Methyldeficient mammalian 4S RNA: evidence for L-ethionine-induced inhibition of N⁶-dimethyladenosine synthesis in rat liver tRNA

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ABSTRACT

The nucleotide composition of 4S RNA from livers of rats fed with a diet containing 0.3 % D-ethionine was found to be identical with that from untreated animals. In contrast, one single modified nucleotide was absent in 4S RNA from livers of rats fed with a 0.3 % L-ethionine diet. The minor nucleotide was also absent in liver 4S RNA from rats fed with a 0.3 % L-ethionine diet followed by ten days of normal food. It was identified after dephosphorylation by ultraviolet absorption spectra, cochromatography with authentic material and mass spectra as N⁶-dimethyladenosine. It is concluded that S-adenosylethionine, the primary product of L-ethionine in the liver, causes strong and selective inhibition of the specific RNA-methylase responsible for adenosine to N⁶-dimethyladenosine methylation in rat liver 4S RNA. Compared to the strong inhibition of N⁶-dimethyladenosine formation described here, L-ethionine-dependent ethylation of liver 4S RNA is far less efficient. The quantitation of 1-methyladenosine, ribothymidine and 3'-terminal adenosine in this 4S RNA as well as its amino acid acceptor activity is typical for tRNA; hence it may be concluded that N⁶-dimethyladenosine is a component of rat liver tRNA. This may demonstrate the first evidence for the existence of specifically methyl-deficient mammalian tRNA. A possible correlation between the activity of L-ethionine as a liver carcinogen and its ability to induce the formation of methyl-deficient tRNA by selectively inhibiting the synthesis of N⁶-dimethyladenosine on the tRNA level in the same organ is discussed.

INTRODUCTION

L-Ethionine, the ethyl analog of the natural aminoacid L-methionine, induces hepatomas in rats after chronic dietary administration^{1,2}. L-Ethionine is of special interest since its mode of action as a carcinogen may be related to its interference with the methionine metabolism. In fact it has been demonstrated that L-ethionine acts as an ethyl donor in the liver, thus competing with methylation. The in vivo ethylation of DNA³, tRNA^{4,5,6} and protein⁷ has been reported.

This report describes a new effect of L-ethionine in rat liver: it selectively inhibits the synthesis of N⁶-dimethyl=adenosine on the 4s RNA level. D-Ethionine, in contrast, does not influence N⁶-dimethyladenosine formation in rat liver 4s RNA.

MATERIALS AND METHODS

L- and D-Ethionine was bought from Fluka AG, RNase T₂ from Calbiochem. (Ethyl-1-¹⁴C) L-ethionine (spec. act. 4.13 ci/M) was from NEN Chemicals GmbH. Precoated thinlayer plates (cellulose without fluorescence indicator) were from Merck AG, Darmstadt; Whatman no. 1 paper was used for twodimensional descending paper chromatography. Solvents for twodimensional thinlayer or paper chromatography were: first dimension (solvent A) isobutyric acid - conc. ammonia - water (577 : 38 : 385) pH 4,3; second dimension (solvent B) t-butanol - conc. HCl - water (7 : 1.5 : 1.5). Other solvents for thinlayer chromatography (table 2) were (solvent C) 2-propanol - conc. ammonia - water (7 : 1 : 2); (solvent D) 1-butanol - acetic acid - water (45 : 5 : 12.5); (solvent E) 1-butanol - water (85 : 15); (solvent F) methanol - water (75 : 25).

Female Wistar rats were fed with a diet containing 0.3 % L-ethionine; two control groups got normal or a 0.3 % D-ethionine diet. After the time indicated in table 1 the animals were killed and their livers immediately frozen in liquid nitrogen.

diet	time schedule			
	4 weeks	4 weeks + 10 days normal diet	3 months	3 months + 10 days normal diet
0.3 % L-ethionine	(12)	(12)	(12)	(12)
0.3 % D-ethionine	(12)	--	--	--
normal	(12)	--	(12)	--

Table 1: time schedule for ethionine feeding; in brackets: number of rats per experiment.-

tRNA from rat liver was prepared as described by Rogg et al.⁸ and checked for purity by polyacrylamide electrophoresis⁹; besides 4s RNA, only 3 - 5 % 5s rRNA was detected, which does not contain modified nucleotides as evidenced by nucleotide analysis. Quantitation of 1-methyladenosine and 3'-terminal adenosine was performed as follows: rat liver 4s RNA, purified according to Rogg et al.⁸, was hydrolyzed with RNase T₂¹⁰ and 284 A₂₆₀ units of the hydrolyzed material were put on a 25 x 1,3 cm DEAE cellulose column equilibrated with 50 mM triethylammoniumbicarbonate (TEAB) buffer pH 8.5. Only 3'-terminal adenosine (3.6 A₂₆₀ units) and 1-methyladenosine-2':3'-cyclophosphate (1.95 A₂₆₀ units) were eluted with the same solvent as separated peaks. Other 3'-terminal nucleotides (3 to 5 % would have been detected in TLC) were not present. Hence 79 A₂₆₀ units of hydrolyzed 4s RNA yielded 1 A₂₆₀ unit of 3'-terminal adenosine and 0.54 A₂₆₀ units of 1-methyladenosine-2':3'-cyclophosphate, indicating that in average one of two rat liver 4s RNA molecules contains 1-methyladenosine.

