Partial purification and characterization of mRNA guanylyltransferase from *Saccharomyces cerevisiae*

Naoto Itoh, Kiyohisa Mizumoto and Yoshito Kaziro

Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

Received 15 March 1983

Abstract not received

Capping enzyme S. cerevisiae *Enzyme–GMP intermediate* *RNA 5′-triphosphatase*

1. INTRODUCTION

Most viral and cellular mRNAs in eukaryotes contain the cap structure, $m^7G(5')pppN_m-$, at their 5′-termini (reviews [1,2]). The mechanism of biosynthesis of the cap structure has been studied with enzyme systems from both viral [3–7] and cellular [8–11] sources. From these studies, the following sequences of reactions are shown to be involved, in general, for the cap formation:

$$pppN- \xrightarrow{\text{RNA triphosphatase}} ppN- + P_i \quad (1)$$

$$\underset{\gamma\beta\alpha}{pppG} + \underset{\beta'\alpha'}{ppN-} \xrightarrow{\text{RNA guanylyltransferase}} \underset{\alpha\beta'\alpha'}{G(5')pppN-} + \underset{\beta\gamma}{PP_i} \quad (2)$$

$$GpppN- + AdoMet \xrightarrow[\text{RNA (ribose-2′-}O\text{-)methyltransferase}]{\text{RNA (guanine-7-)methyltransferase}} m^7GpppN_m- + AdoHcy \quad (3)$$

By studying further the capping reaction (eq.(2)) catalyzed by rat liver guanylyltransferase, we have shown that the reaction proceeds through two partial steps involving a covalent enzyme–GMP intermediate [12]. Formation of the enzyme–GMP complex has been reported also for guanylyltransferase from vaccinia virus [13], HeLa cells [14–16], and calf thymus [17], indicating that the covalent catalysis is a general mechanism for the mRNA capping. More recently, we have found that the RNA 5′-triphosphatase activity catalyzing reaction (1) is closely associated with rat liver guanylyltransferase [18]. Studies on the capping system in yeast are of interest, since it may be possible to isolate t^s mutants of the transcriptional apparatus and analyze the function of the capping enzyme system in the initiation of transcription.

Here, we describe a partial purification and some properties of RNA guanylyltransferase from *Saccharomyces cerevisiae pep4*, a protease-deficient mutant:

(i) Yeast guanylyltransferase of M_r 130000 has been purified ~800-fold by chromatography on Sephadex G-200, CM- and DEAE-Sephadex columns;

(ii) The purified enzyme formed a covalent enzyme–GMP intermediate of app. M_r 45000 on SDS–polyacrylamide gel electrophoresis;

(iii) An RNA 5′-triphosphatase activity was found to be associated with the partially purified enzyme.

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

2. MATERIALS AND METHODS

Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma. [α-^{32}P]GTP and $^{32}PP_i$ were obtained from Amersham Japan and New England Nuclear, respectively. Zymolyase 60000 was the product of Kirin Brewery (Japan). Pepstatin was a kind gift from Dr T. Matsushima in our institute. *Saccharomyces cerevisiae* A364A (*a ade*1,2 *ura*1 *his*7 *lys*2 *tyr*1 *gal*1) and *S. cerevisiae pep*4 (*a ade*3 *leu*1 *pep*4) were provided by Drs Y. Nogi and T. Fukazawa (Keio University, Tokyo) and T. Mizunaga (University of Tokyo), respectively. YEPD broth for yeast growth contained 1% yeast extract, 2% peptone and 2% glucose supplemented with 10 μg/ml each of adenine and uracil.

2.1. *Enzyme assays*

RNA guanylyltransferase was assayed by the cap formation on ppG-terminated RNA, GTP–$^{32}PP_i$ exchange, or the enzyme–[^{32}P]GMP complex formation as in [8,12]. RNA (guanine-7-)methyltransferase was assayed using GpppG as a substrate [8].

