Transmethylation and transguanylylation in 5'-RNA capping system isolated from rat liver nuclei
(nuclear enzymes/mRNA/methyltransferase/guanylyltransferase)

Kiyohisa Mizumoto† and Fritz Lipmann‡

The Rockefeller University, New York, New York 10021

Contributed by Fritz Lipmann, July 9, 1979

ABSTRACT Rat liver nuclei were isolated and sonicated for extraction in order to study the capping of RNA. The guanosine 7-methyltransferase was purified from the extract by hydroxylapatite column chromatography with stepwise addition of phosphate buffer. It was assayed by using as methyl acceptor synthetic G(5')ppp(5')G and S-adenosylmethionine as donor. The enzyme appeared in a sharp peak at 160 mM. The same peak fraction was subsequently found to contain the enzyme that guanylates short synthetic polynucleotides and low molecular weight yeast RNA as acceptors. The two enzymatic activities were separated on Sephadex G-150 chromatography, yielding guanylyltransferase and guanosine 7-methyltransferase with molecular weights of approximately 65,000 and 130,000, respectively. Guanylyltransferase was further purified by CM-Sephadex chromatography, whereby G-7-methyltransferase was completely removed. Dithiothreitol was essential for guanylation, and 2 mM MnCl₂ (optimum) was twice as active as 5 mM MgCl₂ (optimum). The δ-32P of [δ-32P]GTP, but not its β- or γ-32P, was incorporated into the cap structure. By using unlabeled GTP with [δ-32P]pppGppCpC-poly(A₅U₃G) as acceptor, [δ-32P]-GpppG₃ was formed. Our purified transguanylylation enzyme was found to catalyze a [32P]pyrophosphate exchange with GTP, which may be useful as a rapid assay for transguanylylation.

An additional role for GTP in protein synthesis has been added through the discovery of its activity in the capping reaction, which results in a blocking of eukaryote mRNA by guanylation of 5'-terminal phosphate groups to form a G(5')-ppp(5')NpN… bridge. The primary reaction is followed by methylation at position 7 of the external guanosine and at the 2'-ribose of internal nucleotides. Reviews of various aspects of the basic mRNA structure have appeared on the mechanism of biosynthesis (1), on the isolation of preformed cap structures from cell nuclei (2), and on the role of RNA processing in eukaryote mRNA production (3). The most thorough studies on the mechanism have been done with virus core-associated or core-derived transguanylylation and transmethylation enzymes (4–6). Cap formation was studied with HeLa cell nuclei by Groner and Hurwit (7), with L-cell nuclei by Winicov and Perry (8), and with HeLa cell crude nuclear extract by Wei and Moss (9). We chose to use an extract of normal rat liver nuclei as enzyme source in the experiments described here.

MATERIALS AND METHODS

Materials. P-L Biochemicals supplied GpppG, GpppCm, GpppA, GpppAm, GpppCpC, GpppU, their 7-methylguanosine derivatives, and G > p, pC > p, and ppG > p; ppG > p was prepared by incubating ppG > p with pyruvate kinase and phosphoenolpyruvate and was purified by chromatography on DEAE-Sephadex. S-Adenosyl[methyl-3H]methionine (11.5 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) was obtained from New England Nuclear, and [α-32P]GTP (350 Ci/mmol) and [γ-32P]GTP (17.2 Ci/mmol) were from Amersham/Searle. [β-32P]GTP (10 Ci/mmol) was prepared from 32P (carrier-free, New England Nuclear) and GDP by exchange reaction with Escherichia coli polynucleotide phosphorylase (P-L Biochemicals), followed by phosphorylation with pyruvate kinase (10). E. coli alkaline phosphatase (code BAPF), nucleotide pyrophosphatase (type III), and nuclease P1 were obtained from Worthington, Sigma, and Yamasa Shoyu (Choi, Japan), respectively.

Oligo- and Polynucleotide Synthesis. GpppC, ppGppC, ppGppCpC, and pppGppCpC were prepared by a modification of the procedure of Simoncits et al. (11) from (ppp)G > p and CpC by reverse reaction of RNAse T1. 32P was introduced in the following manner: [32P]ppG > p was prepared from ppG > p and 32P, by exchange reaction with Micrococcus luteus primer-dependent polynucleotide phosphorylase, using ApApA as primer (12); after incubation for 6 hr at 37°C, [32P]ppG > p was purified by DEAE-Sephadex column chromatography and [32P]pppGppCpC was prepared as described (11). To synthesize [32P]pppGppCpC-poly(A₅U₃G), [32P]pppGppCpC was added with M. luteus polynucleotide phosphorylase according to the method of Both et al. (13).

