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Isolation and Characterization of a Nucleolar 2′-*O*-Methyltransferase from Ehrlich Ascites Tumor Cells†

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ABSTRACT: A 2′-*O*-methyltransferase that transfers the methyl group from *S*-adenosylmethionine to the 2′-hydroxyl group of ribose moieties of RNA has been purified from Ehrlich ascites tumor cell nucleoli. The partially purified enzyme is devoid of other RNA methylase activities and is free of ribonucleases. The enzyme has optimal activity in tris(hydroxymethyl)aminomethane buffer, pH 8.0, in the presence of 0.4 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, and 50 mM KCl, and has an apparent K_m for *S*-adenosylmethionine of 0.44 μM. Gel filtration studies of this enzyme gave a Stokes radius of 43 Å. Sedimentation velocity measurements in glycerol gradients yield an $s_{20,w}$ of 8.0 S. From these values, a native molecular weight of 145 000 was calculated. The enzyme catalyzes the methylation of synthetic homoribopolymers as well as 18S and 28S rRNA; however, poly(C) is the preferred synthetic substrate, and preference for unmethylated sequences of rRNA was observed. For each RNA substrate examined, only methylation of the 2′-hydroxyl group of the ribose moieties was detected.

Nucleoli not only act as an organizational structure for rRNA genes but also contain the machinery for rRNA transcription, methylation, and processing (Perry, 1976; Attardi & Amaldi, 1970; Hadjilov & Nickolaev, 1976). Methylation of preribosomal RNA is confined to the conserved 18S and 28S sequences destined to become ribosomes (Weinberg & Penman, 1970; Maden & Salim, 1974). Although both base methylation and 2′-O-methylation occur, the extent of 2′-O-methylation is greater (Lane & Tamaoki, 1969; Brown &

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Attardi, 1965; Wagner et al., 1967; Salim & Maden, 1973; Starr & Sells, 1969). The importance of this posttranscriptional modification is supported by the following evidence. Methylation appears to occur concomitantly with or very soon after synthesis of nucleolar 45S preribosomal RNA (Greenberg & Penman, 1966; Muramastsu & Fujisawa, 1968; Zimmerman & Holler, 1967). Selective inhibition of rRNA methylation does not appear to affect transcription but alters processing of the rRNA precursor, preventing normal maturation (Wolf & Schlessinger, 1977; Caboche & Bachellerie, 1977; Dabeva et al., 1976). Vaughn et al. (1967) showed that, during methionine starvation, ribosome production was completely abolished, although 45S precursor RNA continued to be synthesized. More recent results have shown that ethionine, a methionine analogue, may differentially affect 2′-*O*-methylation of pre-rRNA (Wen & Tsukada, 1983) and disrupt ribosomal RNA processing, again establishing the essentiality of this type of posttranscriptional modification.

A variety of physical and chemical studies therefore suggest that rRNA methylation plays an essential role in the metabolism and in the structure of rRNAs. For this reason, we decided to investigate the type of activities responsible for this process and, in particular, to focus on the activity or activities responsible for 2′-O-methylation. In this initial report, we describe the preparation and characterization of a distinct nucleolar activity which carries out exclusively the 2′-O-methylation of RNA.

Materials and Methods

Chemicals. Labeled *S*-[^{3}H]adenosylmethionine (75.4 Ci/mmol) was obtained from New England Nuclear. Hydroxylapatite was purchased from Bio-Rad Laboratories, DEAE-cellulose 52 and P-11 cellulose were from Whatman, and Sephacryl S-300 and DEAE-Sephadex A-25 were from Pharmacia. Blue-agarose was purchased from Bethesda Research Laboratories.

Enzymes. Polynucleotide kinase, bacterial alkaline phosphatase, and all restriction endonucleases were purchased from P-L Biochemicals. Bacteriophage SP6 RNA polymerase was obtained from Boehringer Mannheim.

DNAs. Riboprobe vectors pSP64 and pSP65 were obtained from Promega Biotec. A 1.9-kilobase (kb) *Sal*I–*Eco*RI insert cloned in pBR322 representing essentially 18S mouse rDNA sequence was generously provided by Dr. Norman Arnheim (State University of New York at Stony Brook) and was subcloned into pSP64. A 5.4-kb *Sal*I–*Eco*RI insert in pBR322 representing the external transcribed spacer and 18S mouse rDNA sequence was kindly provided by Dr. Lewis Bowman (University of South Carolina) and was also subcloned into pSP64.

