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5' CAP METHYLATION OF HOMOLOGOUS POLY A(+) RNA BY A RNA (guanine-7) METHYLTRANSFERASE FROM NEUROSPORA CRASSA

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SUMMARY: RNA (guanine-7) methyltransferase, partially purified from N. crassa mycelia, catalyzed the transfer of the methyl group from S-adenosyl-methionine to the 5' terminus of both N. crassa poly A(+) RNA and reovirus unmethylated mRNA. RNase T_2 digestion of the in vitro methylated poly A(+) RNA from N. crassa yielded the "cap" structures $m^7G(5')pppAp$ and $m^7G(5')pppGp$ in a ratio of 2:1 respectively. RNase T_2 digestion of the in vitro methylated reovirus mRNA yielded $m^7G(5')pppGp$ exclusively. The absence of mRNA 2'-O-methyltransferase activity in the enzyme preparation is consistent with the absence of 2'-O-methylation in N. crassa mRNA [Seidel, B. L. and Somberg, E. W. (1978) Arch. Biochem. Biophys. 187, 108-112]. This is the first isolation of an eucaryotic, cellular RNA (guanine-7) methyltransferase that has been shown to methylate homologous substrate.

INTRODUCTION: Eucaryotic cellular and viral mRNAs contain a variety of 5' terminal "cap structures." In most of these, a terminal 7-methyl guanosine residue is linked to the penultimate nucleoside by a 5', 5' triphosphate bridge. In addition, ribose methylation is often found on one or both of the nucleosides adjacent to the 7-methyl guanosine terminus [for review, see Shatkin (1)]. N. crassa mRNA contains the "cap 0" structures $m^7G(5')pppAp$ and $m^7G(5')pppGp$ which lack ribose methylation and occur in vivo in a ratio of 3:1 (2).

Enzymes involved in the methylation of the 5' terminus of viral mRNAs have been partially purified from vaccinia virus (3) and demonstrated in reovirus (4), vesicular stomatitis virus (5), silkworm cytoplasmic polyhedrosis virus (6), and Artemia salina (7). An eucaryotic cellular RNA (guanine-7) methyltransferase has been partially purified from HeLa cells using, as substrate, unmethylated vaccinia virus mRNA (8); a suitable homologous mRNA substrate was unavailable. We report here the first demonstration of an eucaryotic cellular RNA (guanine-7) methyltransferase that is capable of modifying homologous substrate. The undermethylated homologous poly A(+) RNA, used as sub-

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strate, was prepared from a methionine auxotroph of N. crassa which was starved for methionine.

METHODS: Growth Conditions: N. crassa mutant (met-1, 38706, Fungal Genetics Stock Center, Humboldt State College, Arcata, Ca.) was used for both enzyme and substrate preparation. 20L carboys, containing Vogel's medium (9) supplemented with 50 µg/ml DL-methionine were inoculated with 10^{10} conidia/carboy and aerated for 14.5 h at 30°. The mycelia were harvested and washed with 10L H_2O. For undermethylated substrate preparation, the mycelia were re-suspended in the orig. vol. of medium lacking methionine, aerated for an additional 4.5 h and harvested as before. The mycelia were lyophilized and stored at -20°. The yield was approximately 12 g lyophilized powder/carboy for both the methionine-starved and methionine-supplemented cultures.

Substrate Preparation: Undermethylated RNA was prepared from methionine-starved lyophilized mycelia. Two g were suspended in 28 ml of 0.01M Tris-Cl (pH 7.2), 0.01M $MgCl_2$ at 4° and 3 ml of 5% SDS and 1.5 ml of 1M NaCl were added. The suspension was stirred at room temperature for 2 min and an equal volume of redistilled phenol:$CHCl_3$:isoamyl alcohol (12:12:1) was added. Total RNA was extracted as previously described (2), and precipitated with 2 vol ethanol at -20°. Poly A(+) RNA was prepared by dissolving the total RNA (22 mg) in 8 ml of 0.5M NaCl, 0.01M Tris-Cl (pH 7.5), 0.5% SDS, 1mM EDTA, heating to 80° for 2 min and quick-cooling in an ice bath. The treated RNA was applied to a 5 ml column containing 1 g oligo (dT)-cellulose at room temperature and washed with 5 column vol of buffer. The poly A(-) RNA in this eluate was recovered by ethanol precipitation. The poly A(+) RNA was then eluted with 3 x 4 ml of 0.01M Tris-Cl (pH 7.5), 0.05% SDS, 1mM EDTA, lyophilized, dissolved in 2 ml H_2O and precipitated with 2 vol ethanol (-20°). The recovery of poly A(+) RNA was 0.7 mg, representing 3.5% of the total RNA. It was assumed throughout that 1 mg RNA = $20A_{260}$ units.

