INFLUENCE OF POLYAMINES AND SALTS ON CHANGING PATTERNS OF tRNA METHYLATION

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Received 30 April 1971
Revised version received 19 June 1971

1. Introduction

Comparisons of extracts from normal and neoplastic tissues have indicated differences in tRNA methylase activity as measured by rate, extent and patterns of methylation [1]. Recent studies from this laboratory have demonstrated that the rate and extent of methylation produced with rat organ extracts can be changed by addition of polyamines or salts to the reaction mixture [2, 3]. This communication provides evidence that the differences observed upon addition of magnesium ion, ammonium ion or polyamines are a result of differing effects on the formation of specific methylated bases. These results demonstrate the necessity for careful control of ionic conditions when comparing tRNA methylase extracts from different sources.

2. Materials and methods

Methyl-deficient tRNA from *E. coli* HfrC was methylated *in vitro* using $^{14}CH_3$-S adenosylmethionine and an EDTA-treated ammonium sulfate fraction (30–48% saturation) from the supernatant of rat liver homogenates [2]. Incubations were carried out under conditions of excess enzyme and limiting tRNA as described previously [4], with the exception that the incubation mixtures were scaled up to a final volume of 1–2 ml containing 8–16 μg tRNA to ensure at least 10,000 cpm $^{14}CH_3$ incorporated into substrate RNA. After 40 min incubation at 37°, 1 mg yeast tRNA was added as carrier and the RNA re-isolated [4]. The purified RNA was hydrolyzed in 0.2 ml of 0.25 M ammonium acetate, pH 8.8, containing 0.02 M magnesium acetate, 20 μg snake venom phosphodiesterase and 250 μg alkaline phosphatase. Hydrolysis was carried out for 4 hr at 44°. The resulting nucleosides, with 0.2 ml of a mixture of methylated nucleosides (400 μg/nucleoside), were separated on a Dowex 50 column (fig. 1). 5 ml fractions were collected, checked for A_{260} and A_{280}, and dried. The residue was resuspended in 10 ml of scintillation fluid (Aquasol, New England Nuclear Corp.) and counted in a liquid scintillation spectrometer. Counts recovered in methylated nucleoside peaks accounted for at least 90% of the counts applied to the column.

In order to verify the identity of methylated nucleosides eluted from the column, fractions from each radioactive peak were lyophilized and co-chromatographed with authentic methylated nucleosides on MN 300 cellulose plates (500 μ thickness) in solvents A and C of Mittleman et al. [12].

Snake venom phosphodiesterase and bacterial alkaline phosphatase were purchased from Worthington Biochemical Co. Cyclo Chemical Corp. was the source of the following nucleosides: 1-methyladenosine, N^6-methyladenosine, N^6,N^6-dimethyladenosine, 3-methylcytidine, 5-methylcytidine, N^1-methylguanosine, N^2-methylguanosine, N^2,N^2-dimethylguanosine, N^7-methylguanosine, 5-methyluridine and 1-methylinosine. $^{14}CH_3$-S adenosylmethionine (40–50 μCi/μmole) was purchased from New England Nuclear Corp. The Dowex resin was AG 50W-X4, minus 400 mesh, hydrogen form, obtained from Bio-Rad Laboratories.

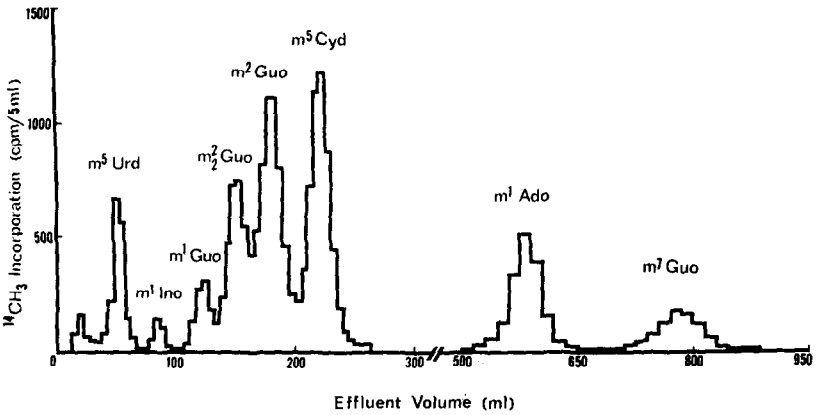

Fig. 1. Separation of radioactive methylated nucleosides on a Dowex 50W-X4 column. Nucleosides were derived from hydrolysis of RNA methylated in the presence of 0.36 M ammonium acetate. The column (24 X 1.5 cm) was eluted with 0.4 M ammonium formate, pH 4.65, at 25° and a flow rate of 1 ml/min [5].