RNAs. Poly(U), poly(C), poly(I), poly(A), and poly(C)·poly(I) were purchased from P-L Biochemicals. Yeast 5.8S rRNA was prepared according to the procedure of Rubin (1975) from frozen yeast cells (*Saccharomyces cerevisiae* X2180-1B). The 5′-^{32}P labeling of yeast 5.8S rRNA was carried out according to the procedure of Donis-Keller et al. (1977) using T4 polynucleotide kinase and [γ-^{32}P]ATP. End-labeled yeast 5.8S rRNA was purified by 8% polyacrylamide–urea gel electrophoresis. Run-off transcripts of pSP64 constructs containing either a *Sal*I–*Eco*RI 5.4-kb insert (Vance et al., 1985) linearized with *Sac*I (external transcribed spacer RNA) or a *Sal*I–*Eco*RI 1.9-kb insert (Arnheim & Kuehn, 1979) linearized with *Eco*RI (pre-18S RNA) of mouse ribosomal DNA were prepared by using SP RNA polymerase. RNA transcripts were purified by DNase I treatment and phenol extraction before being used in methylase assays. Ribosomes were prepared from mouse L cells according to the procedure of Blobel and Potter (1967). Mature 18S and 28S ribosomal RNAs were isolated from ribosomes with phenol and the RNAs fractionated on sucrose velocity gradients.

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 ethylenediaminetetraacetic acid (EDTA), 0.4 mg/mL bovine serum albumin, 0.4 μM *S*-[^{3}H]adenosylmethionine (5×10^4 cpm/pmol), and 1.2 mM RNA. After 60 min at 37 °C, the mixture was chilled at 0 °C 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 of 15% trichloroacetic acid (TCA) were added. After 10 min at 0 °C, the mixture was filtered through GF/C filters (Whatman), washed 5 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.

Assay for Ribonuclease Activity. ^{32}P-Labeled 5.8S rRNA was incubated in a 0.05-mL reaction mixture containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.48 mM RNA, 1.2 mM *S*-adenosylmethionine, and 0.56 unit of fraction VI. The samples were incubated at 37 °C, and aliquots (4 μL) were taken at 0, 15, 30, and 60 min into 4 μL of 5 mg/mL tRNA, 35 μL of 10 M urea, and 7 μL of tracking dye (0.4% bromophenol blue and 0.4% xylene cyanol). The samples were applied to a 20% polyacrylamide-urea gel and electrophoresed. The gel was exposed to X-ray film (Kodak X-Omat R film) plus an intensifying screen (Dupont Cronex screen) for 17 h at −70 °C.

Measurement of Protein. Protein was determined by the method of Schaffner and Weissman (1973).

Results

Purification of Nucleolar 2′-O-Methyltransferase. All steps for this purification of the 2′-*O*-methyltransferase were carried out a 0–4 °C unless otherwise indicated. The preparation of nucleoli from Ehrlich ascites cells was as described earlier by Lasater and Eichler (1984).

Nuclear Extraction. Purified Ehrlich cell nucleoli (from approximately 4×10^{10} nuclei) were suspended in 100 mL of buffer A (0.2 M potassium phosphate, pH 8.2, 1 mM EDTA, and 5 mM DTT). The suspension was mixed gently for 1 h, and the chromatin was collected by centrifugation at 20000g_{av} for 30 min. The supernatant was saved, and the chromatin was suspended in half the previous volume (50 mL) of buffer A. After 30 min of gentle mixing, the chromatin was collected by centrifugation, and the supernatant was pooled with that obtained from the first extraction. The pooled extract was dialyzed against two changes of 1 L of buffer B (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 5 mM DTT) over a 20-h period and clarified by centrifugation at 105000g for 60 min. The supernatant represents fraction I (196 mL).

