Methyl-deficient mammalian transfer RNA: II. Homologous methylation in vitro of liver tRNA from normal and ethionine-fed rats: ethionine effect on 5-methylcytidine synthesis in vivo

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ABSTRACT

Following hydroxyapatite chromatography, rat liver tRNA methylase activity was assayed with liver tRNA from normal rats and with methyl-deficient liver tRNA from ethionine-fed rats. The difference in homologous methylation between normal and methyl-deficient tRNA was maximal in certain fractions in presence of cadaverine, and much less in presence of Mg^{++} or Mg^{++} plus cadaverine. These methylase fractions, which contained endogenous tRNA, were used for preparative homologous methylation of added normal and methyl-deficient tRNA in presence of 30 mM cadaverine. The ^{14}C -methylated tRNA was digested with RNase T_2 and the resulting methylated mononucleotides were characterized and quantitated after twodimensional thinlayer chromatography and autoradiography. The major products of homologous tRNA methylation were m^5C and m^1A . However, the methylase fraction used here did not catalyze the formation of m^2A with m^2A -deficient tRNA as substrate. In addition to the previously described, analytically detectable m^2A -deficiency, a partial m^5C -deficiency was demonstrated in liver tRNA from ethionine-fed rats by measuring the methyl-acceptance in vitro. In presence of cadaverine, with the methylase fraction used here, methyl-deficient tRNA from ethionine-fed rats was a twofold more efficient methyl-acceptor in vitro than normal liver tRNA, while endogenous tRNA isolated from the methylase fraction was a threefold more efficient methyl-acceptor than normal liver tRNA. Homologous methylation of normal tRNA, as observed here, has not been described before.

INTRODUCTION

We have demonstrated recently that N^6 -dimethyladenosine is a component of rat liver tRNA, and that liver tRNA from L-ethionine-fed rats however lacks this minor nucleoside¹, which was also found in B. subtilis tRNA². The methyl-deficiency of liver tRNA from L-ethionine-fed rats with respect to N^6 -dimethyladenosine was explained by a possible inhibition in vivo of the corresponding tRNA methylase by S-adenosyl-ethionine, the primary ethionine metabolite^{3,4,5}. Therefore it

was of interest to find out whether S-adenosylethionine inhibits m_2^6A -methylase alone. A qualitative nucleotide analysis of normal tRNA and tRNA from ethionine-fed rats had allowed this conclusion¹.

In order to establish quantitative differences between normal and methyl-deficient tRNA⁺ we studied and compared the homologous methylation of normal and methyl-deficient rat liver tRNA in vitro.

MATERIALS AND METHODS.

L-Ethionine and cadaverine dihydrochloride were bought from Fluka AG, RNase T₂ (Sankyo) was from Calbiochem. (Methyl¹⁴C) S-Adenosylmethionine (spec. act. 58 ci/M) was from NEN Chemicals GmbH. Precoated thinlayer plates (cellulose without fluorescence indicator) were from Merck AG, Darmstadt. Solvents for cellulose thinlayer chromatography (TLC) were: (solvent A) 2-propanol - conc. ammonia - water (7:1:2); (solvent B) isobutric acid - conc. ammonia - water (66:1:33) pH 3.7; (solvent C) 1-propanol - conc. ammonia - water (55:10:35); (solvent D) 1-butanol - water (85:15); (solvent E) 1-butanol - acetic acid - water (45:5:12.5); (solvent F) methanol - water (75:25); (solvent G) isobutric acid - conc. ammonia - water (577:38:385) pH 4.3; (solvent H) t-butanol - conc. HCl - water (7:1.5:1.5). Hydroxyapatite (Bio-Gel HTP) was from Bio-Rad Laboratories. Methylated nucleosides were bought from PL-Biochemicals Inc. or from Cyclo Chemicals.- A mixed pool of livers from male and female adult rats was used for tRNA and tRNA methylase preparation. tRNA was prepared as described by Rogg et al.⁶. Methyl-deficient tRNA from rat liver was obtained after L-ethionine feeding of female Wistar rats for four weeks as described previously¹. tRNA methylases were partially purified by hydroxyapatite chromatography, ammonium sulfate precipitation and dialysis as described earlier^{7,8,9}. Endogenous tRNA in this methylase preparation was quantitated as follows: 1 ml methylase containing 39 mg protein was twice phenolized, the combined phenol layers were re-extracted with buffer, and the combined

⁺ "methyl-deficient tRNA" refers to submethylated tRNA from the livers of ethionine-fed rats.