The standard reaction mixture for RNA 5′-triphosphatase assay contained in 10 μl; 50 mM Tris–HCl (pH 7.9), 0.5 mM $MgCl_2$, 2 mM dithiothreitol, 16% (v/v) glycerol, 5 μg bovine serum albumin, 1 pmol [^{32}P]$\overset{*}{p}$pppA(pA)$_n$ ($1-4 \times 10^4$ cmp/pmol) and enzyme fraction. After incubation for 30 min at 30°C, the reaction mixture received 2 μl 5 N HCOOH and was applied to polyethyleneimine cellulose thin-layer plate, which was developed with 0.5 M KH_2PO_4 (pH 3.5). The $^{32}P_i$ spots were cut out and the radioactivity was counted. [^{32}P]$\overset{*}{p}$pppA(pA)$_n$ was synthesized from [γ-^{32}P]ATP by *E. coli* RNA polymerase using heat-denatured calf thymus DNA as a template [18].

2.2. *Preparation of yeast crude extracts*

Spheroplasts were prepared from *S. cerevisiae* essentially as in [19] except that Zymolyase 60000 was used instead of glusulase to lyse the cell walls. After washing twice with 1.2 M sorbitol, spheroplasts were suspended in 50 mM Tris–HCl (pH 7.5)/100 mM KCl/5 mM $MgCl_2$/10 mM 2-mercaptoethanol/1 mM PMSF/2 μg pepstatin/ml and homogenized in a glass homogenizer with a motor-driven Teflon pestle for 3 min. To the homogenate was added $(NH_4)_2SO_4$ to 0.3 M and the mixture was homogenized further for 2 min to lyse the nuclei, and centrifuged for 120 min at 100000 × *g*. The supernatant was concentrated by $(NH_4)_2SO_4$ precipitation (80% saturation), and dialyzed against buffer A (40 mM Tris–HCl, pH 7.9/20% glycerol/5 mM 2-mercaptoethanol/0.1 mM EDTA/1 mM PMSF/2 μg pepstatin/ml) containing 50 mM KCl and 1 mM $MgCl_2$.

3. RESULTS

When concentrated crude extracts from *S. cerevisiae pep*4 were chromatographed on a Sephadex G-200 column, both guanylyltransferase and (guanine-7-)methyltransferase were eluted near the void volume (fig.1). This is in contrast to rat liver enzymes in which guanylyltransferase (65 kDa) and methyltransferase (130 kDa) were separated by Sephadex G-150 column chromatography [8]. To characterize yeast guanylyltransferase further, the active fractions in fig.1 were adsorbed onto a CM–Sephadex column and proteins were eluted with a linear gradient of KCl. The guanylyltransferase assayed by the capping activity was eluted as a single peak at 0.15 M KCl, while

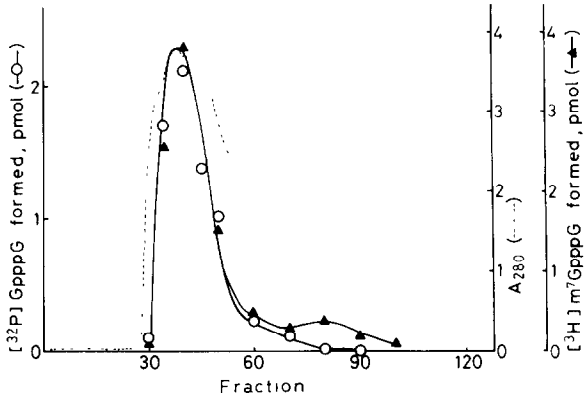

Fig.1. Sephadex G-200 column chromatography of yeast crude extract. S-100 fraction (840 mg protein) from 22 g *S. cerevisiae pep*4 was concentrated and chromatographed on a column of Sephadex G-200 (1.7 × 63 cm) pre-equilibrated with buffer A containing 50 mM KCl and 1 mM $MgCl_2$. Fractions (1.4 ml) were collected, and 5 μl of each fraction was assayed for capping (○) and methyltransferase (▲) activities as in section 2.