Hydroxylapatite Chromatography. A hydroxylapatite column (2.5 × 30 cm) was equilibrated against 1 mM potassium phosphate, pH 6.8. Fraction I was loaded at approximately 50 mL/h. The column was washed with 300 mL of buffer B and then eluted with 0.3 M potassium phosphate, pH 6.8, 5 mM DTT, and 20% glycerol. The peak activity fractions which eluted with the high-salt step were pooled and brought to 70% saturation with a saturated ammonium sulfate solution. After 1 h, the precipitate was collected by centri-

Table I: Purification of Nucleolar 2′-*O*-Methyltransferase[a]

fraction	volume (mL)	mg/mL	units/mL	units/mg[b]	yield (%)
(I) extract	196	1.6			
(II) hydroxyl-apatite	50	4.23	18.2	4.3	100
(III) DEAE-52 cellulose	12.7	6.43	31.4	4.9	44
(IV) Sephacryl S-300	2.5	4.3	104	24	29
(V) P-11 cellulose	11.4	0.18	10.6	61	13
(VI) blue-agarose	1.0	0.42	56	133	6

[a] Purification was carried out as described in the text, starting with nucleoli derived from 4×10^{10} nuclei. One unit of enzyme represents the incorporation of 1 pmol of [^{3}H]methyl group into acid-insoluble material in 60 min at 37 °C in standard assay conditions with poly(A) as the substrate. [b] Fold purification and percent yield are likely underestimated since the standard assay, although biased for detection of ribose methylation, does detect both base and ribose methylation.

fugation at 20000*g* for 20 min. The pellet was suspended in a minimal volume (25 mL) of 0.05 M Tris-HCl, pH 8.0, 0.1 M KCl, 5 mM DTT, 1 mM EDTA, and 20% glycerol and dialyzed overnight against 1 L of this same buffer. The dialyzed material was clarified by centrifugation for 15 min at 20000*g* and represents fraction II (50 mL).

DEAE-cellulose Chromatography. A DEAE-cellulose column (2.5 × 40 cm) was equilibrated against buffer C (0.05 M Tris-HCl, pH 8.0, 0.05 M KCl, 1 mM EDTA, 5 mM DTT, and 20% glycerol). Fraction II was diluted approximately 2-fold such that its conductivity equaled that of buffer C and was loaded. The column was washed with 400 mL of buffer C and then eluted with 0.4 M KCl in buffer C. The peak activity fractions which eluted with the high-salt step were pooled and brought to 70% saturation with a neutralized, saturated solution of ammonium sulfate. After 1 h, the precipitate was collected by centrifugation at 20000*g* for 15 min. The pellet was suspended in a minimal volume of buffer D (0.05 M Tris-HCl, pH 7.5, 0.1 M KCl, 10% glycerol, 2 mM EDTA, and 2 mM DTT) and represents fraction III (12.7 mL).

Sephacryl S-300 Chromatography. A Sephacryl S-300 column (2.5 × 90 cm) was equilibrated against buffer D. Fraction III was loaded onto the sizing column, and fractions (3 mL) were collected. Active peak fractions were pooled and concentrated by vacuum dialysis against buffer E (0.05 M Tris-HCl, pH 7.5, 0.05 M KCl, 2 mM EDTA, 2 mM DTT, and 10% glycerol) and represent fraction IV (2.5 mL).

Phosphocellulose Chromatography. A P-11 cellulose column (1 × 6.4 cm) was equilibrated against buffer E. Fraction IV was loaded, and 1-mL fractions were collected. The column was washed with 10 mL of buffer and eluted with a 50-mL gradient of 0.05–0.5 M KCl in buffer E. The peak activity fractions were pooled and dialyzed against buffer E without KCl and represent fraction V (11.4 mL).

Blue-Agarose Chromatography. A blue-agarose column (3-mL) bed volume) was equilibrated against buffer E without KCl. Fraction V was loaded, and 1-mL fractions were collected. The column was washed with 10 mL of buffer without KCl and eluted with 6-mL volumes of buffer E containing 50 mM, 100 mM, 200 mM, 300 mM, 500 mM, and 1 M KCl. Peak activity fractions which eluted with the 500 mM step were pooled and vacuum dialyzed against buffer E containing 50 mM KCl and represent fraction VI (1 mL). A summary of the purification and yield of the nucleolar 5′-*O*-methyltransferase is presented in Table I. Fraction VI was found