Quantitation of ribothymidine and N⁶-dimethyladenosine compared to 3'-terminal adenosine was done as follows: 18 A₂₆₀ units of RNase T₂-hydrolyzed rat liver 4s RNA were put on three 20 x 20 cm cellulose plates and the nucleotides separated by twodimensional thinlayer chromatography (TLC, compare fig. 1B). The adenosine, rTp and m₂⁶Ap (Np in fig. 1B) spots were scraped out and eluted with 10 mM TEAB buffer pH 8.5. The molar ratios of adenosine : rTp : m₂⁶Ap were found

to be 1.0 : 0.8 : 0.08. The adenosine : rTp ratio is not 1 : 1, since 1 mole of unfractionated rat or rabbit liver tRNA contains 0.1 to 0.2 moles (unpublished) of modified rT, i.e. 2'-O-methylribothymidine, which after RNase T₂ digestion is isolated as the dinucleotide, 2'-O-methyl-rTp^γp (Gross et al., first issue of this journal).- In this quantitation, 1 A₂₆₀ unit of adenosine was found per 75 A₂₆₀ units of hydrolyzed rat liver 4s RNA.- Rabbit liver 4s RNA also, purified as described by Rogg et al., gave the same adenosine: rTp : m₂⁶Ap ratio.- Aminoacid acceptor activities for 15 aminoacids were checked and found to be normal in both rat and rabbit liver 4s RNA preparations.-

Characterization of Np (m₂⁶Ap, fig. 1B): elution of the unknown nucleotide from thinlayer plates or from paper was performed with 10 mM TEAB buffer pH 8.5. The eluted material was put on a 1 x 0.5 cm DEAE cellulose column. Impurities were eluted with the same buffer and the nucleotide was eluted from the column with 1 M TEAB buffer pH 8.5. After evaporation the nucleotide was dephosphorylated with bacterial alkaline phosphatase (Boehringer Mannheim GmbH.) and further purified by TLC in solvent C. Ultraviolet absorption spectra were measured with the Gilford Model 240, mass spectra were obtained with the mass spectrometer CH 7 (Varian MAT).- An adult femal Wistar rat was given 25 μci (ethyl-¹⁴C)L-ethionine by intraperitoneal injection. The rat was killed after 24 hours and the liver tRNA was isolated and analyzed by RNase T₂ digestion and twodimensional TLC as described above. The TLC plate was exposed to Kodak RP Royal X-Omat film for two months (fig. 4).

RESULTS AND DISCUSSION

The minor nucleoside N⁶-dimethyladenosine has been identified in 16s rRNA of E.coli by Fellner and Sanger¹¹, in the 23s rRNA of an erythromycin-resistant strain of Staphylococcus aureus by Lai and Weisblum, while it was not found in a non-resistant strain¹², and it was also detected in yeast

ribosomal RNA¹³. Its presence in rat liver microsomes and in the microsomal supernatant has been shown^{14,15,16}, but it was not found in HeLa, yeast or *E. coli* tRNA by Klagsbrun¹⁷, and it has not been found as a minor component in about sixty tRNAs sequenced until now.

In an attempt to study the ethionine-induced ethylation of rat liver tRNA we compared the nucleotide composition of liver 4s RNA from untreated, D-ethionine or L-ethionine fed rats (table 1). Instead of ethylated nucleotides in the case of 4s RNA from L-ethionine fed rats we found that one minor nucleotide (Np) was absent (fig. 1A). This modified nucleotide Np was present in liver 4s RNA from untreated and D-ethionine fed rats (fig. 1B). The absence of Np was observed after four weeks or three months of L-ethionine feeding. The absence of Np in liver 4s RNA of L-ethionine fed rats is probably not due to an acute toxic effect of the carcinogen, since it was also absent in liver 4s RNA of rats fed L-ethionine for four weeks (or three months) followed by ten days of normal diet.

Fig. 1. Twodimensional TLC of RNase T₂ digests of rat liver 4s RNA. A: Nucleotide analysis of liver 4sRNA from L-ethionine fed rats (four weeks or three months; four weeks or three months followed by 10 days of normal diet (table 1). B: Nucleotide analysis of liver 4s RNA from untreated (four weeks or three months) or D-ethionine fed rats (four weeks, table 1).

Np was isolated by preparative twodimensional thinlayer or paper chromatography of RNase T₂ digests of rat liver 4s RNA. After dephosphorylation the nucleoside N was purified by TLC in solvent C. The ultraviolet absorption spectrum of N (fig. 2B) provided some evidence that N might be identical to N⁶-dimethyladenosine (fig. 2A).