aqueous layers were once more treated with phenol. Phenol from the aqueous phase was extracted with ether and put onto a 2.6 (l) x 0.8 (d) cm DEAE column equilibrated with 0.1 M sodiumacetate pH 5.0. Nucleotide material was eluted with 0.3 M NaCl in the same buffer and 4.1 A₂₆₀ units of tRNA were eluted as a single peak between 0.6 - 0.8 M NaCl with a 60 ml gradient of 0.3 - 1.0 M NaCl in the above buffer. The tRNA was pooled after ethanol precipitation and characterized by electrophoresis in 11 % polyacrylamide in 6 M urea¹⁰. Assay for tRNA methylase activity was performed as before⁸ with the following differences: 120 µl contained 0.63 nmoles (methyl¹⁴C) S-adenosylmethionine (spec. act. 58 ci/mole) and 30 mM cadaverine as indicated in legend to Fig. 1. For preparative homologous tRNA methylations, 2 A₂₆₀ units of rat liver tRNA were incubated for 180 min. at 37°C in 30 mM cadaverine (no Mg⁺⁺) with corresponding amounts of the other components in 4 ml volume; 85 nmoles of (methyl¹⁴C) S-adenosylmethionine and 23.4 mg of protein from the methylase fraction containing 2.46 A₂₆₀ units of endogenous tRNA were used. The *in vitro* methylated tRNA was isolated by phenolization of the incubation mixture followed by adsorption to a 2 (l)x 1(d) cm DEAE cellulose column. The column was washed with 15 ml 0.3 M NaCl and the ¹⁴C-methylated tRNA was eluted with 12 ml of 1 M NaCl and pooled after ethanol precipitation. RNase T₂ digestion of tRNA and twodimensional TLC were performed as before¹. Radioactive spots from twodimensional TLC were located by autoradiography, scraped out and the radioactivity was measured in toluene-PPO (1000:6 - v:w) using a Packard Tricarb Model 3380. The yields of ¹⁴C-methylated nucleotides are presented in detail in Table 2. For further identification the radioactive nucleotides were re-isolated from the scintillation mixture as follows: after filtration through a Pasteur pipette with a cotton wool plug, the remaining cellulose powder was washed with toluene (20 ml), ether (30 ml) and ethanol (1 ml). The radioactive nucleotide was then eluted with 1 ml 50 mM triethylammonium bicarbonate pH 8.5.-

The methyl-acceptance of the endogenous tRNA introduced with the methylase (4.1 A₂₆₀ units of tRNA per 39 mg protein

per ml) was determined by two methylation experiments without added tRNA, without and with ethidium bromide, which selectively inhibits tRNA methylation but does not affect protein methylation⁸.

RESULTS AND DISCUSSION

We have shown recently that N⁶-dimethyladenosine is absent in liver tRNA of L-ethionine-fed rats¹; however, other differences in the nucleotide composition between normal and methyl-deficient tRNA were not detected by the methods applied.

Fig. 1. A: HAP chromatography of rat liver methylase^{7,8} and assay for methylase activity in presence of 30 mM cadaverine. ▲---▲ Protein methylation (no rat liver tRNA added to the assay); ■---■ liver tRNA from normal rats added to the assay; ●---● liver tRNA from L-ethionine-fed rats added to the assay.

B: The difference between methyl-acceptance ("Δcpm") of normal and methyl-deficient tRNA from A was plotted in order to locate those fractions which give optimal methylation of methyl-deficient tRNA. Fractions 94 to 106 were pooled, precipitated with ammonium sulfate, dialyzed as described⁸ and used for preparative methylations. The Δcpm were less than half of the above values if the enzymatic methylation was performed in presence of 10 mM Mg⁺⁺ or 10 mM Mg⁺⁺ plus 30 mM cadaverine (not shown here).

In order to find out whether liver tRNA from L-ethionine-fed rats is methyl-deficient with respect to other methylated nucleotides and whether N⁶-dimethyladenosine can be synthesized on the tRNA level in vitro, we compared homologous methylation of normal and methyl-deficient rat liver tRNA. The fractionation of rat liver tRNA methylase is shown in Fig. 1A. The assay allowed us to locate those methylase fractions which give optimal methylation of methyl-deficient rat liver tRNA compared with normal tRNA. The difference in methyl-acceptance of normal and methyl-deficient rat liver tRNA was optimal in presence of cadaverine without Mg⁺⁺ (Fig. 1B); preparative homologous methylations were therefore performed in 30 mM cadaverine. In vitro methylated normal and methyl-deficient tRNA (including endogenous tRNA from the methylase) was digested with RNase T₂ and the mononucleotides were separated by twodimensional TLC followed by autoradiography. Six ¹⁴C-methyl nucleotides were found from both types of tRNA (Fig.2.).

Fig.2. Twodimensional TLC of RNase T₂ digests of rat liver tRNA methylated with rat liver methylase in vitro. A: tRNA from normal rats; B: tRNA from L-ethionine-fed rats. Solvent G was used for the first dimension, solvent H for the second dimension. The radioactive nucleotides (shaded spots) were scraped out and the radioactivity was measured (Table 1). White spots: nucleotides visible in UV light (from added carrier nucleotides). Nucleotides 1,2,3 and 4 are unidentified.

