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Synthesis of Gp_4N and Gp_3N compounds by guanylyltransferase purified from yeast

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ABSTRACT

Guanylyltransferase that catalyzes mRNA capping by the reaction, ppNpN + GTP ⟶ GpppNpN was purified from S. cerevisiae. The enzyme forms a nucleotidyl intermediate by phosphoamide linkage of GMP. Two guanylylated polypeptides of M_R ~52,000 and 46,000 were obtained, the latter apparently by proteolysis of the larger component. Both forms transferred the covalently bound GMP to ppApG, yielding GpppApG. Dinucleoside tri- and tetraphosphates of the type Gp_3N and Gp_4N were also produced by using ribonucleoside 5'-di and triphosphates as acceptors. The purified yeast guanylyltransferase contained little or no RNA 5'-triphosphatase or methyltransferase.

INTRODUCTION

One of the key enzymes necessary for generating mRNAs in a variety of eukaryotic cellular and viral systems is mRNA guanylyltransferase (1). It selectively converts the 5'-termini of nascent pre-mRNAs to GpppN ends. As a consequence of guanylylation, transcripts are stabilized (2,3) and become substrates for methyltransferase(s) that complete the characteristic cap structure, $m^7GpppN^{(m)}$.

mRNA guanylyltransferase has been isolated from many sources including vaccinia virus (4,5), wheat germ (6), HeLa cells (7-9), rat liver (10), calf thymus (11) and, most recently, yeast (12,13). As reported first for the viral enzyme, cellular guanylyltransferases also form enzyme-GMP covalent complexes that are intermediates in the capping reaction:

$$E + GTP \rightleftarrows E\text{-}pG + PPi$$

$$E\text{-}pG + ppNpN \longrightarrow GpppNpN + E.$$

Because mRNA capping constitutes a fundamental and distinguishing feature of eukaryotic genetic expression, further study of this process including isolation and characterization of guanylyltransferase genes seems warranted. An initial experimental approach involving the preparation of specific antibody to guanylyltransferases from HeLa cells and calf thymus was hampered by the low amount of enzyme in animal cells. As an alternative,

guanylyltransferase has been purified from S. cerevisiae pursuant to amino acid sequencing as a basis for synthesis of specific gene probes. The properties of the purified enzyme including a capacity to catalyze formation of the cap-like compounds, Gp_4N and Gp_3N are the subject of this report.

METHODS

Cell culture and extraction

Saccharomyces cerevisiae strain SKQ-2N was inoculated into medium consisting of 1% yeast extract, 2% peptone and 2% glucose by addition of a 1/200 volume of seed culture (A_{700nm} = 1-2). After aerated growth for 18 hr at 28°C, the A_{700nm} was again 1-2, and cells were harvested by centrifugation. A portion (20 gm) of the wet cell pellet was suspended in 40 ml of 30 mM Hepes buffer-pH 7.4 containing 2 mM dithiothreitol and 10% glycerol (buffer A); 0.3 mM phenylmethylsulfonyl fluoride was also present to inhibit proteolysis. Cells were broken by shaking with glass beads for 3 min at 5,000 rpm in a mechanical cell disrupter. This procedure and subsequent ones were carried out at 0-4°C. A crude extract was obtained as the supernatant fraction after centrifugation for 1 hr at 25,000 rpm (Beckman SW-28 rotor).

Enzyme purification

The extract (36 ml) was applied to DEAE-cellulose DE-52 (2.6 x 22 cm column) that had been pre-equilibrated with buffer A. Samples eluted with a gradient of NaCl (0-1.0 M in buffer A, 200 ml each) were collected in 5 ml fractions, and every other tube was assayed for guanylyltransferase activity. A broad peak of activity eluted between 0.3 and 0.7 M. These fractions were combined (160 ml), dialysed against buffer A, and loaded onto heparin-Sepharose (1.5 x 3 cm column) equilibrated with buffer A. Fractions of 2.5 ml from a gradient elution (0-0.7 M NaCl in buffer A, 50 ml each) were assayed, pooled (30 ml, 0.23-0.34 M NaCl), dialysed and re-applied in buffer A to cellulose phosphate P11 (0.7 x 6 cm column). The same elution conditions were used, and the pooled fractions (25 ml, 0.2-0.4 M NaCl) were dialysed against 0.025 M tris-acetate-pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol (buffer B) containing 0.05 M NaCl and applied to GTP-Sepharose (0.7 x 2 cm column, buffer B). A gradient of 0.05 to 1.0 M NaCl (25 ml each in buffer B) was used, and 1.6 ml fractions were collected and assayed. The peak of activity eluted between 0.14 and 0.34 M NaCl (16 ml pool).