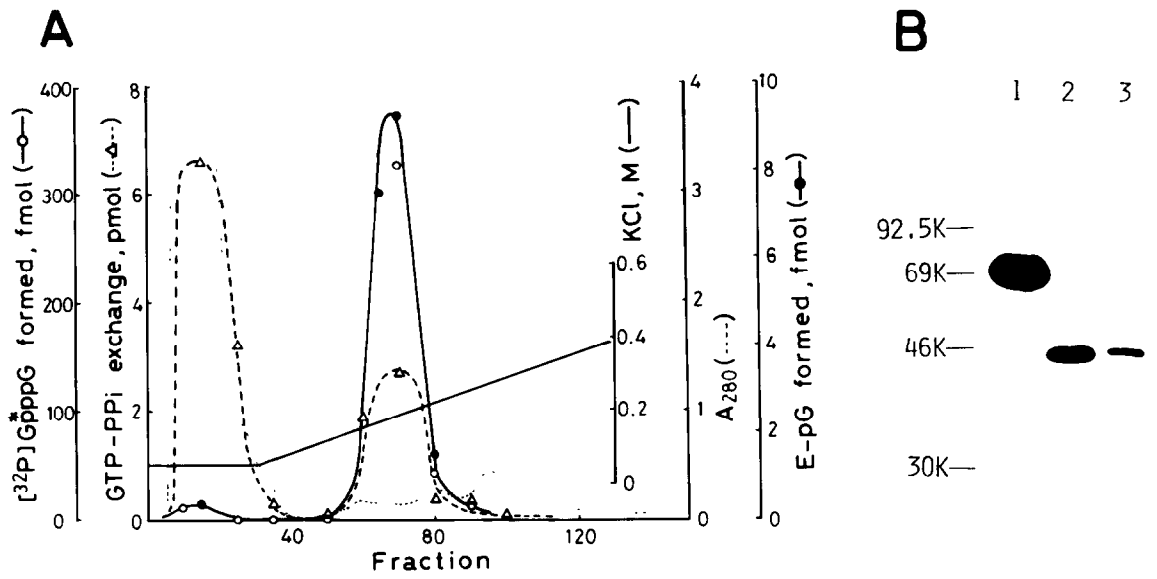

Fig.2. (A) Purification of guanylyltransferase by CM–Sephadex column chromatography. The guanylyltransferase fractions from Sephadex G-200 were pooled and directly applied onto a column of CM–Sephadex C-50 (1.5 × 40 cm) equilibrated with buffer A containing 50 mM KCl. The proteins were eluted with a 400 ml 50–500 mM KCl gradient in buffer A. Fractions (2.5 ml) were collected, and 1 μl of each fraction was assayed for cap formation (○), GTP–PP_i exchange (△), and protein–[^{32}P]GMP complex formation (●) activities. The amount of the protein–[^{32}P]GMP complex was determined by measuring the radioactivity in the M_r 45000 bands in a similar experiment as in (B).

Fig.2. (B) Demonstration of the enzyme–[^{32}P]GMP complex on SDS–polyacrylamide gel electrophoresis. Enzyme frations were incubated with [α-^{32}P]GTP to generate the enzyme–[^{32}P]GMP complex [12]. The proteins were precipitated with cold 5% trichloroacetic acid and subjected to electrophoresis as in [12]. Lanes: (1) 1.3 μg rat liver guanylyltransferase purified through CM–Sephadex [8]; (2) 4.5 μg Sephadex G-200 step guanylyltransferase from *S. cerevisiae* A364A; (3) 1 μl of pooled CM–Sephadex fractions (60–73) in (A).

most proteins were not adsorbed to the column (fig.2A). Exactly co-chromatographed was a GTP–PP_i exchange activity, suggesting the presence of an enzyme–GMP reaction intermediate as observed for rat liver capping enzyme [12]. The presence of such an intermediate was demonstrated by incubating the column fractions with [α-^{32}P]GTP followed by electrophoresis of ^{32}P-labeled proteins on SDS–polyacrylamide gel. A single radioactive band that migrated to the position of app. M_r 45000 was found with the pooled capping enzyme fraction from the CM–Sephadex column (fig.2B(3)). By this assay, the amount of ^{32}P incorporated into the 45 kDa enzyme–[^{32}P]guanine nucleotide complex was found to be proportional to the capping or the GTP–PP_i exchange activities of the corresponding fractions from the CM–Sephadex column (fig.2A). Mild acid treatment (for 30 min, at 37°C in 0.1 N HCl) of the complex released all the ^{32}P radioactivity as GMP. A phosphoamide-type linkage between the enzyme and GMP was suggested from its acid-labile, alkali-stable nature and the susceptibility to the cleavage by acidic hydroxylamine (not shown).

