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SPECIFICITY OF A NUCLEOLAR 2'-O-METHYLTRANSFERASE FOR RNA SUBSTRATES

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SUMMARY: In this study we examined the specificity of a nucleolar 2'-O-methyltransferase isolated from nucleoli of Ehrlich ascites tumor cells. The nucleolar methyltransferase was capable of methylating each of the four nucleosides of RNA, however, the level of methylation at a particular nucleoside varied with the type of RNA. Both kinetic analysis and the stimulation of methylation by polyamines suggested that the structure of RNA was critical in influencing the discrimination and apparent specificity of nucleolar 2'-O-methyltranferase.

In mammalian cells, newly synthesized pre-ribosomal RNA is methylated before it is processed to cytoplasmic rRNA (1-3). Methylation of pre-ribosomal RNA occurs on both specific bases and ribose moieties following transcription, and appears to be confined to regions in the precursor RNA destined to become mature ribosomal RNA species (4-7) and to play an essential role in ribosomal function (8-13). In particular, greater than 80-90% of the methylation represents 2'-O-methylation of ribose (4-6,14-15) which has been shown to affect the proper maturation of precursor to mature 28S and 18S rRNA.

We have undertaken a study of the enzymatic machinery involved in the process of 2'-O-methylation of precursor ribosomal RNA. Recently, we reported on the partial purification and characterization of a nucleolar 2'-O-methyltransferase from

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nuclei of Ehrlich ascites tumor cells (16). In this paper, we will describe the effect of various RNA substrates on catalysis and the possible implications of this effect relative to the biological function of the enzyme.

MATERIALS AND METHODS

Chemicals- Labeled S-adenosyl-L-[methyl-^{3}H]methionine (75.4 Ci/mmol) was obtained from New England Nuclear. S-adenosyl-L-methionine, spermine, spermidine, and putrescine were all obtained from Sigma.

Enzymes- The nucleolar 2'-O-methyltransferase represents fraction VI as described previously by Eichler et al. (16). Bacterial alkaline phosphatase and phosphodiesterase I (Crotalus atrox venom) were purchased from P.L. Biochemicals, and pancreatic RNase was obtained from Sigma Chemical Company.

RNAs- Poly(A) and poly(C) were purchased from P-L Biochemicals. Bacteriophage f_2 RNA was prepared essentially according to the procedure of Glitz (17) for MS2 RNA.

Methylase Assay- The assay measures the incorporation of labeled methyl groups into acid-insoluble RNA. The reaction mixture (0.05 ml) contained 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, 50 mM KCl, 2 mM dithiothreitol (DTT), 0.4 mM EDTA, 0.4 mg/ml bovine serum albumin, 0.4 uM S-adenosyl-L-[methyl-^{3}H]methionine ($5x10^4$ cpm/pmol) and 1.2 mM RNA. After 60 min. at 37 ^oC the mixture was chilled at 0^oC in an ice bath, and 0.2 ml of 1 mg/ml tRNA, 10 mM $Na_4P_2O_7$, 10 mM EDTA and 1 ml 15% trichloracetic acid were added. After 10 min at 0^oC, the mixture was filtered through GF/C filters (Whatman), washed five times with 1.5 ml of 5% TCA followed by ethanol and dried 10 min under a heat lamp. The radioactivity on the filter was determined by counting in 4 ml of 3a20 toluene scintillation fluid (Research Products International Corp.). One unit of enzyme is that amount which incorporates 1 pmol of methyl group in acid-insoluble RNA in 60 min.

RESULTS AND DISCUSSION

In order to determine whether the methylase showed any apparent specificity for the nucleoside methylated, the relative distribution of the 2'-O-methylated nucleosides in an RNA substrate after reaction with the methyltransferase was examined using high pressure liquid chromatography under conditions which would distinguish the four 2'-O-methylated nucleosides. As shown