Guanylyltransferase assay

Enzyme fractions were incubated in a 40 ul mixture consisting of 25 mM tris-HCl-pH 7.5, 5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dithiothreitol, 2.5 ug bovine

serum albumin, 5 uCi α-^{32}P-GTP (specific activity = 200-410 Ci/mmole) and 7 µM ppApG where indicated. After 30 min at 37°C (standard conditions), samples were adjusted to 0.01 M EDTA digested sequentially with nuclease P1 and calf intestine alkaline phosphatase, and analyzed by high voltage paper electrophoresis in pyridine acetate-pH 3.5 and/or thin layer chromatography, all as described previously (8).

Isolation of ^{32}P-labeled enzyme-GMP intermediate

Guanylyltransferase purified through step 5 (GTP-Sepharose) was incubated in a 0.5 ml reaction mixture under standard conditions with α-^{32}P-GTP but without acceptor. Also added were 2 ug inorganic pyrophosphatase to prevent the back reaction, E-pG + PPi $\longrightarrow$ E + GTP and the protease inhibitors, 0.5 mM phenylmethylsulfonyl fluoride and 100 units Trasylol. The reaction was stopped by addition of 30 µl 0.2 M EDTA, and the sample was treated with 20 units calf intestine alkaline phosphatase (37°-30 min) before purification of the enzyme-GMP by heparin-Sepharose chromatography (8).

RESULTS

Crude extract from 20 gm of cells was fractionated by sequential chromatography on DEAE-cellulose, heparin-Sepharose. cellulose phosphate and GTP-Sepharose. Column fractions were assayed for guanylyltransferase based on the utilization of α-^{32}P-GTP to convert ppApG to G*pppApG. The pooled, broad peak of activity eluting from DEAE-cellulose between 0.3 and 0.7 M NaCl was 25-fold enriched in guanylyltransferase (Table 1). In addition, the total activity was increased several-fold at this first step in the purification, presumably due to removal of inhibitor(s), e.g. phosphatase(s) that degraded the donor GTP and/or the 5'-terminal diphosphate on the acceptor.

Table 1. Summary of Purification of Yeast mRNA Guanylyltransferase.

Step	Pooled Fraction	Protein (mg)	Enzyme Activity (total U)	Sp. Act. (U/mg)	Fold Purification
1	Crude extract	1468.5	185	0.13	1
2	DEAE-cellulose DE-52	305.7	1003	3.3	25
3	Heparin-Sepharose	31.3	186	5.9	45
4	Cellulose phosphate P11	6.5	127	19.5	150
5	GTP-Sepharose	1.5	45	30.0	231

1 unit is equivalent to conversion of 1 pmole of ppApG to GpppApG under standard conditions.