Enzyme Purification: 24 g of lyophilized mycelia from 40L of non-starved growth medium was extracted for enzyme by stirring for 20 min with 400 ml of 0.01M Tris-HCl (pH 7.2), 0.01M NaCl, 1.5 mM $MgCl_2$, 1 mM dithiotheitol and 0.01 mg/ml S-adenosylmethionine. S-adenosylmethionine was included to enhance methylation of endogenous substrate and was removed during the dialysis steps (see below). The extract was then centrifuged for 10 min at 10,000 g. The 10,000 g supernatant was further fractionated according to the procedure of Ensinger and Moss (8). This involved differential centrifugation at 30,000 g and 100,000 g, phase partition of the 100,000 g supernatant using polyethylene glycol/dextran-500 and dialysis of the polyethylene glycol phase followed by ammonium sulfate precipitation. The ammonium sulfate precipitate was further dialyzed and applied to a DEAE-cellulose column (2.5 x 35 cm) to further reduce nucleic acid contamination. The DEAE-cellulose excluded material was further fractionated on a DNA-agarose column (2.2 x 16 cm) and eluted with a linear gradient (2 x 150 ml) from 0.05 to 0.5M NaCl (8) (Fig 1). The DNA-agarose fractions were pooled as indicated in Fig 1, concentrated 10 fold by ultrafiltration and stored in small aliquots at -20°. Attempts to purify the enzyme further using carboxymethyl-cellulose or Sephadex G-200 column chromatography were unsuccessful since less than 10% of the enzyme activity was recovered. The DNA-agarose purified fraction (P_9B) was, however, stable for at least several months when frozen at -20° in 10% glycerol. This fraction contained 40% of the original activity in the crude extract and represented a 100 fold purification.

Protein Determination: Protein was determined according to Shaffner and Weissman (10).

Methyltransferase assays:

(1) CCl_3COOH filtration: Total methyltransferase activity was measured by

incubating the enzyme in 0.1 ml of a reaction mixture containing 2.5 mM $MgCl_2$, 2.5 mM GTP, 1 mM dithiotreitol and 0.5 μCi [^{3}H-methyl] S-adenosylmethionine (70 Ci/m mole) in 0.05M Tris-Cl (pH 7.8). After incubation for 60 min at 37°, the reaction was stopped by addition of 0.1 ml of redistilled phenol and 0.1 ml H_2O. The mixture was agitated and centrifuged at room temperature for 5 min at 1,000 g; 0.18 ml of the aqueous phase was removed and added to 0.05 ml of 2 mg/ml carrier poly A . An equal volume of 10% CCl_3COOH was added, the precipitate recovered on a 2.5 cm Millipore filter (0.45 μ pore-size) and washed on the filter with 5 x 5 ml of 5% CCl_3COOH. The filter was dried and counted in Aquosol-2 fluor.

(2) DEAE-cellulose: When the methylated RNA product was further characterized, the standard reaction mixture was scaled up 5 fold (0.5 ml) and the aqueous phase from the phenol treatment was first extracted with 3 x 5 ml of diethyl ether. The ether was removed with N_2 and 0.25 ml of 2 mg/ml polyA carrier was added. RNA was precipitated by the addition of 2 vol of ethanol (-20°) and maintained for at least 3 h at -20°. The RNA was recovered by centrifugation, dissolved in 1 ml of 0.2M Na acetate and the ethanol precipitation repeated two times. The final precipitate was dissolved in 0.5 ml of 0.01M Na acetate (pH 4.5) and incubated for 16 h at 37° with 4 units of RNase T_2. The pH of the solution was adjusted to 6.0 with 0.1M NaOH and 0.25 ml was removed. To the remaining 0.25 ml was added 10 units of P_1 nuclease and the incubation was continued for an additional 30 min. The samples were diluted 4 fold with 0.05M Tris-Cl (pH 8.0) containing 0.05M NaCl and 7M urea, applied separately to a 1.2 x 20 cm DEAE-cellulose column and eluted with a linear gradient (2 x 70 ml) of 0.05 to 0.3M NaCl in the same buffer. Oligonucleotide markers from a partial alkaline hydrolysate (0.1M NaOH, 1 h, 37°, 2 mg) of polyA were used to standardize the columns according to charge.