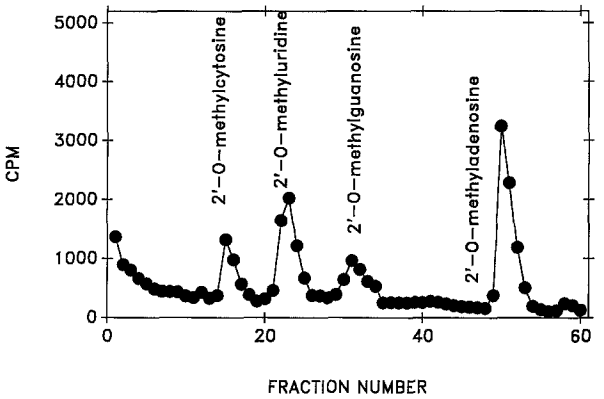

Figure 1. **HPLC Analysis of the 2'-O-Methylated Nucleosides of f_2 RNA:** The reaction mixtures (0.1 ml) contained 0.05 M Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/ml bovine serum albumin, 1.6 uM S-adenosyl-L-[methyl-^{3}H]methionine (12 x 10^4 cpm/ml), and 0.2 mM f_2 RNA. The reaction mixtures were started by the addition of 0.24 units of enzyme. After 60 min at 37 ^{o}C, the reaction mixture was passed through a spun-column, and the eluant treated with 100 units pancreatic RNase for 60 min at 37 ^{o}C. The reaction mixture was then adjusted to 0.1 M Tris-HCl pH 9.0, 10 mM $MgCl_2$, and incubated at 37^{o}C for 12 hr with 0.12 units of bacterial alkaline phosphatase, and 30 units of snake venom phosphodiesterase. Protein was removed from the reaction mixture by centrifugation through an Amicon filter, and run on a C-18 reverse phase (Rainin) HPLC column (0.4 x 10 cm) at 1 ml per min with a linear gradient starting with 95% solvent A (25% acetonitrile / 75% H_2O) and 5% solvent B (0.01 M potassium acetate, pH 5.0) programmed over 30 min. to 100% solvent B. Fractions (0.2 ml) were collected and counted in 2 ml aqueous scintillation fluid.

in Fig. 1, the nucleoside most frequently methylated was adenosine, then uridine, and cytosine and guanosine, respectively, These results could be interpreted initially as suggesting some base specificity by the 2'-O-methyltransferase. However, when ribohomopolymers were used as the RNA substrate, poly(C) was preferred, and poly(A) and heterologous RNA were utilized less effectively (Table I). Thus, the the apparent preference for methylation of adenosine residues in heterologous RNA was not reflected in the preference for homopolymers of RNA. In both situations, evidence supported the fact that the methyltransferase could 2'-O-methylate each of the four different nucleosides, although at significantly different levels.

How does the enzyme discriminate against a substrate? In order to further examine the basis for the enzyme specificity and

TABLE I

Methylation of RNA by the Nucleolar 2'-O-Methyltransferase

SUBSTRATE	METHYL GROUPS INCORPORATED (pmol)
f_2 RNA	1.0
Poly(A)	1.5
Poly(C)	5.0
Poly(I)	0.15
Poly(U)	0.6

All assays were carried out as described in the text for a standard methylase assay. The reactions were started by the addition of 0.84 unit of nucleolar methyltranferase. Aliquots (0.01 ml) were removed at 0, 15, 30 and 60 min and then processed and counted as for a standard assay. The values shown in the table are for the 60 min time point in each assay.

to resolve the apparent inconsistency of the previous results, we carried out a kinetic analysis using various RNA substrates. As shown in Fig. 2, the apparent preference for poly(C) appears in the V_{max} parameter and not in the K_m. In fact, poly(C) was the poorest substrate tested relative to K_m values. Although the

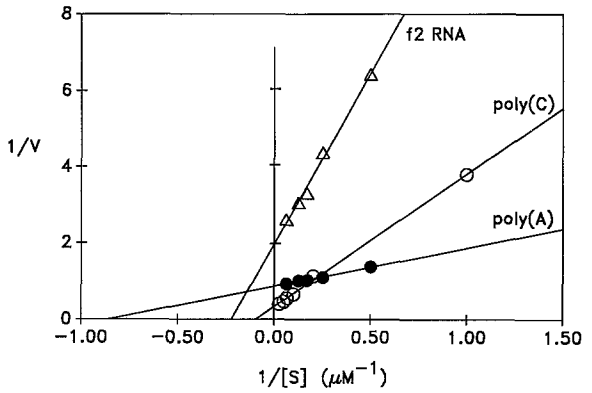

Figure 2. **Kinetic Analysis.** The reaction mixtures (0.05 ml) contained 0.05 M Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/ml bovine serum albumin, 1.6 uM S-adenosyl-L-[methyl-^{3}H]methionine (12 x 10^4 cpm/pmol), and RNA concentrations ranging from 1 to 100 uM. Reactions were started by the addition of 0.24 units of enzyme and samples were processed as described for a standard assay.