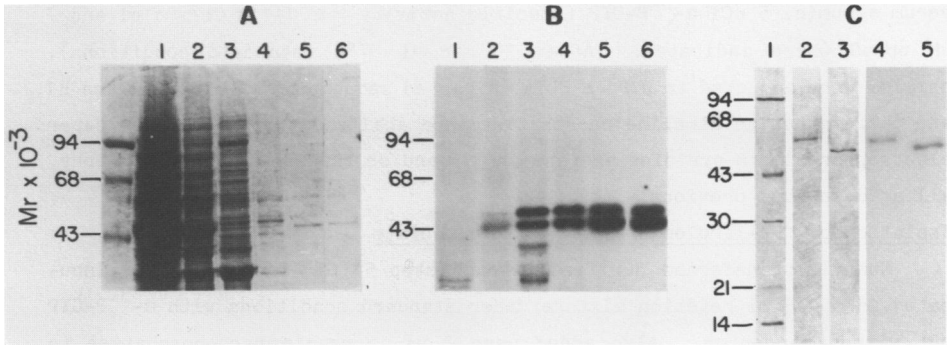

Fig. 1. Polyacrylamide gel electrophoresis of guanylyltransferase at different purification steps. Pooled enzyme fractions (10 ul) were incubated under standard conditions with α-^{32}P-GTP but no acceptor and analyzed under denaturing conditions in a 10% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate (8,17). A. Coomassie-blue stained; B Autoradiogram of the same gel. Lanes and amounts of protein: 1. crude extract, 0.4 mg; 2. DEAE-cellulose eluate, 20 ug; 3. Heparin-Sepharose eluate, 10.4 ug; 4. Cellulose phosphate eluate, 2.6 ug; 5. GTP-Sepharose eluate, 0.9 ug; 6. step 5 sample radiolabeled and repurified by heparin-Sepharose chromatography. C. Another sample of radiolabeled step 5 enzyme eluted from a gel as in lane 5 was re-analysed by silver staining (18) followed by autoradiography. Lanes 2,3: 0.1 ug, 0.13 ug protein estimated from amino acid analyses.

Subsequent chromatography steps removed most of the extraneous protein and resulted in a 231-fold purification with a 24% recovery relative to the guanylyltransferase activity in the crude extract (Table 1).

To determine if yeast guanylyltransferase resembles the corresponding mammalian enzyme, i.e. forms a guanylyl covalent complex, pooled samples from each purification step were incubated with α-^{32}P-GTP but without acceptor and analyzed by SDS-polyacrylamide gel electrophoresis. The stained gel profiles were progressively decreased in polypeptide complexity, most notably after step 4 (Fig. 1A, lane 4). Autoradiography of the gel revealed two radioactive bands that were prominent after the heparin-Sepharose step (Fig. 1B, lane 3). They remained the only polypeptides that were ^{32}P-labeled by incubation of step 5 enzyme with α-^{32}P-GTP (lane 5), and both radiolabeled bands were obtained after rechromatography on heparin-Sepharose (lane 6). The individual radiolabeled bands eluted from a gel corresponding to lane 5 also were re-analyzed; the two silver stained, ^{32}P-labeled polypeptides had apparent molecular weights of ca. 52,000 and 46,000 (Fig. 1C).

The nature of the ^{32}P-labeled ligand and its covalent linkage to the polypeptides were determined. Radiolabeled polypeptides obtained as in Fig.

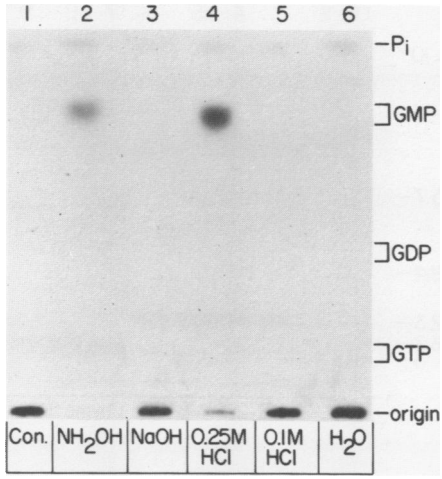

Fig. 2. Stability of protein-GMP complex. ^{32}P-labeled samples corresponding to the upper and lower bands in Fig. 1B, lane 6, were extracted with H_2O, combined and treated as follows: 1. 0.2 M sodium acetate-pH 4.8, 37°C, 20 min; 2. same as 1 but in the presence of 3.8 M hydroxylamine; 3. 0.25 M NaOH, 70°C, 10 min; 4. 0.25 M HCl, 70°C, 10 min; 5. same as 4 but 0.1 M; 6. H_2O, 37°C, 20 min. Samples were neutralized and spotted on PEI-cellulose TLC plates which were developed in 0.7 M KH_2PO_4-pH 4.3 before autoradiography.

1, lane 6 were eluted, combined, and analyzed by thin layer chromatography. As shown in Fig. 2, treatment with 3.8 M hydroxylamine at pH 4.8 or with 0.25 M HCl released most of the radiolabel as GMP. The guanylyl-polypeptide complex was relatively stable in 0.25 M NaOH or 0.1 M HCl and remained at the origin. These properties indicate that the yeast enzyme, like guanylyltransferases from other sources, forms enzyme-GMP complexes via phosphoamide-linkage. The Km determined for the reaction, E + GTP $\longrightarrow$ E-pG + PPi was 1 x 10^{-5}M.

A simple explanation for the presence of two ^{32}P-labeled polypeptides, rather than a single gel band, is that the faster migrating component is derived from the 52,000 dalton polypeptide by proteolytic cleavage during the extraction or purification procedures. This suggestion is supported by the results of partial digestion with S. aureus V8 protease and chymotrypsin. Digests of the two gel-purified radiolabeled polypeptides yielded the same profiles of P^{32}-peptides (Fig. 3).