High voltage paper electrophoresis: Fractions from the DEAE-cellulose columns were desalted by diluting 10-fold with H_2O and adsorbing to a stack of 10 Whatman DE-81 filters under vacuum. The filter stack was washed with 20 ml of 0.01M triethylammonium carbonate (pH 7.5) and the material was eluted with 7 ml of 0.5M triethylammonium carbonate (pH 7.5) and lyophilized. The samples were dissolved in 0.2 ml of 0.02M Tris-Cl (pH 7.5) containing 1mM $MgCl_2$. Nucleotide pyrophosphatase (0.05 Units/ml) was added to an aliquot and the samples were incubated for 15 min at 37°. High-voltage electrophoresis was conducted at pH 3.5 on Whatman 3MM filter paper (11). Radioactive material that comigrated with m^7GMP was eluted from the paper with 0.2 ml of 0.01M Tris-Cl (pH 8.0) and digested with 50 μg of bacterial alkaline phosphatase for 15 min at 37° to remove the phosphate group. This incubation was repeated 3 times and the material applied to 3MM paper and electrophoresis repeated. 1.5 cm strips were counted in toluene-base scintillation fluid. Marker compounds were visualized under U.V. light.

Materials: P_1 nuclease, bacterial alkaline phosphatase, $m^7G(5')pppA$, $m^7G(5')pppG$, polyA, m^7GMP and m^7G, were from P-L Biochemicals. Nucleotide pyrophosphatase, RNase T_2 and S-adenosylmethionine were from Sigma, and oligo (dT)-cellulose (T-3) was from Collaborative Research. DE-81 filters (2.3 cm dia) and 3MM filter sheets were from Whatman; [^{3}H-methyl] S-adenosylmethionine (70 Ci/m mole) and Aquosol-2 were obtained from New England Nuclear. Ultrafiltration was performed under pressure using an Amicon U-10 filter. Reovirus unmethylated mRNA (12) was generously supplied by Dr. Y. Furuichi, Roche Institute of Molecular Biology, Nutley, N. J.

RESULTS: Fig 1A shows the enzyme elution profile from the DNA-agarose column. Various fractions were tested for total methyltransferase activity versus unmethylated reovirus mRNA in the TCA filtration assay. Three peaks of

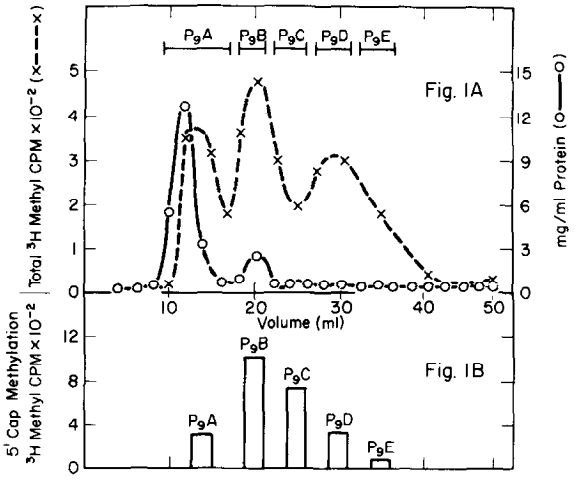


Figure 1: DNA-agarose elution profile of RNA (guanine-7) methyltransferase activity.

1A. Total methylation of reovirus mRNA (TCA filtration assay). Mg/ml protein (o—o). Total ^3H methyl CPM incorporated into 1 μg RNA using 1/100 (0.05 ml) of each fraction (x---x).