The yeast [^{32}P]enzyme–GMP complex was isolated in a native form through a Sephadex G-50 column to test whether the 45 kDa complex is an active intermediate of the guanylylation reaction. When the isolated [^{32}P]enzyme–GMP complex was incubated with ppG-RNA, the enzyme-bound GMP moiety was quantitatively transferred to the acceptor RNA molecule to form [^{32}P]G$\overset{*}{p}$ppG-RNA. This was confirmed by isolating [^{32}P]G$\overset{*}{p}$ppG on paper electrophoresis after digestion of the product RNA with nuclease P1.

The size of the yeast enzyme–GMP complex on the SDS–polyacrylamide gel electrophoresis was

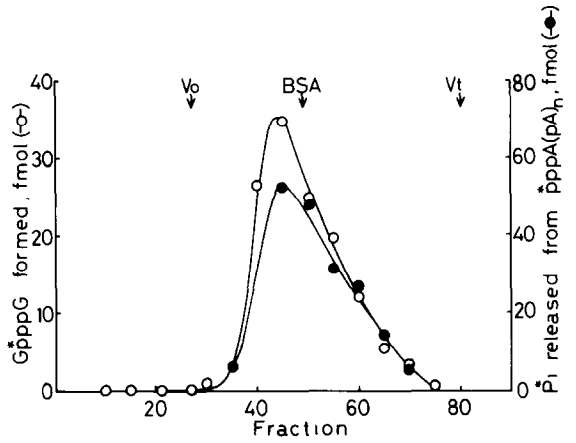

Fig.3. Second Sephadex G-200 column chromatography of yeast guanylyltransferase. A portion of the concentrated CM–Sephadex step enzyme (140 μg protein) was chromatographed on a column of Sephadex G-200 (0.7 × 33 cm) pre-equilibrated with buffer A containing 50 mM KCl and 1 mM $MgCl_2$. Fractions (0.16 ml) were collected at 4 ml/h, and assayed for capping (○) and RNA triphosphatase (●) activities, using 5 μl and 0.5 μl aliquots, respectively.

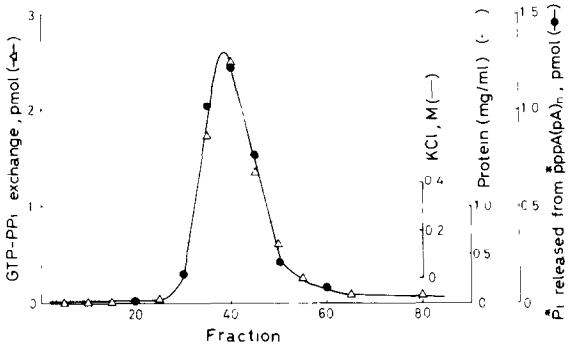

Fig.4. DEAE–Sephadex column chromatography of yeast guanylyltransferase. A portion of concentrated CM–Sephadex step enzyme (1.1 mg protein) was applied to a column of DEAE–Sephadex A-50 (0.6 × 18 cm), and the proteins were eluted with a 40 ml 50–400 mM KCl gradient in buffer A. Fractions (0.5 ml) were collected, and 1 μl each fraction was assayed for GTP–PP_i exchange (△) and RNA triphosphatase (●).

considerably smaller than that of the rat liver enzyme (69 kDa, see fig.2B(1)). An identical band of the enzyme–[^{32}P]GMP complex of 45 kDa was obtained for guanylyltransferase from yeast strain A364A (fig.2B(2)). Since the undenatured form of the enzyme was estimated to be 130 kDa by gel filtration on Sephadex G-200 (fig.3) and Sephacryl S-200 columns (not shown), the yeast capping enzyme may consist of several subunits.

Another peak of the GTP–PP_i exchange activity was eluted in the flow-through fractions of CM-Sephadex column chromatography but this activity was not related to guanylyltransferase, since neither the capping nor the enzyme–GMP complex forming activity was detected in these fractions (fig.2A). RNA (guanine-7-)methyltransferase activity was eluted from the CM–Sephadex column in two peaks, one in the flow-through fractions and the other at 0.25 M KCl (not shown). They were not characterized further.