If, as the data suggest, the 46,000 dalton polypeptide is a proteolytic product of the higher molecular weight component, it nevertheless retains

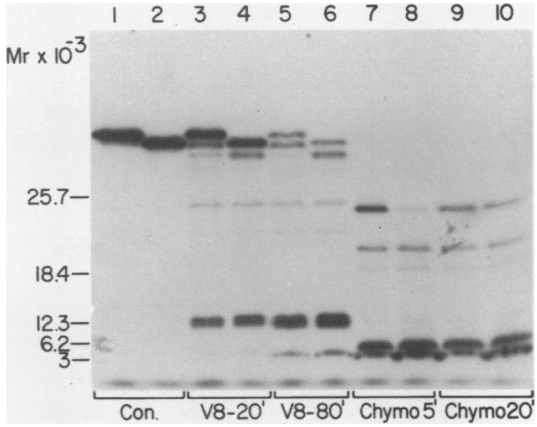

Fig. 3. Partial peptide maps of purified guanylyltransferase. ^{32}P-labeled enzyme-GMP was extracted from an SDS-polyacrylamide gel as in Fig. 1B, lane 6. The separated upper band (odd numbered lanes) and lower band (even numbered lanes) were incubated at 37°C with S. aureus V8 protease or chymotrypsin (25 µg/ml). Digests were analyzed by electrophoresis in a 15% polyacrylamide gel (19). Lanes 1,2: control without protease, 80 min; 3,4: V8, 20 min; 5,6: V8, 80 min; 7,8: chymotrypsin, 5 min; 9,10: chymotrypsin, 20 min.

enzymatic activity as an intermediate in the capping reaction. Step 5 guanylyltransferase was incubated with α-^{32}P-GTP without acceptor and repurified by heparin-Sepharose chromatography. The putative enzyme-GMP^{32}

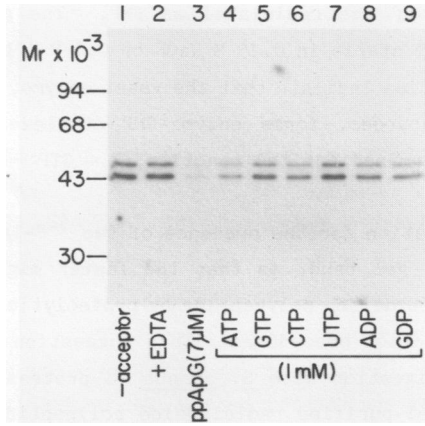

Fig. 4. SDS-polyacrylamide gel electrophoresis of ^{32}P-labeled purified enzyme-GMP after incubation under standard conditions. Lanes: 1. no acceptor; 2. 7 uM ppApG + 30 mM EDTA; 3. 7 µM ppApG; 4-9. 1 mM of ATP, GTP, CTP, UTP, ADP and GDP, respectively.