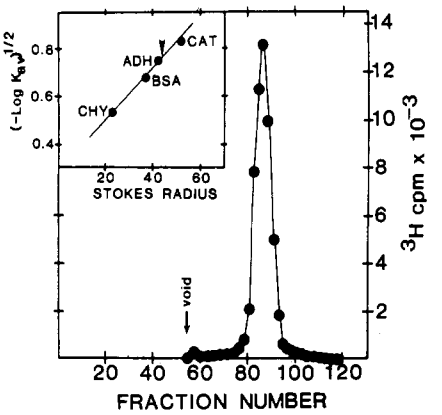

FIGURE 1: Gel filtration of the nucleolar methyltransferase. A Sephacryl S-300 column (2.5 × 90 cm) was equilibrated against 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 10% glycerol, 2 mM EDTA, and 2 mM dithiothreitol. Fractions (3 mL) were collected, and 25-μL aliquots were assayed. "Void" arrow indicates the position of blue-dextran elution. The fractional elution volumes (K_{av}) of various standard proteins used to calibrate the column were determined by reading the optical density at 280 nm of each fraction. CAT, catalase (52 Å); ADH, yeast alcohol dehydrogenase (41.7 Å); BSA, bovine serum albumin (37 Å); and CHY, chymotrypsinogen (22 Å). The arrow indicates the fractional elution position of the nucleolar methyltransferase.

to be stable for several months when stored at 0 °C.

Since the assay procedure for methylase activity was not, in itself, selective for the methylation of 2′-hydroxyl groups on RNA, we initially investigated the use of various RNA substrates as potential reagents to differentiate base methylation from ribose methylation. From alkaline digest patterns of methylated RNA substrates analyzed on DEAE-Sephadex A-25 columns, we found that poly(A) was predominantly methylated on the ribose rather than the base of crude nucleolar extracts. The results of such an analysis were conducted by the same procedure as described in the legend for Figure 5B. For this reason, poly(A) was then used as the substrate throughout the purification procedure to better follow the fractionation of the nucleolar 2′-*O*-methyltransferase. Although this approach permitted identification of 2′-*O*-methylation, base methylation was also observed with the early fractions. However, most of the apparent base methylase activity, using poly(A) as the substrate, appeared to be lost at the DEAE-cellulose step (fraction III) and, in part, explains the apparent excessive loss in total methylase units at this step. In this regard, the fold purification was also diminished by the fact that the methylase assay was not initially specific for one enzyme.

To examine for the presence of ribonuclease activity, aliquots of fraction VI were incubated with 5′-^{32}P-labeled yeast 5.8S rRNA for 60 min under assay conditions for the nucleolar methylase and were then subjected to electrophoresis on a 20% polyacrylamide–urea gel. No significant degradation of the labeled RNA by fraction VI was observed relative to the control mixture to which no enzyme was added (data not shown).

Physical Properties. To determine the apparent molecular weight for the partially purified nucleolar methyltransferase, the enzyme and chromatographed on a Sephadex S-300 molecular sieving column that was standardized with proteins of known molecular weight. A single peak of methylase activity eluted at a position close to alcohol dehydrogenase which has a molecular weight of 150000 (Siegel & Monty, 1966) (Figure 1), and a Stokes radius of 43 Å was determined from the elution volume of the methylase (Laurent & Killander, 1964). A sedimentation coefficient of 8.0 S for the nucleolar me-

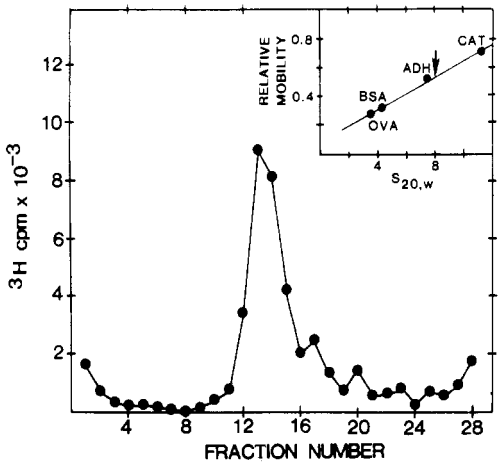

FIGURE 2: Glycerol gradient centrifugation of nucleolar methyltransferase. An aliquot (0.1 mL) of enzyme was layered on a 3.8-mL 10–30% (v/v) glycerol gradient containing 50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 2 mM dithiothreitol, and 2 mM EDTA, and the gradients were centrifuged for 12 h at 50 000 rpm at 4 °C in an SW60 rotor. After centrifugation, fractions (0.15 mL) were collected from the bottom of the tube. An aliquot (0.025 mL) of each fraction was assayed for methylase activity as described for a standard assay. Protein standard peaks were determined by reading the optical density at 280 nm of each fraction with catalase [11.3 S (CAT)], alcohol dehydrogenase [7.6 S (ADH)], bovine serum albumin [4.3 S (BSA)], and ovalbumin [3.5 S (OVA)] as markers in parallel gradients.

