shows the amount of radioactivity recovered in each nucleotide. The presence of m^1A in *M. smegmatis* tRNA is evident, because the nucleotide of 6-methyladenosine was detected in an alkaline digest of the tRNA, but not in an RNase T2 digest. 6-Methyladenosine must be formed from m^1A in alkali (5). m^1A itself will not be evident in an RNase T2 digest, since the corresponding radioactive spot will merge with that of adenosine 3′-phosphate. From these data it is reasonable to conclude that in *M. smegmatis* almost all the tRNA species lack ribothymidine, and that a

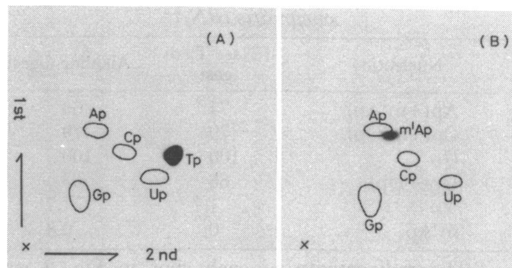

FIG. 1. *Two-dimensional chromatographic pattern of RNase T2 digests of the products of in vitro methylation. Composite tracing of autoradiogram. (A) tRNA of M. smegmatis methylated by E. coli S-100. (B) tRNA of E. coli methylated by M. smegmatis S-100.*

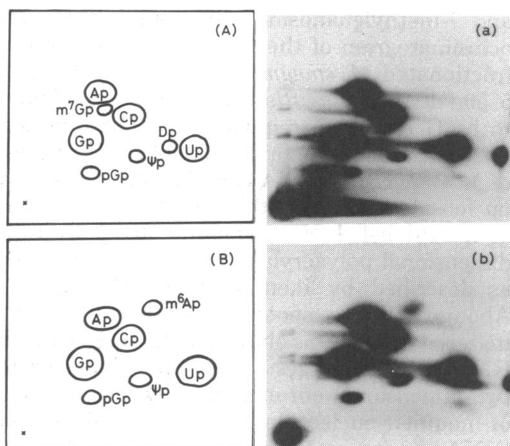

FIG. 2. *Analysis of ^{32}P-labeled nucleotides from RNase T2 and alkaline digests of M. smegmatis tRNA. Autoradiogram of thin-layer chromatogram of (a) RNase T2 digests; (b) alkaline digests. (A) and (B) Schematic diagrams of (a) and (b), respectively. Alkaline hydrolysis was carried out in 0.3 N KOH. The hydrolysate was neutralized with Dowex 50 (H^+ form) and spotted onto a thin-layer plate. m^6A, 6-Methyladenosine; m^7G, 7-methylguanosine; D, dihydrouridine; ψ, pseudouridine.*

TABLE 1. *Comparison of the methylating activities of E. coli and M. smegmatis extracts with different tRNA's as substrate*

Source of enzyme	Source of tRNA	Methyl groups incorporated into tRNA (pmol/min)
E. coli S-100	*E. coli* (normal)	0
	E. coli (methyl deficient)	3.8
	M. smegmatis	2.3
M. smegmatis S-100	*E. coli* (normal)	2.2
	E. coli (methyl deficient)	5.1
	M. smegmatis	0

TABLE 2. *Content of major nucleotides in M. smegmatis tRNA* [a]

Nucleotide	RNase T2 digest	Alkaline digest
Ap(+m^{1}Ap)	74	66
Cp(+m^{7}Gp)	79	79
Gp	100	100
Up(+Dp)	66	63
Ψp	1.7	1.7
m^{6}Ap	0	0.8

[a] The radioactivity of each spot in Fig. 2 was counted and calculated relative to Gp (100).

large proportion of the tRNA species contain m^{1}A.

It is also noteworthy that *M. smegmatis* tRNA contains fewer modified nucleosides than *E. coli* tRNA: only m^{1}A, pseudouridine, dihydrouridine, and 7-methylguanosine were found in a radiochromatogram of the RNase T2 digest of unfractionated *M. smegmatis* tRNA (Fig. 2). The 5′ end of *M. smegmatis* tRNA was usually found to be G and, less frequently, U. The question arises whether a minor fraction of tRNA species of *M. smegmatis* tRNA contains other modified nucleosides. To clarify this problem, we fractionated ^{32}P-labeled *M. smegmatis* tRNA by two-dimensional polyacrylamide gel electrophoresis as described by Ikemura and Dahlberg (7). About 18 distinct spots were obtained, and the material in each was eluted and hydrolyzed with RNase T2. The digests were then subjected to two-dimensional chromatography for detection of modified nucleotides. None of the digests of tRNA species contained the 3′ nucleotide of 2-methyladenosine, 6-methyladenosine, 4-thiouridine, 5-methylaminomethyl-2-thiouridine, uridine-5-oxyacetic acid, or 2′-methylguanosine. In addition, no unknown modified nucleoside was detected in any digest. However, small amounts of *N*-[*N*-(9-*β*-D-ribofuranosylpurin-6-yl)carbamoyl]threonine, N^6-isopentenyladenosine (we cannot exclude the possibility that this material may be 2-methylthio-N^6-isopentenyladenosine), and Q-nucleoside were detected in chromatograms of some of the digests.

DISCUSSION

The most unusual feature of modified nucleosides in *M. smegmatis* tRNA is the complete absence of ribothymidine. It is reasonable to speculate that ribothymidine in the TψC region is replaced by unmodified uridine in *M. smegmatis* tRNA, since *E. coli* tRNA methylase can catalyze the formation of ribothymine in *M. smegmatis* tRNA. Ribothymidine is present in the tRNA of most organisms, and among procaryotes so far only *Methanococcus vannielii* (2) and *Micrococcus luteus* (3) are reported to lack ribothymidine. (Schmidt et al. [10] reported that *M. luteus* contains ribothymidine.)

m^{1}A was commonly present in *M. smegmatis* tRNA. The location of this m^{1}A in *M. smegmatis* tRNA was not determined, but it probably occupies the 19th position from the CCA and where m^{1}A is generally located. m^{1}A is more common in eucaryotic tRNA, but it is also known to be present in the procaryotes *Bacillus subtilis* (1), *M. vannielii* (2), and *Thermus thermophilus* (13).

The contents of other modified nucleosides in *M. smegmatis* tRNA are very low. In this respect, *M. smegmatis* tRNA is similar to mycoplasma tRNA (6). It will be interesting to find out whether the unique features of *M. smegmatis* tRNA have any influence on the function of tRNA in this bacterium. It will also be interesting to know whether, in connection with the low content of modified nucleosides, the secondary structure of *M. smegmatis* tRNA is specifically different from those of tRNA's from other sources. Work on these problems is in progress in our laboratories.

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