1B. Cap methylation of Neurospora poly A(+) RNA (DEAE-cellulose assay). 7.5 μg RNA was methylated with 0.05 ml of each pooled, concentrated enzyme fraction (P_9A-P_9E). The RNA was digested with RNase T_2 and applied to DEAE-cellulose columns. The bar graphs represent the total ^3H methyl CPM that eluted at -4.2 charge. See Methods for assay conditions, nuclease digestion and chromatography.

methyltransferase activity were evident. The fractions were pooled as indicated and further tested versus Neurospora undermethylated poly A(+) RNA in the DEAE-cellulose assay. In this assay, the methylated poly A(+) RNA was isolated by phenol extraction, digested with RNase T_2 and applied to a DEAE-cellulose column in 7M Urea. RNase T_2 digestion of "cap-0" type mRNA molecules yields a product of the general structure $m^7G(5')pppXp$ which elutes from DEAE-cellulose at a charge of approximately -4.2 (2, 11). The amount of radioactivity that eluted at -4.2 charge is shown in the bar graphs in Fig 1B. Fraction P_9B contained the highest concentration of RNA (guanine-7) methyltransferase activity. An aliquot of the RNase T_2 digest of the poly A(+) RNA was further digested with P_1 nuclease which removes the 3' phosphate group from the cap structure and causes a shift in the elution pattern from -4.2 to -2.5 charge. Fig 2A shows the elution profiles of Neurospora poly A(+)RNA, methylated with the P_9B enzyme fraction after RNase T_2 digestion (solid line)

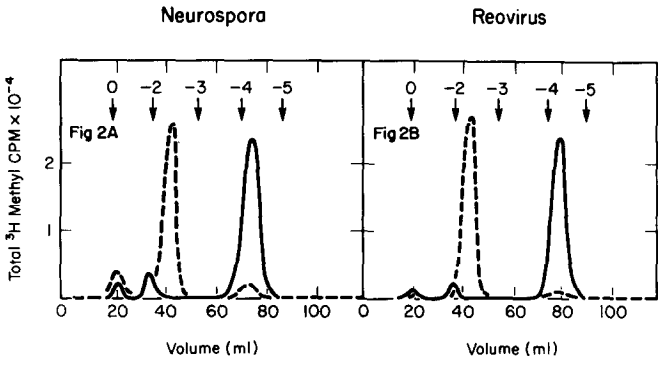
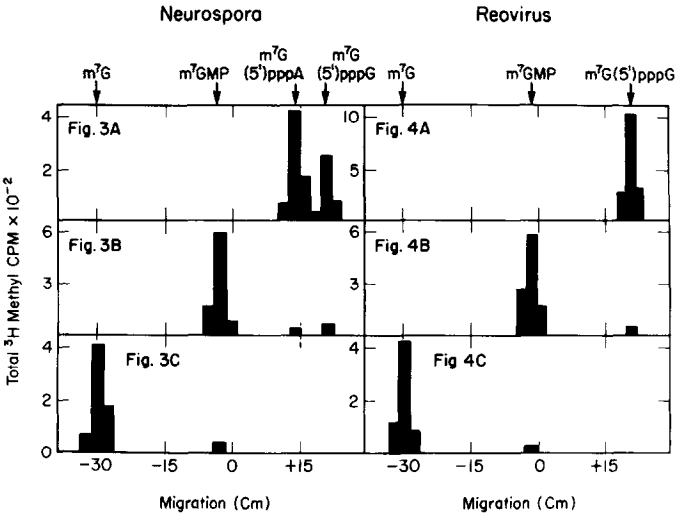


Figure 2: DEAE-cellulose elution profiles of N. crassa poly A(+) RNA (Fig 2A) and reovirus mRNA (Fig 2B) after RNase T_2 digestion (solid lines) and after RNase T_2 + P_1 nuclease digestion (dotted lines). The P_9B enzyme fraction (0.09 mg) was used to methylate both the Neurospora RNA (25 μg) and the reovirus RNA (2 μg). Positions of oligonucleotide markers are indicated by charge at top of figure. See Methods for condition of methylation, nuclease digestion, and chromatography.

and after RNase T_2 + P_1 nuclease digestion (dotted line). When reovirus mRNA was used as substrate with the P_9B enzyme fraction, similar results were obtained (Fig 2B). The -2.5 material from Neurospora poly A(+)RNA comigrated with $m^7G(5')pppA$ and $m^7G(5')pppG$ in a ratio of 2:1 respectively when subjected to high-voltage paper electrophoresis (Fig 3A). The -2.5 material from reovirus mRNA comigrated with $m^7G(5')pppG$ (Fig 4A). Verification of the cap structure was obtained by treatment of an aliquot of the -2.5 materials from Figs 3A and 4A with nucleotide pyrophosphatase. This treatment released m^7GMP in both cases (Figs 3B and 4B). Further treatment of this material with bacterial alkaline phosphatase gave m^7G (Figs 3C and 4C). This material also co-chromatographed with m^7G when subjected to ascending paper chromatography in isobutyric acid: 0.5M NH_4OH (10:6) (data not shown).