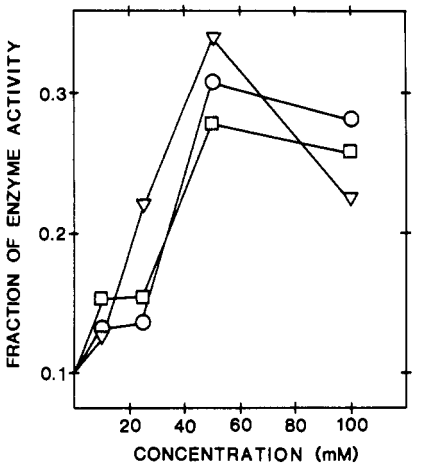

FIGURE 3: Effect of monovalent cations on the methylation of RNA by the nucleolar methyltransferase. The reaction mixtures (0.05 mL) contained 50 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/mL bovine serum albumin, 0.1 mM *E. coli* RNA, *S*-[^{3}H]adenosylmethionine (5.8 × 10^2 cpm/pmol), and monovalent cation as indicated. Reactions were started by the addition of 0.3 unit of enzyme, and samples were processed as described for a standard assay. (○) KCl; (□) NH_4Cl; (▽) NaCl.

Table II: Properties of Partially Purified 2′-*O*-Methyltransferase[a]

reaction condition	relative activity[b]
complete	1.00
minus bovine serum albumin	0.57
minus dithiothreitol	0.82
minus EDTA	1.00
minus EDTA, plus 1 mM $MgCl_2$	0.34

[a] The complete reaction mixture (0.05 mL) contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/mL bovine serum albumin, 0.1 mM *E. coli* RNA, and 2 μM *S*-[^{3}H]adenosylmethionine (5.3 × 10^5 cpm/pmol). [b] Relative activity is the ratio of picomoles of [^{3}H]methyl groups incorporated in RNA relative to the complete reaction mixture.

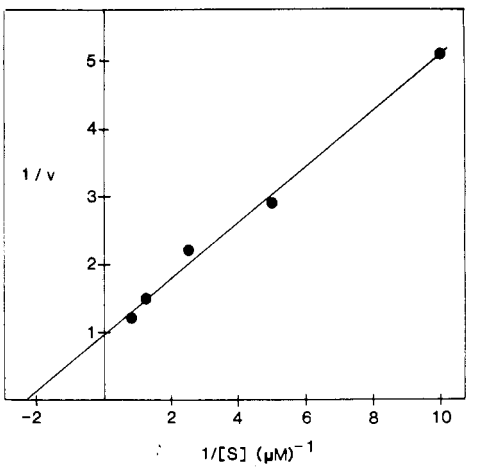

FIGURE 4: S-Adenosylmethionine K_m determination for the nucleolar methyltransferase. The reaction mixtures (0.05 mL) contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/mL bovine serum albumin, 0.3 mM *E. coli* RNA, and *S*-[^{3}H]adenosylmethionine (5.3 × 10^5 cpm/pmol) with concentrations ranging from 0.1 to 1.2 μM. Reactions were started by the addition of 0.84 unit of enzyme, and samples were processed as described for a standard assay.

thyltransferase was estimated according to the method of Martin and Ames (1961) using catalase, bacterial alkaline phosphatase, and bovine serum albumin ($s_{20,w}$ = 11.3, 7.6, and 4.3 S, respectively) (Siegel & Monty, 1966; Le Maire et al., 1980) as markers on a glycerol gradient (Figure 2). When the values for the Stokes radius (43 Å) and the sedimentation coefficient (8 S) were substituted into the Svedberg equation, an apparent molecular weight of approximately 145 000 was calculated by assuming a partial specific volume of 0.73 for the enzyme.