Purification of Yeast Low Molecular Weight RNA. Yeast soluble RNA (type III, lot 15C-8250, Sigma) was passed through a Sephadex G-75 column and the material that eluted at Vᵣ/V₀ = 1.8–2.3 was lyophilized. This RNA fraction of 10–20 nucleotides in chain length was found to be a very active guanylyl acceptor.

Preparation of Nuclei and Crude Nuclear Extract. Nuclei were isolated from 30 livers of Holtzman male rats (140–160 g), and the extract was prepared by sonication according to Roeder and Rutter (14).

Assay for Methyltransferase. Methyl transfer from S-adenosyl[methyl-3H]methionine with synthetic GpppCm as acceptor was measured as follows. The incubation mixture contained in 10 μl: 50 mM Tris-HCl (pH 7.6), 1 mM MgCl₂, 1 mM dithiothreitol, 2 μM S-adenosyl[methyl-3H]methionine (6000 cpm/pmol), 15% (vol/vol) glycerol, 50 mM (NH₄)₂SO₄, 90–150 μM GpppGm, and the protein fraction to be assayed. After incubation for 40 min at 37°C, the reaction mixture was applied to polyethyleneimine cellulose thin layer chromatography sheets (Brinkmann Instruments), and the sheets were desalted by soaking in absolute methanol and then developed with 0.4 M LiCl together with the mGpppGm marker. The chrom.

Abbreviations: mG, 7-methylguanosine; Gm, 2'-O-methylguanosine; AdoMet, S-adenosylmethionine; p, denotes 32P label in that position; G > p, cyclic GMP; m, denotes 32H label in methyl group.
† Present address: The Institute of Medical Science, University of Tokyo, Tokyo 108, Japan.
‡ To whom reprint requests should be addressed.
Biochemistry: Mizumoto and Lipmann

FIG. 1. Cap formation with crude rat liver nuclear extract. Low molecular weight yeast RNA (1.5 A₂₆₀) was incubated with [α-³²P]GTP and rat liver nuclear extract (150 μg protein) in the presence or absence of 0.2 mM AdoMet under standard conditions, except with 2.5 mM MgCl₂, 0.5 mM MnSO₄, and 15 μM A₅''pppp₅''A. AppppA added to inhibit non-specific nucleotide pyrophosphatase, a possible contaminant. After incubation for 80 min at 37°C, RNA was extracted and processed. (A) Nuclease P₁- and alkaline phosphatase-resistant material was subjected to electrophoresis on Whatman DE-81 paper and the electropherogram was then radioautographed. (B) Analyses of bands 1, 2, and 3 in A by polyethyleneimine cellulose thin layer chromatography. Materials from the labeled molecular weight yeast RNA were incubated for 30 min at 37°C with or without 0.03 unit of nucleotide pyrophosphatase (NPPase) in 10 μl of 50 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate. The hydrolysate was chromatographed on polyethyleneimine cellulose thin layer plates in 1.0 M LiCl (bands 1 and 2) or 0.4 M LiCl (band 3). The positions of authentic markers under ultraviolet light are indicated by the dotted circles.

matogram was cut into 0.5-cm pieces which were eluted with 0.5 ml of 0.5 M (NH₄)₂CO₃, and the overlap with the marker was assayed by scintillation counting in Aquasol (New England Nuclear).