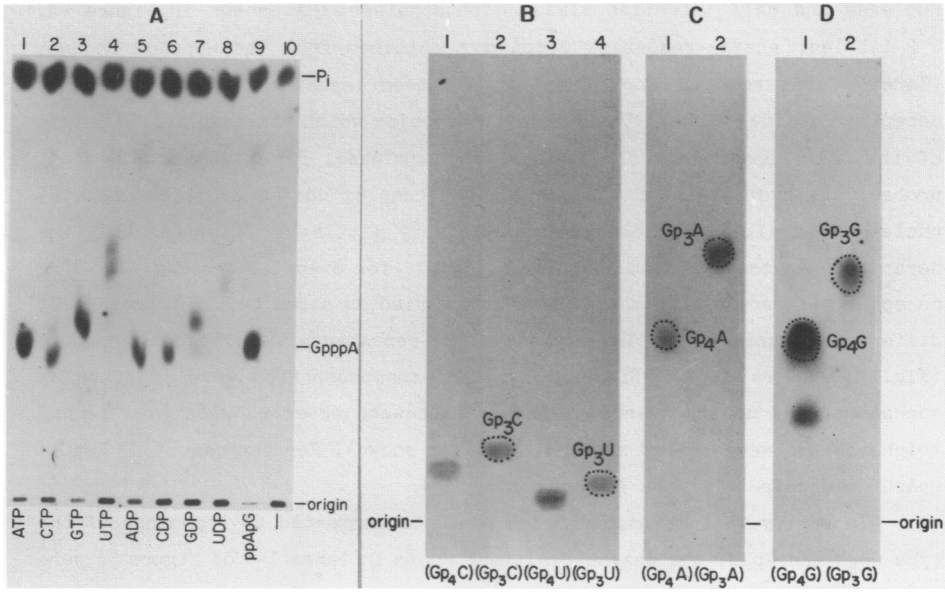

Fig. 5. Analyses of products formed by incubation of ^{32}P-labeled enzyme-GMP with different acceptors. A. Purified intermediate was incubated with 1 mM mononucleotide or 7 µM ppApG under standard conditions except that 0.2 µg inorganic pyrophosphatase was also present. Samples were treated with P1 nuclease and phosphatase and analyzed by high voltage paper electrophoresis. Enzyme-resistant spots migrating in the vicinity of GpppA were eluted with H_2O and analyzed further by TLC with marker compounds. B. Cellulose, isobutyric acid/NH_4OH/H_2O (14:3:3, v/v). Lanes 1-4 correspond to samples eluted from panel A-lanes 2, 6, 4 and 8, respectively. C. PEI-cellulose, 0.7 M KH_2PO_4-pH 4.3. Samples from panel A-lanes 1 and 5. D. DEAE-cellulose; 0.2 M ammonium formate, 9 M urea, 1 mM EDTA. Samples from panel A-lanes 3 and 7.

intermediates were reincubated with ppApG. Other nucleotides were also tested as potential GMP acceptors. Analysis by SDS polyacrylamide gel electrophoresis demonstrated that the bands of 46,000 and 52,000 daltons both retained GMP after incubation under standard conditions without acceptor or in the presence of 30 mM EDTA (Fig. 4, lanes 1,2). The radiolabel in both bands was markedly decreased following incubation under conditions of $Gppp\overset{*}{A}pG$ formation (lane 3). In contrast to HeLa cell guanylyltransferase (8), the yeast enzyme intermediate also transferred the covalently bound GMP to ribonucleoside 5'-di and triphosphates which were present at a concentration of 1 mM as compared to 7 uM ppApG (Fig. 4, lanes 4-9)

The guanylylated products resulting from transfer of GMP^{32} were analyzed by high voltage paper electrophoresis after digestion with P1

nuclease and calf intestine alkaline phosphatase. As shown in Figure 5A, ^{32}P-labeled, enzyme-resistant $\overset{*}{G}$pppA was obtained from the $\overset{*}{G}$pppApG product (lane 9), but reaction mixtures that had been incubated in the absence of acceptor retained most of the enzyme-GMP complex which remained at the origin of the electropherogram (lane 10). In each case, Pi was also generated, probably by hydrolysis of the protein-GMP complex during treatment with P1 nuclease and alkaline phosphatase since very little Pi was present in the heparin-Sepharose purified starting material (for example, see Fig. 2). The acceptor efficacy of the mononucleotides varied as shown by the formation of different amounts of nuclease/phosphatase-resistant radiolabeled products (Fig. 5A, lanes 1-8). Ribonucleoside 5'-monophosphates were inactive as acceptors, and no enzyme-resistant products were observed when the di- and triphosphates were tested at 7 μM (data not shown). For the reaction, E-pG + ppApG $\longrightarrow$ GpppApG + E, the Km was 2 x 10^{-6} M.

To verify that mononucleotides could be converted to compounds of the type Gp_4N and Gp_3N, the enzyme-resistant spots in lanes 1-8 of Figure 5A were eluted and analyzed by thin layer chromatography. Putative Gp_3C and Gp_3U obtained by incubating ^{32}P-labeled enzyme intermediate with CDP and UDP comigrated with authentic samples; products obtained with CTP and UTP migrated slightly more slowly, as expected for the corresponding tetraphosphates (Fig. 5B). The dipurine tri- and tetraphosphate compounds also migrated with marker compounds, although an unidentified spot was also obtained with GTP (Figs. 5C and D). Quantitation of the acceptor activities indicates that, among the mononucleotides tested, ATP and ADP are preferred by the yeast guanylyltransferase (Table 2).