Fig. 2. Ultraviolet spectra of authentic N⁶-dimethyladenosine (A) and N isolated from rat liver 4s RNA (B).

Further support for this idea was obtained by the comparison of R_f values of N with authentic N⁶-dimethyladenosine as shown in table 2.

	Solvents and R _f values			
	C	D	E	F
N from 4s RNA	0.71	0.65	0.62	0.64
N ⁶ -dimethyladenosine	0.71	0.65	0.62	0.63

Table 2. R_f values of N and N⁶-dimethyladenosine.

Final evidence that N is identical to N⁶-dimethyladenosine was provided by the low resolution mass spectra of these two nucleosides (fig. 3).

Fig. 3. Mass spectra of N⁶-dimethyladenosine (A) and N from rat liver 4s RNA (B).

A molecular ion peak for N at 295, corresponding to the formula $C_{12}H_{17}N_5O_4$ (calculated 295.3) was found (fig. 3B); under identical conditions authentic N⁶-dimethyladenosine showed a molecular ion peak at 295. The fragmentation patterns of both nucleosides are identical and the same as those described by Hecht *et al.*¹⁸.— The presence of m₂A⁶ in rat liver 4s RNA does not necessarily mean that it is a minor component of transfer RNA. However, the absence of high molecular weight ribosomal RNA in our 4s RNA preparations, the identification of adenosine alone as the 3'-terminal nucleoside, and the reasonable rTp to 3'-terminal adenosine ratio (also in rabbit liver 4s RNA) suggests that N⁶-dimethyladenosine may be a minor component of rat (and rabbit) liver tRNA. It should be kept in mind that most modified nucleosides have been isolated from unfractionated tRNA before they were identified as components of individual tRNA

species¹⁹.

The presence of N⁶-dimethyladenosine in liver tRNA (or 4s RNA) of untreated or D-ethionine fed rats, as well as its absence in liver tRNA of L-ethionine fed rats was reproduced in several independent experiments. However, no ethylated nucleotides were observed in these experiments as shown in fig. 1A.

Fig. 4. Twodimensional TLC and autoradiography of nucleotides from rat liver 4s RNA ethylated in vivo (see material and methods). White spots: nucleotides visible in UV light. Dark spots: ethylated nucleotides located by autoradiography.

Fig. 4 shows the influence of intraperitoneally injected (ethyl-¹⁴C)L-ethionine on the nucleotide composition of rat liver 4s RNA: after RNase T₂ digestion and twodimensional TLC, eight ethylated nucleotides were located after the TLC plate had been exposed to X-ray film for two months. These ethylated nucleotides are not visible among the nucleotides from 4s RNA of rats fed L-ethionine for one or three months (compare fig. 1A), although in this case N⁶-dimethyladenosine (Np in fig. 1B) is completely absent. The absence of visible amounts of ethylated nucleotides and the absence of N⁶-dimethyladenosine in fig. 1A demonstrates that ethylation of 4s RNA is a weak effect, and that inhibition of N⁶-dimethyladenosine formation on the 4s RNA level is a strong and drastic effect of the liver carcinogen, L-ethionine.

The inhibition of N⁶-dimethyladenosine formation after dietary L-ethionine administration may be explained by the accumulation in liver of S-adenosylethionine^{20,21,22}, which is an inhibitor of tRNA methylation in vitro^{23,24}. In contrast to L-ethionine, D-ethionine can not be activated to S-adenosylethionine²⁵. Hence the inhibition of N⁶-dimethyladenosine formation in rat liver tRNA after L-ethionine administration may be caused by S-adenosylethionine, while it is not generally accepted until now that S-adenosylethionine is the ethyl donor for DNA, tRNA and protein ethylation in vivo⁴.

The carcinogenic activity of L-ethionine could not yet be related to one of its effects in liver, i.e. a) DNA³⁾, tRNA^{4,5,6} and protein⁷ ethylation and b) induction of increased tRNA methylase activity²⁶. The first effect, ethylation, is very weak compared to the strong inhibition of N⁶-dimethyladenosine formation in tRNA. The second, induction of tRNA methylase, may not be related to carcinogenesis, since a few cases are known where no difference in the qualitative and quantitative composition of methylated nucleotides from tRNA of normal tissue and tissue with increased tRNA methylase activity was found²⁷.

The specific L-ethionine-dependent inhibition of N⁶-dimethyladenosine formation in liver tRNA may, better than ethylation, explain changes in certain rat liver tRNA species observed after L-ethionine feeding²⁸. Attempts to follow the degree of inhibition of N⁶-dimethyladenosine formation after L-ethionine administration, to identify the methyldeficient tRNA species, and to use them as substrates for specific adenosine to N⁶-dimethyladenosine methylation in vitro as well as to investigate their function in protein biosynthesis in vitro are in progress.

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