Assay for Guanylyltransferase by Using [α-³²P]GTP as Donor in GpppN-RNA Synthesis. The standard reaction mixture (0.1 ml) contained: 50 mM Tris-HCl (pH 7.7), 2 mM MnSO₄, 0.5 mM MgCl₂, 10 mM dithiothreitol, 9% (vol/vol) glycerol, 6 μM [α-³²P]GTP (7,000–30,000 cpm/pmol), 0.5–1.5 A₂₆₀ units of purified yeast soluble RNA as acceptor, and enzyme fraction. Incubation was for 60 min at 37°C. RNA was extracted with phenol and passed through a Sephadex G-25 column (0.8 × 20 cm) in 40 mM CH₃COONa, and the void volume fractions were lyophilized. The lyophilized sample was digested with nuclease P₁ in 15 μl of 20 mM Na acetate (pH 5.5) containing 10 μg of enzyme for 60 min at 37°C followed by alkaline phosphatase treatment in 25 μl of 80 mM Tris-HCl (pH 8.5) containing 5 μg of bacterial alkaline phosphatase for 60 min at 37°C. Nuclease P₁- and alkaline phosphatase-resistant materials were analyzed by paper electrophoresis on a 20-cm-long Whatman DE-81 sheet for 2 hr at 900 volts in pyridine/acetate buffer, pH 3.5. The electropherogram was radioautographed and the radioactivity in the GpppN marker spot was assayed.

FIG. 2. Separation of methyltransferase and guanylyltransferase on hydroxylapatite column. Rat liver crude nuclear extract (333 mg of protein) from 30 rats was applied to a column of hydroxylapatite (1.0 × 28 cm) and developed stepwise with buffer A (20% glycerol/1 mM dithiothreitol/0.1 mM EDTA/20 mM Tris-HCl, pH 7.7/1 mM MgCl₂) containing 1, 5, 16, 20, and 50 mM potassium phosphate buffer, pH 6.8. Each eluate was pooled and concentrated by (NH₄)₂SO₄ precipitation. Methyltransferase was assayed with 180 μM GpppG as acceptor and [³²P]AdoMet as methyl donor. Guanylyltransferase was assayed with 1.0 A₂₆₀ unit of low molecular weight yeast RNA and [α-³²P]GTP. Activity is expressed as the total activity present in pooled fractions.

RESULTS

Capping Activity and Methyl Transfer in Crude Nuclear Extract. When low molecular weight yeast RNA was incubated with crude nuclear extract and [α-³²P]GTP, nuclease P₁- and bacterial alkaline phosphatase-resistant ³²P-labeled materials appeared in the absence of (band 1) and in the presence of S-adenosylmethionine (AdoMet) (band 2 and 3) as shown in Fig. 1A. For further identification, extracts of these bands were analyzed by polyethyleneimine cellulose thin layer chromatography, as shown in Fig. 1B. The band 1 material comigrated with GpppG marker, and band 2 material gave two spots corresponding to GpppG and GpppG₃. Band 3 also gave two spots, m⁷GpppG and m⁷GpppG₃. Furthermore, [⁵-³²P]GMP was formed from bands 1 and 2, and 7-methyl-[⁵-³²P]GMP from band 3 after treatment with nucleotide pyrophosphatase, indicating that the cap structures m⁷GpppG[m] had been formed. There was no significant difference between the amounts of cap structure formed either in the presence or absence of methyl donor. Thus, three enzymatic activities were found to be present: (i) incorporation of [α-³²P]GMP and methylation from AdoMet to form (ii) m⁷GpppN₃ and (iii) m⁷GpppN₅. In our purified preparation, only the m⁷G transmethylation has been found and no attempts were made to separate the additional ribose methylase activity from crude extracts.

Chromatographic Purification and Separation of Guanosine 7-Methyl- and Guanylyltransferases. The crude nuclear extract was applied to a hydroxylapatite column, and
proteins were successively eluted with 1, 55, 160, 250, and 500 mM potassium phosphate buffer. As shown in Fig. 2, both methylation and guanylation activities were eluted by addition of 160 mM buffer. This eluate was concentrated and then chromatographed on a Sephadex G-150 column, and methyl- and guanylyltransferases eluted separately as shown in Fig. 3. From the calibration curve with standard proteins, the molecular weights of these fractions were estimated to be approximately 130,000 and 65,000, respectively.

As summarized (15), by using the hydroxylapatite eluate peak at 160 mM potassium phosphate (see Fig. 2), the first experiments on transmethylation were carried out with GpppG, GpppC, GpppA, and GpppU as acceptors. [3H]Methyl was accepted from AdoMet on guanosine at C-7 with greatest abundance when guanosine was in the second position, diminishing in amount from cytosine to uridine and adenosine. Methylation of guanosine at C-7 was unexpectedly stimulated by preparations that were ribose-2'-O-methylated on the second guanosine, and particularly on adenosine which was increased 5-fold.