These spots were scraped out and the radioactivity was determined. The major products of homologous tRNA methylation in vitro were characterized as m^5C and m^1A by cochromatography with authentic material (Table 1) after re-isolation from the scintillation liquid.

	solvents and R_f values					
	A	B	C	D	E	F
" m^1Ap " from 2-D TLC (Fig.2)	0,1	0,52	0,50	-	-	-
authentic m^1Ap	0,1	0,52	0,50	-	-	-
" m^5Cp " from 2-D TLC (Fig.2) after dephosphorylation	0,51	0,76	-	0,14	0,20	0,61
authentic m^5C	0,51	0,76	-	0,14	0,21	0,61

Table 1. R_f values, on 5 cellulose thinlayer plates, of authentic m^1Ap and m^5C compared with values of radioactive material isolated from twodimensional TLC (Fig.2). " m^5Cp " (Fig. 2) was dephosphorylated with bacterial alkaline phosphatase, since only the m^5C nucleoside marker was available.

The yields of ^{14}C -methylated nucleotides are summarized in Table 2. From this table as well as from Fig. 2 it can be seen that N^6 -dimethyladenosine was not a product of homologous tRNA methylation under the conditions applied here, albeit this nucleoside is absent in methyl-deficient tRNA from ethionine-fed rats¹. The lack of N^6 -dimethyladenosine synthesis on the tRNA level in vitro may be due to the following reasons, (a) we may have lost the m_2^6A methylase in those HAP fractions (Fig. 1) which have not been pooled; (b) the preparative tRNA methylations were performed in presence of cadaverine which might inhibit the m_2^6A methylase.-- m^1A formation was found to be identical in normal and methyl-deficient tRNA. However, significantly more m^5C was formed in methyl-deficient rat liver tRNA (Table 2) compared with normal tRNA. This indicates that S-adenosyl-ethionine in the livers of ethionine-fed rats^{3,4,5} does not inhibit the m^1A methylase. In contrast, the presence of

	A cpm from the TLC plate Fig. 2A (normal tRNA)	B cpm from the TLC plate Fig. 2B (methyldeficient tRNA)
m^5Cp	812	1315
m^1Ap	870	877
1	38	28
2	268	272
3	50	108
4	10	24
total cpm in 0.27 A_{260} units tRNA	2048	2624
cpm (methylation of 0.15 A_{260} units of endogenous tRNA)	1610 ⁺	1610 ⁺
Δ cpm (methylation of 0.12 A_{260} units of added tRNA)	438 ⁺⁺	1014 ⁺⁺

Table 2. Incorporation of ^{14}C -methyl groups into m^5Cp , m^1Ap and four unidentified nucleotides by homologous enzymatic methylation of liver tRNA from normal (A) and L-ethionine-fed rats (B). 0.27 A_{260} units of in vitro methylated rat liver tRNA (0.12 units A_{260} of added tRNA plus 0.15 units of endogenous tRNA from the methylase preparation) were digested with RNase T_2 , identified as shown in Fig. 2, and quantitated.

⁺ In a separate experiment 0.15 A_{260} units of endogenous tRNA in the methylase accepted 1610 cpm (^{14}C -methyl). This was measured in two methylation experiments without added tRNA, without and with ethidium bromide, which selectively inhibits tRNA methylation but does not affect protein methylation⁸.

⁺⁺ see in text below.

m^5C in methyl-deficient liver tRNA from ethionine-fed rats as observed earlier¹ and the increased m^5C formation in vitro with methyl-deficient liver tRNA as methyl-acceptor indicates that, this tRNA is partially m^5C -deficient.

Hence, ethionine feeding of rats causes complete m_2^6A - and partial m^5C -deficiency in liver tRNA. From Table 2 we can conclude that, in presence of cadaverine, methyl-deficient tRNA from ethionine-fed rats (B++) accepts twice as much methyl groups as normal tRNA (A++). The methyl-acceptance of endogenous (A+) compared with added (A++) tRNA shows that the former tRNA is a more efficient methyl-acceptor than the latter. Hence the methylase fraction seems to contain more methyl-accepting tRNA species per A_{260} unit than the added tRNA. This may be a consequence of hydroxyapatite chromatography (Fig. 1) or due to preferential binding of endogenous methyl-deficient tRNA to tRNA methylase. The mechanism and the biological significance of homologous tRNA methylation in vitro is yet unexplained. This reaction may partly occur due to cadaverine-induced conformational changes of tRNA tertiary structure as shown in a preceding paper by Wildenauer et al.¹¹. More significant information will be available as soon as pure rat liver tRNA species have been used as substrates for homologous tRNA methylation in vitro.

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