Properties of Partially Purified Methyltransferase Enzyme Fraction. The nucleolar methyltransferase was found to be optimally active in 50 mM Tris buffer at pH 8.0. Monovalent cations are stimulatory (Figure 3) with maximal activity at concentrations of 50 mM KCl. Maximal activity and stability of the methyltransferase required the presence of bovine serum albumin in the assay mixture in addition to a sulfhydryl reagent such as dithiothreitol (Table II). The enzyme was inhibited by divalent cations; therefore, EDTA was included in all reaction mixtures.

Kinetics of Methyltransferase Activity. The influence of enzyme and substrate concentrations on the methylation of RNA was examined. The reaction was linear over an enzyme concentration of 0.1–5.0 units for at least 60 min. As shown in Figure 4, the nucleolar methyltransferase gave an apparent K_m for *S*-adenosylmethionine of 0.44 μM. Nonsaturating levels of RNA substrate were not observed until concentrations were below 3 μM in a standard assay mixture. An absolute K_m for an RNA substrate was not determined.

Mode of Methylation. To demonstrate that methylation occurs at the 2′-OH position of ribose moieties of RNA, the following experiments were performed. Since the phosphodiester bond adjacent to the 2′-OCH_3 is resistant to alkaline hydrolysis, methylated RNA samples were subjected to alkaline hydrolysis, and the products of hydrolysis were chromatographed on DEAE-Sephadex A-25. Elution of ^{3}H-labeled methylated nucleotides at the position of mononucleotide was taken to indicate base methylation. Elution at positions corresponding to dinucleotides or larger was taken to indicate alkaline resistance and thus 2′-O-methylation. A typical experiment is shown in Figure 5B, demonstrating that all the ^{3}H label associated with methyl group incorporation eluted at positions of dinucleotide or greater. To ensure that this later eluting material was not due to incomplete hydrolysis, a ^{32}P-labeled RNA standard was added to each experiment at the

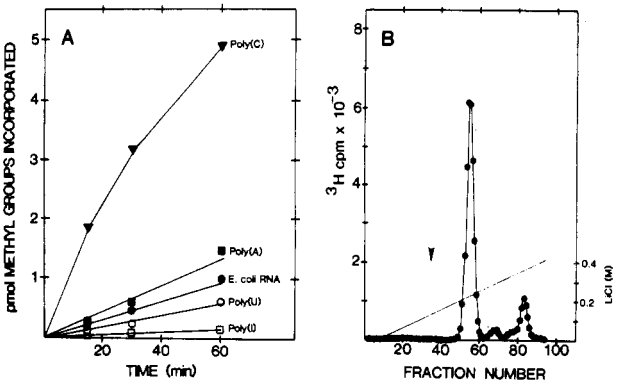

FIGURE 5: Comparative methylation of various homoribopolymer substrates by the nucleolar methyltransferase. (A) The reaction mixtures (0.05 mL) contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/mL bovine serum albumin, 0.4 μM *S*-[^{3}H]adenosylmethionine(3.0 × 10^4 cpm/pmol), and a 1.2 mM sample of the designated homoribopolymer. The reactions were started by the addition of 0.84 unit of nucleolar methyltransferase. Aliquots (0.01 mL) were removed at the indicated times and then processed and counted as for a standard assay. (B) The specificity of poly(C) methylation was investigated by using alkaline hydrolysis and DEAE-Sephadex A-25 chromatography. To the remainder of the reaction mixture at 60 min was added 0.2 mL of 1 mg/mL tRNA, 10 mM $Na_4P_2O_7$, and 10 mM EDTA. The reaction was precipitated with 1 mL of 15% trichloroacetic acid at 0 °C for 10 min, and the pellet was collected by centrifugation. The pellet was washed twice with 1-mL volumes of −20 °C 70% ethanol and taken up in 0.1 mL of 0.3 M KOH. Approximately 20 000 cpm of ^{32}P-labeled RNA was added to each digest as an internal standard. After 16 h at 37 °C, the sample was diluted to 3 mL with 5 mM Tris-HCl, pH 7.5, and 7 M urea and loaded onto a 3.5-mL DEAE-Sephadex A-25 column equilibrated against this same buffer at 65 °C. The column was washed with 5 mL of buffer, and a 100-mL gradient of buffer from 0 to 0.4 M LiCl in column buffer was run. One-milliliter fractions were collected, and 0.5 mL of each fraction was counted. The arrow indicates the position at which the ^{32}P-labeled mononucleotide marker elutes.

time of alkaline hydrolysis. All ^{32}P-labeled nucleotide eluted at the position of mononucleotide indicated by the position of the arrow. Using this type of analysis, differences were also observed in the distribution of ^{3}H label, and therefore methyl groups, in hydrolysis products. These differences were dependent on the type of RNA substrate used [i.e., poly(A) vs. *Escherichia coli* RNA].