Capping of cellular RNAs is a process restricted to RNA polymerase II products, presumably due to selective association of the guanylyltransferase with this polymerase or to some other type of intracellular compartmentalization. However, uncapped, specific run-off transcripts can be formed by RNA polymerase II in vitro (14,15), consistent with reports that the capping enzyme and polymerase can be separated during purification (11,16). The guanylyltransferase also does not copurify with the methyltransferase that alkylates the N-7 position of the 5'-terminal guanosine of capped transcripts. Purified yeast enzyme was incubated with ppApG and α-^{32}P-GTP in the presence or absence of the methyl donor, S-adenosylmethionine. Digestion of the incubation products with P1 nuclease and phosphatase yielded GpppA but no m^7GpppA (Fig. 6).

Other activities associated with partially purified guanylyltrans-

Table 2. Nucleotide Acceptor Activity for Yeast Guanylyltransferase

Acceptor	Product	(cpm)	Residual Donor (cpm)	% Conversion
None	--	--	1290	--
ppApG	GpppApG	1170	178	87
ATP	GppppA	916	414	69
CTP	GppppC	685	506	58
GTP	GppppG	643	669	49
UTP	GppppU	217	997	15
ADP	GpppA	686	625	61
CDP	GpppC	405	811	33
GDP	GpppG	535	610	47
UDP	GpppU	281	719	28

Step 5 guanylyltransferase was incubated with α-^{32}P-GTP, and the resulting radiolabeled enzyme-GMP was repurified by heparin-Sepharose chromatography. The complex was incubated under standard conditions with 1 mM acceptor except 7 uM ppApG, and the products were analyzed by high voltage paper electrophoresis and TLC.

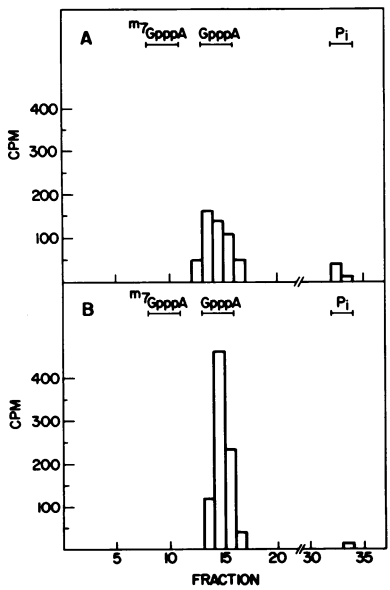

Fig. 6. Absence of methyltransferase activity in purified guanylyltransferase. Step 5 enzyme was incubated with ppApG and α-^{32}P-GTP under standard conditions in the absence (A) or presence (B) of 0.1 mM S-adenosylmethionine. Samples were digested with P1 nuclease and phosphatase and analyzed by high voltage paper electrophoresis.

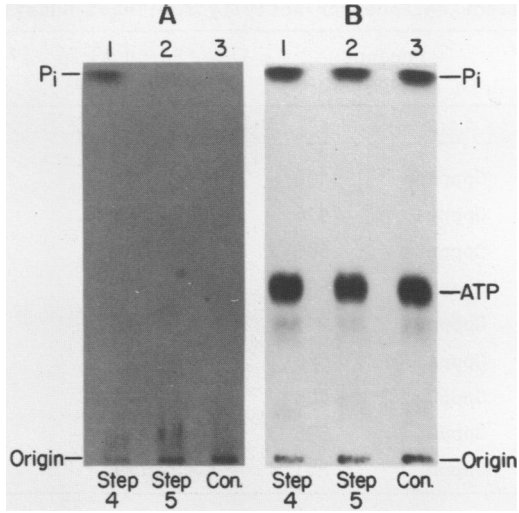

Fig. 7. Analysis of guanylyltransferase pools for phosphatase and RNA 5'-triphosphatase. Fractions were tested for RNA 5'-triphosphatase (panel-A) and phosphatase (panel B) by incubation with 5'-^{32}P-labeled polyadenylic acid and γ-^{32}P-ATP, respectively. For A. reaction mixtures (10 ul) consisting of 5 µl enzyme (control, 5 ul H_2O), 50 mM tris-HCl-pH 8.0, 0.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 µg bovine serum albumin and 5% glycerol were incubated for 30 min at 30°C with 300 cpm of pppA(pA)$_n$ that was prepared from γ-^{32}P-ATP with E. coli RNA polymerase (20). After adding 2 ul 5 N formic acid to stop the reaction, samples were spotted on a PEI plate which was developed in 0.7 M KH_2PO_4-pH 4.3. Pi and origin regions were cut and counted; conversion to Pi was 38% in lane 1 as compared to 1% in lane 2. The same incubation procedure was used for panel B except that poly(A) was replaced by 3,000 cpm of γ-^{32}P-ATP.