It would be of interest to examine whether or not an RNA 5′-triphosphatase is associated with the yeast capping enzyme. Since the enzyme after CM–Sephadex column chromatography was still contaminated with an ATP-hydrolyzing activity, it was difficult to demonstrate the presence of the specific RNA 5′-triphosphatase activity. Therefore, we purified further guanylyltransferase through a DEAE-Sephadex column. An RNA triphosphatase activity was co-chromatographed with guanylyltransferase and eluted at 0.2 M KCl (fig.4). Association of the RNA triphosphatase activity with the guanylyltransferase activity was also demonstrated by gel-filtration on a Sephadex G-200 column (fig.3). The RNA triphosphatase activity specifically removed ^{32}P as inorganic

Table 1

Substrate specificity of yeast RNA triphosphatase

Substrate	$^{32}PP_i$ released (fmol)
5′-[γ-^{32}P]ATP-terminated poly(A)	1160
5′-[β-^{32}P]ATP-terminated poly(A)	5
[γ-^{32}P]ATP	2

The reactions were carried out for 30 min under standard conditions (section 2) using guanylyltransferase purified through DEAE–Sephadex column (1 μl fraction 37 in fig.3). The substrates used were 40 nM [γ-^{32}P]pppA(pA)$_4$, 100 nM [β-^{32}P]pppA(pA)$_n$, or 40 nM [γ-^{32}P]ATP. [β-^{32}P]pppA(pA)$_n$ was synthesized from [β-^{32}P]ATP [20] instead of [γ-^{32}P]ATP as described for [γ-^{32}P]pppA(pA)$_n$ synthesis in section 2.

phosphate from [γ-^{32}P]$\overset{*}{p}$ppA(pA)$_n$, but neither from [β-^{32}P]p$\overset{*}{p}$pA(pA)$_n$ nor from [γ-^{32}P]ATP (table 1). The enzyme required Mg^{2+} for its activity with an optimal 0.5 mM. This is in contrast to the rat liver RNA triphosphatase which does not require Mg^{2+} [18].

4. DISCUSSION

Capping enzyme has been isolated from vaccinia virus [21,22] and various eukaryotic cells including rat liver [8], HeLa cells [23], wheat germ [24], and calf thymus [17]. Vaccinia virus capping enzyme was reported to contain the activities of RNA guanylyltransferase, RNA (guanine-7-)methyltransferase and RNA triphosphatase in a 6 S complex of 127 kDa consisting of 2 major polypeptides of 95 and 31.4 kDa [21]. On the other hand, RNA (guanine-7-)methyltransferase was easily separated from guanylyltransferase in an early stage of purification of the cellular capping enzyme. Guanylyltransferase from cellular sources seemed to consist of a single polypeptide with 65–69 kDa [8,14,17,24]. We have found that the 69-kDa protein purified as guanylyltransferase from rat liver possesses both mRNA capping and RNA triphosphatase activities [18].

Since guanylyltransferase from *S. cerevisiae* catalyzes the GTP–PP_i exchange and the enzyme–GMP intermediate formation, the reaction mechanism of the yeast enzyme appears to be the same as other cellular systems:

(i) GTP + E $\rightleftharpoons$ E–GMP + PP_i

(ii) E–GMP + ppN– $\longrightarrow$ GpppN– + E.

However, the yeast enzyme has a unique feature in its molecular structure. The enzyme purified through CM–Sephadex column chromatography was 130 kDa as measured by gel filtration, while the enzyme–GMP complex was detected as a 45-kDa band on SDS–polyacrylamide gel. These results indicate that the yeast capping enzyme possesses subunit structures.