3. Results and discussion

Fig. 1 depicts a representative pattern of radioactive nucleosides eluted from Dowex 50 at 25°. Eight nucleosides were identified as being radioactive. In addition to these, the position of several other standard methylated nucleosides was determined using this system: N^6-methyladenosine was eluted between N^2-methylguanosine and 5-methylcytidine, N^6,N^6-dimethyladenosine came off the column at 400–460 ml eluting buffer, and 3-methylcytidine eluted with 1-methyladenosine.

No unidentified peaks of radioactivity were observed after Dowex chromatography. However, the possibility cannot be excluded that a small amount of $O^{2'}$-methylated nucleosides may have been formed, since 1–5% of the radioactivity was routinely recovered in the void volume. This radioactivity may represent sequences containing methyl substituents on the ribose moiety which remained unhydrolyzed with the relatively low level of phosphodiesterase employed [7].

Methylation of tRNA was carried out in the presence of polyamine, magnesium and ammonium ion concentrations reported to be optimal for stimulation [2]. Putrescine and magnesium acetate, which showed no sharp peaks of optimal concentration, were tested at two concentrations. All methylation reactions were carried out under conditions designed to determine "extent of methylation" (excess enzyme and limiting tRNA) in order to assess most accurately the spectrum of methylases present. For each stimulatory factor, patterns of methylation were determined using at least two rat liver enzyme preparations and two preparations of methyl-deficient *E. coli* tRNA. These variables produced no detectable differences in the pattern of methylation. Therefore, the results presented are average values of at least four independent determinations.

The effect of each stimulatory factor on the formation of the 8 methylated nucleosides is indicated in table 1. There is a general similarity between the results obtained with polyamines and ammonium acetate, when contrasted with the effect of magnesium ion. Incubation in the presence of 1 or 10 mM magnesium acetate produced almost exclusively methylated guanine derivatives, while the polyamines and ammonium ion permitted formation of significant amounts of 1-methyladenosine and 5-methylcytidine in addition to the guanine compounds.

The formation of several nucleosides (N^2-methylguanosine, N^7-methylguanosine, N^1-methyladenosine) was not greatly altered by changing the polyamine or substituting ammonium acetate. In contrast, 5-methylcytidine formation was markedly diminished with 0.05 mM spermine, and the highest levels of methyl-

Table 1
Concentration of methylated nucleosides in *E. coli* tRNA after methylation *in vitro* in the presence of polyamine, ammonium or magnesium acetate.

Additions to incubation	Concentration (pmoles $^{14}CH_3$/10 μg tRNA) in							
	m_2^2Guo	m^2Guo	m^7Guo	m^1Guo	m^1Ado	m^5Cyd	m^5Urd	m^1Ino
0.05 mM spermine	15	70	18	4	57	6	4	0
0.5 mM spermidine	31	89	17	7	50	33	5	0.5
10 mM putrescine	38	89	13	7	51	33	6	0.5
20 mM putrescine	30	88	17	12	50	61	5	0.5
360 mM ammonium acetate	36	78	18	12	58	61	22	3.5
10 mM magnesium acetate	30	21	2	4	0.5	1	2	0
1 mM magnesium acetate	7	18	8	2	1	3	2	0

Values were calculated from cpm present in each Dowex 50 peak and measurement of total cpm/μg tRNA as determined in a paired incubation; 100 cpm was equivalent to 1 pmole $^{14}CH_3$.

ated cytidine were found after incubation with high concentrations of putrescine or ammonium acetate. Methylation yielding the nucleosides 5-methyluridine and 1-methylinosine was stimulated by 0.36 M ammonium acetate to levels higher than were observed with any of the polyamines. Among the eight nucleosides detected, only one, N^2,N^2-dimethylguanosine, was formed in the presence of magnesium in amounts comparable to those seen with polyamines or ammonium ion.