TABLE II

Summary Table of K_m and V_{max} Parameters for the Nucleolar 2'-O-Methyltransferase

Substrate	K_m (uM)	V_{max} (pmol/min)	V_{max}/K_m
Poly(A)	1.2	0.014	0.12
Poly(C)	10.5	0.006	0.0006
f_2RNA	8.7	0.028	0.0032

All assays were carried out as described in the text for a standard methylase assay and, specifically as stated in legend to Fig. 2.

apparent K_m for poly(C) was almost 10-fold higher than for poly(A), the higher kinetic rate more than compensated for the lower binding. As shown in Table II, by taking the ratio of V_{max} to K_m, the preference for poly(C) can be clearly demonstrated and a rationale for substrate specificity demonstrated.

Important to note was that each of these homopolymers has unique structures in solution (18), and it was possible that this variation in homopolymer structure played a major role in affecting the catalytic efficiency of the 2'-O-methyltransferase. Since, it has been established that polyamines tend to affect the structure of nucleic acid molecules, and that polyamines have also been previously reported to stimulate RNA methylase activities (19), we carried out the following experiments to establish the effects of polyamines on the nucleolar 2'-O-methyltransferase. As shown in Fig. 3A, the most pronounced effect was observed with spermine which possesses the greatest polybasic character of the polyamines tested, having four positive groups per molecule. Stabilization of RNA structure is known to be a property of polyamines (20) and may account for the fact that the effect of polyamines varied greatly with the type of RNA substrate (Fig. 3B). The greatest observable effect was

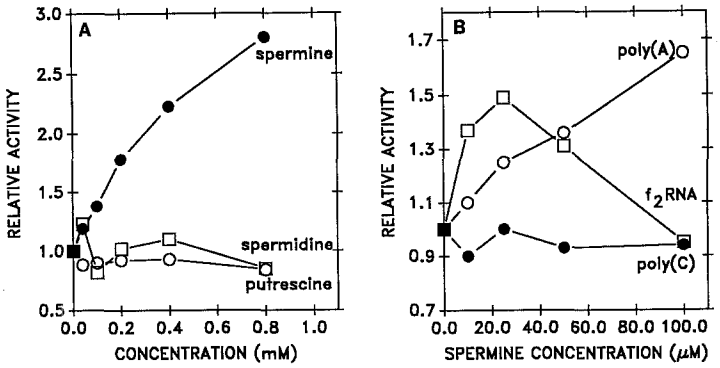

Figure 3. **A:Effect of Polyamines on the Activity of the Nucleolar 2'-O-Methyltransferase.** The reaction mixture (0.05 ml) contained 0.05 M Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/ml bovine serum albumin, 1.6 uM S-adenosyl-L-[methyl-^{3}H]methionine (12 x 10^4 cpm/pmol), 1.2 mM poly(A) and the designated concentrations (0.04 to 0.8 mM) of polyamine. Reactions were started by the addition of 0.24 units of enzyme and samples were processed as described for a standard assay. **B:Effect of Spermine on the 2'-O-methylation of Various RNA's.** The reaction mixtures (0.05 ml) contained 0.05 M Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/ml bovine serum albumin, 1.6 uM S-adenosyl-L-[methyl-^{3}H]methionine (12 x 10^4 cpm/pmol), and the designated RNA at 100 uM. Reactions were started by the addition of 0.24 units of enzyme and samples were processed as described for a standard assay.

with poly(A) and the least (no stimulation) was with poly(C) as the RNA substrate. The degree to which polyamines could stimulate methyl group incorporation relative to the type of RNA substrate, therefore, was directly related to the ratio of V_{max} to K_m shown in Table I. The least catalytically efficient substrate, poly(A), was affected to the greatest extent by spermine. Poly(C), the most kinetically efficient substrate, was not affected by spermine. These results suggest that polyamines affect the structure of poly(A) in some way to facilitate catalysis; a structure similar to that which was already provided by poly(C) in the absence of polyamines.

Previously, we established that this enzyme possessed activity specific for the 2'-O-methylation of RNA. However, the question remained as to whether this activity or a combination of 2'-O-methylating activities may be responsible for *in vivo* methylation of precursor ribosomal RNA. In this regards, the

enzyme was capable, in vitro, of methylating each of the four nucleosides, although to various different levels depending on the RNA. If this were the case in vivo, what then are the features of the biological system which selects for the non-random incorporation of the 2'-O-methyl groups in precursor rRNA? To this end, we explored the effect of various RNA substrates and have established that structural properties of RNA may play a significant role in the recognition process by affecting the efficiency of catalysis. Therefore, the specificity of the 2'-O-methyltransferase, that is the ability to discriminate between specific residues and regions of an RNA molecule, may depend on structural organization of the RNA. In addition, these results may also be taken to suggest that additional features of the processing system and precursor RNA substrate, such as the formation of the ribonucleoprotein particle, contribute to the process for 2'-O-methylation in vivo.

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