Radioactive Phosphate Incorporation from α-, β-, or γ-[32P]-Labeled GTP into Various Acceptor RNAs. As shown in Table 1, α-phosphate radioactivity of GTP was found in the cap structure with all three acceptor RNAs. This indicates a GMP transfer by pyrophosphate displacement, and, in experiment 2, that a 5'-diphospho-poly(A) is a good acceptor with our enzyme preparation.

Table 1. Cap synthesis with α-, β-, or γ-phosphate-labeled GTP

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low molecular weight yeast RNA</td>
<td>837</td>
<td>0.7</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>ppA(pA)n</td>
<td>113</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Poly(C)</td>
<td>96</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(a) Without treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(b) Alkaline phosphatase-treated</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(c) Alkaline phosphatase-treated/ T4 polynucleotide kinase-treated</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reaction was carried out with 3.5 μg of hydroxylapatite-purified enzyme and with differently labeled GTP under the standard conditions. Incubation was for 90 min. RNAs used were: low molecular weight yeast, 1.5 A₂₆₀ units; ppA(pA)n (a generous gift from B. Moss, National Institutes of Health, 0.7 A₂₆₀ unit; poly(C) (Miles, No. 11-304, lot no. 48), 1.1 A₂₆₀ units; alkaline phosphatase-treated poly(C), 1.0 A₂₆₀ unit; alkaline phosphatase/T4 polynucleotide kinase-treated poly(C), 1.0 A₂₆₀ unit (650 pmol of terminal [32P]P). Specific activities and GTP concentrations were: [α-32P]GTP, 1.5 × 10⁴ cpm/pmol, 3 μM; [β-32P]GTP, 1.4 × 10⁴ cpm/pmol, 2.4 μM; [γ-32P]GTP, 5.4 × 10⁴ cpm/pmol, 2.8 μM. Enzyme treatment of poly(C) was carried out as follows: 1.5 mg of poly(C) was dephosphorylated with 4.3 units of alkaline phosphatase for 90 min in a 200-μl reaction mixture containing 5 mM MgCl₂ and 0.1 M Tris-HCl, pH 8. After incubation, the mixture was treated twice with an equal volume of phenol, passed through a Sephadex G-25 column, lyophilized, and then dissolved in H₂O. The incubation mixture for T4 polynucleotide kinase contained in 200 μl: 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM 2-mercaptoethanol, 50 μM [γ-32P]GTP (specific activity 8000 cpm/pmol), 5 units of T4 polynucleotide kinase, and the above dephosphorylated poly(C). After incubation for 90 min at 37°C, RNA was extracted with phenol, passed through Sephadex G-25, and precipitated twice with ethanol. In experiment 3c, both acceptor RNA and donor GTP were labeled with 32P.
Purification of Guanylyltransferase on CM-Sephadex Column. The guanylyltransferase from Sephadex G-150 was further purified on a CM-Sephadex column as shown in Fig. 4. The transguanylylation activity was eluted at 0.27 M KCl; furthermore, this peak was found to contain the activity that promotes pyrophosphate exchange with GTP. About 350-fold purification and 49% recovery was obtained compared with the crude extract at 4°C. At ~70°C, this preparation remained active for several months. As mentioned, included in this figure is an experiment showing that this preparation of guanylyltransferase catalyzes a \([32P]p\) pyrophosphate exchange with GTP. This indicates a mechanism of GMP transfer from GTP by a pyrophosphate displacement reaction to a diphosphate 5′ terminus of RNA. The perfect overlap of the two assay curves in Fig. 4 indicates that the GTP-PPi exchange assay can be used for assaying guanylyltransferase.

Comparison of Guanylylation to ppGpCpC and pppGpCpC as Acceptors. As shown in Fig. 5, with ppGpCpC guanylyl transfer reached a plateau at 15 μM, whereas with pppGpCpC only one-third as much was incorporated, increasing to about one-half at 50 μM. The comparably much slower rate of reaction in the latter case could be explained by assuming that 5′-pp-RNA is the acceptor and that it is formed by a relatively slow dephosphorylation reaction possibly through a contaminating phosphohydrolase. Because we have not attempted to verify such a mechanism, no more can be concluded than that, in this system, at limited acceptor concentration and 30°C, the 5′-pp-RNA reacts much more rapidly than the 5′-ppp-RNA. It should be mentioned that at 150 μM acceptor concentration and 37°C, in an analogous experiment, nearly equal amounts were guanylylated with di- and triphospho-5′-RNA as acceptor after incubation for the same period. Under these assay conditions, one would have to assume that a fast enough conversion of the triphosphate termini to diphosphate has taken place to reach a similar plateau. As would be predicted from Table 1, GpCpC and pGpCpC were completely inert (data not shown).