Estimates of the distribution of $^{14}CH_3$ in the four major nucleotides after incubation of dialyzed mouse organ extracts with ammonium acetate have been reported previously [4]. A similar study has been made using EDTA-treated rat liver extracts incubated with Mg, NH_4 and polyamines [3]. Both of these investigations involved measurement of radioactivity in the nucleotide spots after high voltage electrophoresis. While the results reported previously are, in general, consistent with those reported here, estimates of the amount of methylated cytosine tended to be higher than is seen by Dowex chromatography. Since the presence of a methyl substituent at the N^7 position of guanine results in a net positive charge [8], N^7-guanylic acid might be expected to run at pH 3.5 with an electrophoretic mobility similar to that of cytidylic acid. Therefore, measurements of methyl distribution in nucleotides separated by electrophoresis would probably lead to an overestimate of cytosine methylation and corresponding underestimate of guanine methylation.

Differences in patterns of methylation may be due, not only to differential stimulation of methylation by added factors, but also to selective inhibition. Magnesium acetate has been shown to diminish the specific activity of polyamine-stimulated methylases [2]. Preliminary results using magnesium in combination with polyamines suggests that the divalent cation inhibits the formation of N^2-methylguanosine, 1-methyladenosine and 5-methylcytidine. Several laboratories have reported detecting natural inhibitors of tRNA methylases [9, 10]. A dialyzable inhibitor from rat liver has recently been identified as nicotinamide [10].

The data dealing with patterns of methylation of various organ extracts *in vitro* have customarily relied on experiments carried out using crude undialyzed extracts incubated in the presence of magnesium salts at concentrations between 4 and 50 mM. Based upon experiments carried out under these conditions, several groups have reported differences in patterns of methylation when extracts from tumors are compared with their non-neoplastic counterparts [11–13]. Comparisons of tumor and normal tissue have also indicated that the intracellular polyamines levels are altered [14]. Differences in polyamines might be expected to be retained in undialyzed crude extracts, and therefore provide some explanation for the differences in methylation patterns noted.

Variations in methylation patterns caused by changes in stimulatory factors will be magnified if results are presented as ratios or percent distribution,

since the relative absence of one methylated nucleoside will result in apparent elevation of others. For example, while 10 mM Mg and 10 mM putrescine both produce 30 μmole of N^2,N^2-dimethylguanosine, this amount represents 51% of the total counts found with Mg and only 16% of the $^{14}CH_3$-nucleosides formed with putrescine. The extreme sensitivity of 5-methylcytidine formation to incubation conditions means that comparisons of undialyzed extracts from various sources, expressed as ratio to 5-methylcytidine [11], may be misleading.

Pegg [15] has recently published information on percent distribution of methylated bases, both of rat liver tRNA methylated *in vivo* and of *E. coli* tRNA methylated *in vitro* by rat liver enzymes in the presence of spermidine. Patterns of methylation after incubation in the presence of spermidine as reported here are, in general, similar to those found by Pegg under similar conditions. The absence of closer agreement may be due to possible differences resulting from the use of crude vs. partially purified enzyme preparations as well as to the fact that Pegg studied tRNA methylation under conditions in which enzyme rather than tRNA was limiting. Both the data of Pegg [15] and earlier reports [1] indicate that rat liver tRNA methylated *in vivo*, like bacterial tRNA methylated *in vitro* in the presence of polyamine, contain significant amounts of m^5-cytosine and m^1-adenosine. The observation that methylation of cytosine and adenine is minimal in the presence of Mg and elevated by the other stimulatory factors would suggest that polyamines and/or monovalent ions such as NH_4^+ may play a significant role in methylation *in vivo*.

Acknowledgement

This work was supported by NIH grant CA-10586. I wish to acknowledge the excellent technical assistance of Pamela Enslin, as well as the invaluable help of two students, Carlo Morris and Gail Martin, who were supported by the HEPUP program of the University of Pennsylvania.

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