DISCUSSION: To our knowledge, this is the first isolation of an eucaryotic, cellular RNA (guanine-7) methyltransferase that has been shown to modify homologous substrate. The only other eucaryotic cellular RNA (guanine-7) methyltransferase that has been isolated was partially purified from HeLa cells by Ensinger and Moss (8). Their fractionation procedure was the basis for our partial purification. Whereas Ensinger and Moss used heterologous unmethylated vaccinia virus mRNA as substrate, our fractionation procedure was based on the methylation of N. crassa undermethylated poly A(+) RNA, prepared from a methionine auxotroph that was starved for methionine.



Figures 3 & 4: High-voltage paper electrophoresis of cap structures. An aliquot of the material that eluted at -2.5 charge from Figs 2A and 2B after T_2 plus P_1 nuclease digestion was further analyzed by peper electrophoresis: N. crassa mRNA (Fig 3A); reovirus mRNA (Fig 4A). Another aliquot was also treated with nucleotide pyrophosphatase before electrophoresis; N. crassa (Fig 3B), reovirus (Fig 4B). The material that comigrated with m^7GMP in Figs 3B and 4B was eluted from the paper, treated with bacterial alkaline phosphatase, and electrophoresed again (Figs 3C and 4C). See Methods for conditions of nuclease digestion and electrophoresis.

The partially purified N. crassa enzyme fraction (P_9B) catalyzed the transfer of the methyl group from S-adenosylmethionine to the 5' terminus of both N. crassa undermethylated poly A(+) RNA and unmethylated reovirus mRNA. In both cases, virtually all of the ^3H-methyl radioactivity was shown to be contained in the cap structures by RNase T_2 digestion and DEAE-cellulose chromatography (Figs 2A and 2B). The material that eluted at -4.2 charge from DEAE-cellulose was shown to contain a triphosphate bond by the shift in the elution pattern from -4.2 charge to -2.5 charge after further treatment with P_1 nuclease (Figs 2A and 2B). Had the -4.2 material contained ribose-methylated trinucleotides of the general structure $N_1^mpN_2^mpN_3p$ which might have been released by RNase T_2 digestion, further treatment of this material with P_1 nuclease would have released only radioactive nucleoside monophosphates which would have eluted from DEAE-cellulose at a -2 charge.

High-voltage paper electrophoresis of the -2.5 material from N. crassa poly A(+)

RNA gave the caps, $m^7G(5')pppA$ and $m^7G(5')pppG$ in a ratio of 2:1 (Fig 3A). This was similar to the results obtained *in vivo* (2). The cap structures were verified by treatment with nucleotide pyrophosphatase which released m^7GMP (Fig 3B). Verification of the m^7GMP was obtained by removal of the phosphate group with bacterial alkaline phosphatase (Fig 3C). High-voltage electrophoresis of the -2.5 material from the *in vitro* methylated reovirus mRNA yielded $m^7G(5')pppG$ exclusively (Fig 4A); this structure was also verified by pyrophosphatase and alkaline phosphatase treatment as above (Figs 4B and 4C). In contrast, the product obtained when reovirus mRNA was methylated with the reovirus core enzyme preparation was $m^7G(5')ppp\overset{m}{G}$, which contained 2'O ribose methylation on the penultimate guanosine residue (4). The absence of 2'O methyltransferase activity in the *N. crassa* enzyme preparation was expected, since ribose methylation was not found in *N. crassa* mRNA *in vivo* (2). Thus, the *in vitro* methylation by the purified cap methylase faithfully reflected the the *in vivo* methylation. In addition, the lack of mRNA ribose methylating activity in the *N. crassa* enzyme preparation distinguished it from both the HeLa cell (8) and the vaccinia virus (3) enzyme preparations, both of which contained traces of this contaminant.

The partially purified RNA (guanine-7) methyltransferase which we have prepared may serve as a useful probe in the further study of mRNA methylation and its function. The ability of the enzyme preparation to methylate homologous as well as heterologous substrate and its complete lack of mRNA ribose methylating activity distinguishes it from any other RNA (guanine-7) methyltransferase that has been reported.

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