Guanylation onto the diphosphate end of RNA was confirmed by using \(\beta^{32P}ppGpCpC\)-poly(A\(_9\), U\(_9\), G) as acceptor to obtain the formation of \(\beta^{32P}pppGpCpC\) (see also Table 1, exp. 2). As shown in Fig. 6A, when the \(\beta^{32P}\)-labeled pp-5′-RNA was incubated with purified guanylyltransferase and GTP and digested with nuclease P1, the \(32P\)-label was found in GpppG. In Fig. 6B, efficient methylation on this preformed cap was observed when methyltransferase and AdoMet were added subsequently. The position of \(32P\) in these cap structures was con-
formed by the release of all $^{32}$P as inorganic phosphate with nucleotide pyrophosphatase (data not shown).

DISCUSSION

The capping reaction of short-chain yeast RNA or synthetic polynucleotides studied here in sonicates of rat liver nuclei appears to follow the most frequently reported pattern—i.e., G$^{\text{32pp}}$ + pN$^{\text{32}}$ $\rightarrow$ G$^{\text{32ppp}}$N$^{\text{32}}$ + PP$^{\text{32}}$(4, 6, 9). The G$^{\text{32ppp}}$N$^{\text{32}}$ structure was found to be derived from the condensation of the $\alpha$-phosphate of GTP with terminal 5'-$\alpha'$-, $\beta'$-phosphates of RNA. After prior or simultaneous removal of $\gamma$-phosphate, a 5'-p$^{\text{32}}$pp$^{\text{32}}$RNA was also capped. This reaction appears to be a pyrophosphate displacement in GTP, and this is confirmed by the fact that transguanylylation activity was found to overlap with a GTP $\rightarrow$ [32P]PP$^{\text{32}}$ exchange reaction (Fig. 4) in the CM-Sephadex chromatogram. Furthermore, such a mechanism was indicated by a slower capping reaction of 5'-pp$^{\text{32}}$pp$^{\text{32}}$RNA as compared with 5'-pp$^{\text{32}}$pp$^{\text{32}}$RNA at 30°C and at limiting concentrations (Fig. 5). This is tentatively explained by a conversion by contaminating enzyme, or by the intrinsic activity of our guanylyltransferase, of triphospho-termini to yield diphospho-RNA. By use of a highly purified vaccinia virus capping system (5), an exclusive capping of the triphosphate terminal of RNA was observed with liberation of equivalent amounts of P$^{\text{32}}$ and PP$^{\text{32}}$. However, an easy reversal by pyrophosphorylase was obtained. This indicates a pyrophosphate displacement reaction which might, in this case, be obligatorily linked with RNA phosphohydrolase to remove the terminal $\gamma$-phosphate.

In addition to the capping reaction, the methyltransferase was also partially purified. However, the ribose-2'-methyltransferase was lost during the hydroxylapatite column chromatography. In an experiment (Fig. 6) we first capped a short RNA chain, and then methylated it with AdoMet, showing that capping may be followed by guanosine C-7 methylation. Because, in addition to m$^{7}$G$^{\text{32ppp}}$N, m$^{7}$G$^{\text{32ppp}}$N$^m$ was also formed in the crude extract experiment (Fig. 1), the two methylations must be due here to different enzymes. This was shown by Barbosa and Moss (16) for the vaccinia core enzymes from which they identified and purified the second methyltransferase.

In our experiments, no indication was found for a GppG cap structure which has been reported to be present in Novikoff hepatoma cell nuclei (2) and also as a product with crude extracts of HeLa cell nuclei (7). Furthermore, no indication was found in our system of the unique mechanism of capping indicated in the reaction: G$^{\text{32ppp}}$ + pN$^{\text{32}}$ $\rightarrow$ G$^{\text{32ppp}}$N$^{\text{32}}$ + PP$^{\text{32}}$, which is reported to be the capping mechanism in the vesicular stomatitis virus system (17).

These studies were supported by Grant GM-13972 from the National Institutes of Health.