Specificity for Methylation. The base specificity of the nucleolar enzyme was tested by using single-stranded homoribopolymers as substrates. As shown in Figure 5A, the enzyme would methylate each of the ribopolymers; however, the apparent level of activity with poly(C) was significantly greater than for the other RNA substrates tested. To ensure that all label incorporated was 2′-O-methylation, each ribopolymer substrate was subjected to alkaline hydrolysis and analysis by DEAE-Sephadex A-25 chromatography. Shown in Figure 5B are the results of such an analysis for poly(C). All label eluted at positions greater than mononucleotide, indicating that only 2′-O-methylation had occurred.

The effect of secondary structure was examined by using duplex ribopolymers. Assays using poly(C)·poly(I) showed that methylation of the duplex ribopolymer was substantially lower than poly(C) alone but greater than poly(I) (Figure 6). Subsequently, experiments using poly(A)·poly(U) as a substrate gave essentially identical levels of methylation as for poly(A) alone. These results were taken to indicate that duplex substrates could be methylated and that observed differences between single- and double-stranded substrates are possibly due to differences in recognition processes relating to substrate features other than secondary structure.

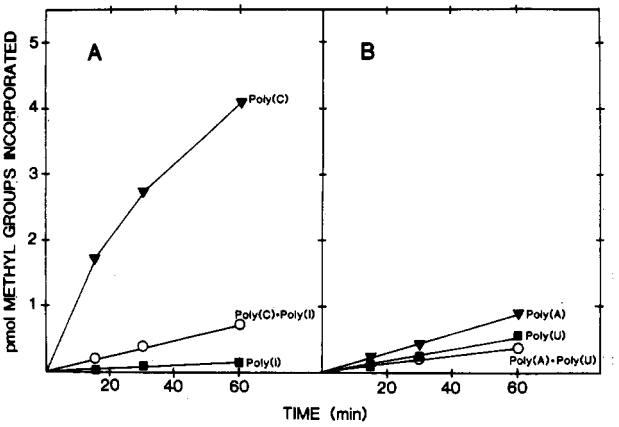

FIGURE 6: Methylation of duplex vs. single-stranded homoribopolymers. The reaction mixtures (0.05 mL) contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/mL bovine serum albumin, 0.4 μM *S*-[^{3}H]adenosylmethionine (3.0 × 10^4 cpm/pmol), and a 1.2 mM sample of the designated RNA substrate. The reactions were started by the addition of 0.84 unit of nucleolar methyltransferase. Aliquots (0.01 mL) were removed at the indicated times and then processed and counted as for a standard assay.

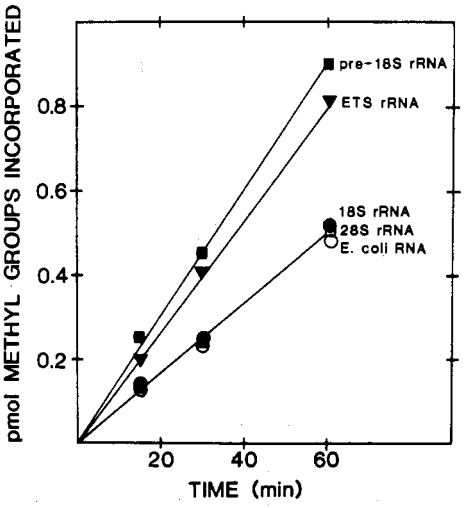

FIGURE 7: Specificity of nucleolar methyltransferase. The reaction mixtures (0.05 mL) contained 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 0.4 mg/mL bovine serum albumin, 0.4 μM *S*-[^{3}H]adenosylmethionine (3.0 × 10^4 cpm/pmol), and a 0.34 μM sample of the designated RNA. The reactions were started by the addition of 0.56 unit of nucleolar methyltransferase. Aliquots (0.01 mL) were removed at the indicated times and then processed and counted as for a standard assay.