In purification of enzymes from yeast, care must be taken to prevent proteolysis during preparation [27]. To minimize proteolytic artefacts, we have chosen the protease-less mutant, *S. cerevisiae pep4* [26] as the enzyme source, and PMSF and pepstatin were included in all buffer solutions used for the purification. Under these conditions, we reproducibly observed only the 45-kDa enzyme–GMP complex with guanylyltransferase prepared from *pep4* as well as from A364A, a wild-type strain with regard to proteases. Furthermore, preincubation of the purified rat liver guanylyltransferase with the yeast S-100 fraction did not cause any detectable fragmentation of the rat liver enzyme. From these facts, it seems unlikely that the 45-kDa enzyme–GMP intermediate we have isolated is generated by a proteolysis of a larger polypeptide during preparation. Recently, an enzyme–[^{32}P]guanine nucleotide complex has also been isolated from *Saccharomyces uvarum* and *S. globosus* (J. Spy, a personal communication). According to him, the M_r of the complex estimated by SDS–polyacrylamide gel electrophoresis was 50000, while the M_r of the enzyme obtained by sucrose gradient centrifugation was 150000.

Since we have found an association of RNA triphosphatase activity with the purified capping enzyme, it is of interest to assign the former activity to any of the polypeptides that make up the yeast 130 kDa enzyme. Further purification and characterization of the enzyme is now in progress.

REFERENCES

[1] Shatkin, A.J. (1976) Cell 9, 645–653.
[2] Banerjee, A.K. (1980) Microbiol. Rev. 44, 175–205.
[3] Furuichi, Y. and Miura, K. (1975) Nature 253, 374–375.
[4] Furuichi, Y., Muthukrishnan, S., Tomasz, J. and Shatkin, A.J. (1976) J. Biol. Chem. 251, 5043–5053.
[5] Martin, S.A. and Moss, B. (1976) J. Biol. Chem. 251, 7313–7321.
[6] Monroy, G., Spencer, E. and Hurwitz, J. (1978) J. Biol. Chem. 253, 4490–4498.
[7] Venkatesan, S., Gershowitz, A. and Moss, B. (1980) J. Biol. Chem. 255, 903–908.
[8] Mizumoto, K. and Lipmann, F. (1979) Proc. Natl. Acad. Sci. USA 76, 4961–4965.
[9] Ensinger, M.J. and Moss, B. (1976) J. Biol. Chem. 251, 5283–5291.
[10] Venkatesan, S. and Moss, B. (1980) J. Biol. Chem. 255, 2835–2842.
[11] Lansberg, S.R. and Moss, B. (1981) J. Biol. Chem. 256, 10054–10060.

[12] Mizumoto, K., Kaziro, Y. and Lipmann, F. (1982) Proc. Natl. Acad. Sci. USA 79, 1693–1697.
[13] Shuman, S. and Hurwitz, J. (1981) Proc. Natl. Acad. Sci. USA 78, 187–191.
[14] Venkatesan, S. and Moss, B. (1982) Proc. Natl. Acad. Sci. USA 79, 340–344.
[15] Shuman, S. (1982) J. Biol. Chem. 257, 7237–7245.
[16] Wang, S., Furuichi, Y. and Shatkin, A.J. (1982) Mol. Cell. Biol. 2, 993–1001.
[17] Nishikawa, Y. and Chambon, P. (1982) EMBO J. 1, 485–492.
[18] Yagi, Y., Mizumoto, K. and Kaziro, Y. (1983) EMBO J. in press.
[19] Gasior, E., Herrera, F., Sadnik, I., McLaughlin, C.S. and Moldave, K. (1979) J. Biol. Chem. 254, 3965–3970.
[20] Kaufmann, G., Choder, M. and Groner, Y. (1980) Anal. Biochem. 109, 198–202.
[21] Martin, S.A., Paoletti, E. and Moss, B. (1975) J. Biol. Chem. 250, 9322–9329.
[22] Monroy, G., Spencer, E. and Hurwitz, J. (1978) J. Biol. Chem. 253, 4481–4489.
[23] Venkatesan, S., Gershowitz, A. and Moss, B. (1980) J. Biol. Chem. 255, 2829–2834.
[24] Keith, J.M., Venkatesan, S., Gershowitz, A. and Moss, B. (1982) Biochemistry 21, 327–333.
[25] Pringle, J.R. (1982) Methods Cell Biol. 12, 149–184.
[26] Jones, E.W. (1977) Genetics 85, 23–33.