ferase include RNA 5'-triphosphatase that converts nascent 5'-terminal pppNpN to the guanylyl acceptor ppNpN and non-specific phosphatase(s) that interfere with the detection of this activity. Step 3 pooled fractions contained phosphatase(s) that released radiolabeled Pi from γ-^{32}P-ATP, but this hydrolytic activity was no longer detectable after further purification by phosphocellulose chromatography (Fig. 7B). Step 4 fractions contained RNA 5'-triphosphatase as measured using γ-^{32}P-labeled polyadenylic acid as substrate (Fig. 7A, lane 1). However, after chromatography on GTP-Sepharose, the pooled fractions of guanylyltransferase contained little or no RNA 5'-triphosphatase (Fig. 7A, lane 2).

DISCUSSION

Like other mRNA guanylyltransferases of cellular (6-11) and viral

(4,5,21) origin, the enzyme purified from yeast forms a covalent intermediate with GMP via phosphoamide linkage (Fig. 2). The location and exact nature of the modified amino acid(s) in the enzyme active site remain to be determined, but a single guanylylated peptide of approximately 3,000 daltons was obtained by gel analysis following exhaustive tryptic digestion of the purified enzyme intermediate (data not shown).

In contrast to the intermediate from other sources, two radiolabeled polypeptides of M_R 52,000 and 46,000 were obtained, despite the use of phenylmethylsulfonyl fluoride, when the yeast enzyme was incubated with α-^{32}P-GTP without a guanylyl acceptor (Fig. 1). Derivation of the ~46,000 dalton component by proteolysis of the larger polypeptide is clearly indicated by their essentially identical peptide-GMP profiles (Fig. 3). Itoh et al. (12) obtained a M_R ~45,000 intermediate but not the larger polypeptide-GMP in guanylyltransferase preparations purified from wild type and a protease-deficient mutant of S. cerevisiae. Although protease inhibitors were also used, extracts were prepared after conversion of the cells to spheroplasts by enzymatic digestion of cell walls--a process that may lead to loss of the higher molecular weight band.

RNA 5'-triphosphatase was found associated with yeast guanylyltransferase that was purified by ammonium sulfate precipitation followed by chromatography on Sephadex G-200, CM-Sephadex and DEAE-Sephadex (12). In the present study, both activities were present in the step 4 pooled fractions, but a comparable amount of step 5 guanylyltransferase was nearly devoid of RNA 5'-triphosphatase (Fig. 7), suggesting that they are different proteins and can be separated by chromatography on GTP-Sepharose. Partially purified wheat germ (6), HeLa cell (22) and calf thymus (11) guanylyltransferases also were reported to be free of RNA 5'-triphosphatase activity. From these and other studies (23,24), it appears that the cellular enzymes that modify mRNA 5'-termini i.e. RNA 5'-triphosphatase, guanylyltransferase and methyltransferases, and act coordinately with RNA polymerase II in vivo are nevertheless resolved by fractionation of cell-free extracts.

Purified yeast guanylyltransferase resembles the comparable enzyme activities in vaccinia virus and insect cytoplasmic polyhedrosis virus (25) in its ability to guanylylate mononucleotides and form Gp_3N and Gp_4N from $pp^{5'}N$ and $ppp^{5'}N$, respectively. This reaction requires a higher level of mononucleotide than ppApG and thus may reflect an in vitro activity resulting from separation of the guanylyltransferase from RNA polymerase II rather than a physiologically significant reaction. However, Gp_4A was recently isolated

from S. cerevisiae as well as from rat liver and Physarum polycephalum (26). In addition, Gp_4G has been obtained from lower eukaryotes (27,28) including apparently several fungi (29). It comprises almost half of the total nucleotide pool in dormant cysts of brine shrimp (Artemia salina) where it was first identified as a major purine storage product (27,30). The intracellular biosynthesis of $Gp_{3-4}N$ compounds by yeast guanylyltransferase, for example during a block in initiation by RNA polymerase II, remains to be explored. Correspondingly little is known about the possible functional significance of Gp_4G and other similar compounds for eukaryotic gene replication (31) and expression (32).

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