Further analysis of methylase specificity was conducted toward natural rRNAs. As shown in Figure 7, no significant difference was observed between mature 18S and 28S ribosomal RNAs as the substrate for the purified nucleolar methyltransferase. some preference, however, was observed when run-off transcripts from cloned mouse rDNA sequences representing either the external transcribed spacer (ETS) region of precursor rRNA or the pre-18S (unmethylated) rRNA sequence were used. These differences could possibly be attributed to the lack of preexisting methylated groups in the run-off transcripts.

Discussion

We have developed a fractionation procedure for the partial purification of a nucleolar methyltransferase based on an assay which allows for the differential detection of 2′-O-methylation of RNA in crude nucleolar extracts. By several criteria, this enzyme bears little resemblance to other RNA methyl-

transferases previously described in the literature. Long et al. (1983) described a partially purified methylase which carried out the methylation of base and ribose moieties in hypomethylated nuclear rRNA. This enzyme has an apparent molecular weight of about 30 000, whereas the enzyme described in this paper was approximately M_r 145 000 and did not carry out detectable methylation of base residues in RNA. Obara et al. (1982) reported on the partial purification of a methylase from Ehrlich cell nucleoli which had an estimated molecular weight of 130 000 and an apparent K_m for *S*-adenosylmethionine of 0.4 μM. Although that particular report did not address the type of methylation relative to sugar or base residues, a subsequent report (Obara et al., 1983) suggested that his enzymatic activity carried out the transfer of methyl groups from *S*-adenosylmethionine to the 5-position of cytosine residues in RNA. In agreement with their work, we also found a very potent base methylation activity (data not shown) in crude nucleolar extracts which appeared to have preference for methylation at the 5-position of cytosine residues in hypomethylated RNA.

To examine the substrate specificity of the partially purified nucleolar 2′-*O*-methyltransferase, we first examined homoribopolymer substrates since the use of one such substrate, poly(A), was initially used in the purification procedure to distinguish this enzyme. Interestingly, this particular enzyme shows a strong preference for poly(C). Because of previous reports of base methylases with this same specificity, we felt it necessary to establish that all methylation was at the 2′-OH position of ribose and not at the 5-position of the cytosine base. The former expectations were confirmed by subjecting the methylated poly(C) to alkaline hydrolysis and analyzing the products of hydrolysis by separation on DEAE-Sephadex A-25. All label was found associated with material eluting at positions larger than mononucleotide. The basis for the apparent preference of poly(C) is not yet understood. In addition, the patterns of methylated alkaline hydrolysis products varied significantly depending on the RNA substrate used. For example, *E. coli* RNA appears to be methylated in such a way that methylation of adjacent sugar residues was significantly more frequent than that observed using poly(A) or poly(C). These results were indicated by the amount of label eluting at positions greater than dinucleotide.

The enzyme will also carry out methylation of mature, and therefore, already-methylated rRNAs. The almost 2-fold preference for the unmethylated vs. methylated 18S RNA sequences is probably not sufficient to define the specificity observed in vivo for ribosomal RNA methylation. The enzyme catalyzed the methylation of the external transcribed spacer sequence nearly as well as the unmodified 18S RNA. In vivo, the external transcribed spacer sequences of the 45S precursor rRNA are not methylated. We calculate that approximately 6% of the nucleotides of pre-18S rRNA were methylated in a 1-h period under conditions of the assay. In vivo, 2′-O-methylated nucleotides account for 1.2% of the total nucleotides in mouse 18S rRNA (Lane & Tamaoki, 1969). The level of in vitro methylation is, therefore, far greater than that observed in vivo for the pre-18S rRNA sequences. This difference would be even greater if the in vitro assay was extended longer than 1 h. Taken together, these results suggest that the extent and specificity of in vivo 2′-O-methylation may depend, in part, on other components that make up the precursor ribosomal ribonucleoprotein particle rather than on RNA sequence and/or structure alone.

Registry No. Poly(C), 30811-80-4; *S*-adenosylmethionine, 29908-03-0; 2′-*O*-methyltransferase, 39369-30-7.

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