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Mechanism of the mRNA guanylyltransferase reaction: isolation of N$^{\epsilon}$-phospholysine and GMP (5′ → N$^{\epsilon}$) lysine from the guanylyl-enzyme intermediate

Reiko Toyama, Kiyohisa Mizumoto*, Yuko Nakahara[1], Takashi Tatsuno[1] and Yoshito Kaziro

Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108, and [1]Institute of Physical and Chemical Research, 2-1, Hirosawa, Wako-city, Saitama 351, Japan

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The mRNA capping reaction catalyzed by rat liver mRNA guanylyltransferase proceeds through an enzyme-GMP intermediate in which GMP is linked to the enzyme by a phosphoamide linkage. The studies described here show that GMP is bound to the ε-amino group of lysine of rat liver guanylyltransferase. The enzyme-[^{32}P]GMP intermediate was digested with pronase to a [^{32}P]GMP-peptide which was then converted to [^{32}P]phosphoryl-peptide through periodate oxidation followed by β-elimination. After alkaline hydrolysis of the [^{32}P]phosphoryl-peptide, the major radioactive product co-electrophoresed with the authentic N$^{\epsilon}$-phospholysine on DEAE-cellulose paper. Neither [^{32}P]N^{imid}-phosphohistidine nor N^{guanido}-phosphoarginine was detected in the hydrolysates. Furthermore, formation of N$^{\epsilon}$-guanylyl-lysine linkage on the enzyme was more directly shown by isolation of [^{32}P]GMP(5′ → N$^{\epsilon}$)lysine when the steps of periodate oxidation and β-elimination were omitted. The results indicate that the nucleophile in the guanylyltransferase to which the guanylyl residue is linked is the ε-amino group of a lysine residue. [^{32}P]Phosphoryl-lysine was also isolated from the vaccinia virus capping enzyme-[^{32}P]GMP intermediate. Guanylyltransferase from HeLa cells, wheat germ, *Artemia salina* and yeast also formed the enzyme-GMP complex and, from the stability of the complex, the linkage between the enzyme and GMP was suggested to be a phosphoamide.

Key words: capping enzyme/covalent catalysis/phosphoamide bond/rat liver/vaccinia virus

Introduction

Most viral and cellular mRNAs contain, at their 5′ termini, the 'cap' structure which is required for the efficient translation of eukaryotic mRNA molecules (for reviews, see Shatkin, 1976; Filipowicz, 1978; Banerjee, 1980). From the studies with virion-associated and cellular capping enzymes, it was demonstrated that the capping occurs by the following sequence of reactions (for a review, see Banerjee, 1980):

$$\text{pppG} + \text{ppN}^1\text{pN}^2\text{p-} \xrightarrow{\text{guanylyltransferase}} \text{GpppN}^1\text{pN}^2\text{p-} + \text{PPi} \quad (1)$$

$$\text{GpppN}^1\text{pN}^2\text{p-} + \text{AdoMet} \xrightarrow[\text{(ribose-2′-O-)methyltransferase}]{\text{(guanine-7-)methyltransferase}} \text{m}^7\text{GpppN}^1\text{mpN}^2\text{mp-} + \text{AdoHcy} \quad (2)$$

Guanylyltransferase has been purified from vaccinia virus (Martin *et al.*, 1975; Monroy *et al.*, 1978), HeLa cells (Venkatesan *et al.*, 1980; Wang *et al.*, 1982; Shuman, 1982), rat liver (Mizumoto and Lipmann, 1979; Mizumoto *et al.*, 1982), wheat germ (Keith *et al.*, 1982) and calf thymus (Nishikawa and Chambon, 1982). We have shown that the capping reaction catalyzed by rat liver guanylyltransferase proceeds through the following two partial reactions *via* an enzyme-GMP covalent intermediate (Mizumoto *et al.*, 1982).

$$\text{Enzyme} + \text{pppG} \longrightarrow \text{Enzyme-pG} + \text{PPi} \quad (1a)$$

$$\text{Enzyme-pG} + \text{ppN-RNA} \longrightarrow \text{GpppN-RNA} + \text{Enzyme} \quad (1b)$$

From the stability of the enzyme-GMP intermediate, the linkage between GMP and the enzyme was suggested to be a phosphoamide type (Mizumoto *et al.*, 1982). Enzyme-GMP intermediates with similar properties have also been identified for guanylyltransferase from vaccinia virus (Shuman and Hurwitz, 1981), HeLa cells (Venkatesan and Moss, 1982; Wang *et al.*, 1982; Shuman, 1982) and calf thymus (Nishikawa, 1982). Thus, covalent catalysis involving the enzyme-GMP intermediate seems to be the general mechanism of the capping reaction. Here we report that the amino acid residue to which GMP is linked in the guanylyltransferase-GMP complexes from rat liver and vaccinia virus is lysine.

Results

Isolation of N$^{\epsilon}$-phospholysine from rat liver guanylyltransferase-GMP

Strategy. The studies on the stability of the enzyme-GMP complex suggested that GMP is linked to a lysine, histidine or arginine residue of the enzyme through a phosphoamide type linkage (Mizumoto *et al.*, 1982). In this work, we identified the amino acid to which GMP is linked as phosphoamino acid after β-elimination of the guanosine moiety from the GMP-peptide. The strategy used for the experiments is depicted in Figure 1.

In brief, the guanylyltransferase-[^{32}P]GMP [I] was digested with pronase to a peptide-[^{32}P]GMP [II]. The peptide-[^{32}P]GMP was then subjected to sodium periodate oxidation followed by β-elimination to yield [^{32}P]phosphoryl-peptide [III]. The [^{32}P]phosphoryl-peptide was hydrolyzed with alkali and hydrolysates were analyzed for [^{32}P]N-phosphoryl-lysine, -arginine and -histidine by paper electrophoresis.

Proteolytic digestion of the enzyme-GMP complex. Rat liver guanylyltransferase-[^{32}P]GMP complex was prepared by incubating the partially purified enzyme with [α-^{32}P]GTP, and digested with pronase or proteinase K. Digests were subjected to paper electrophoresis on DEAE-cellulose paper in two different buffer systems. As seen in Figure 2, digestion with pronase (A,C) and proteinase K (B,D) gave a single radioactive spot on paper electrophoresis both at pH 3.5 (A,B) and pH 8.25 (C,D), indicating that GMP is bound to a single site of the enzyme molecule. The spot obtained by pronase digestion always moved faster than that obtained with proteinase K. When the peptide-[^{32}P]GMP complex formed by digestion with proteinase K was isolated and further digested with pronase, all the radioactivity was converted to the same peptide-[^{32}P]GMP complex as was obtained by digesting the enzyme-[^{32}P]GMP directly with pronase (data not shown), indicating that pronase produced a smaller peptide than proteinase K. The pronase-derived peptide-[^{32}P]GMP seemed to consist of 6–8 amino acids from the

*To whom reprint requests should be sent.

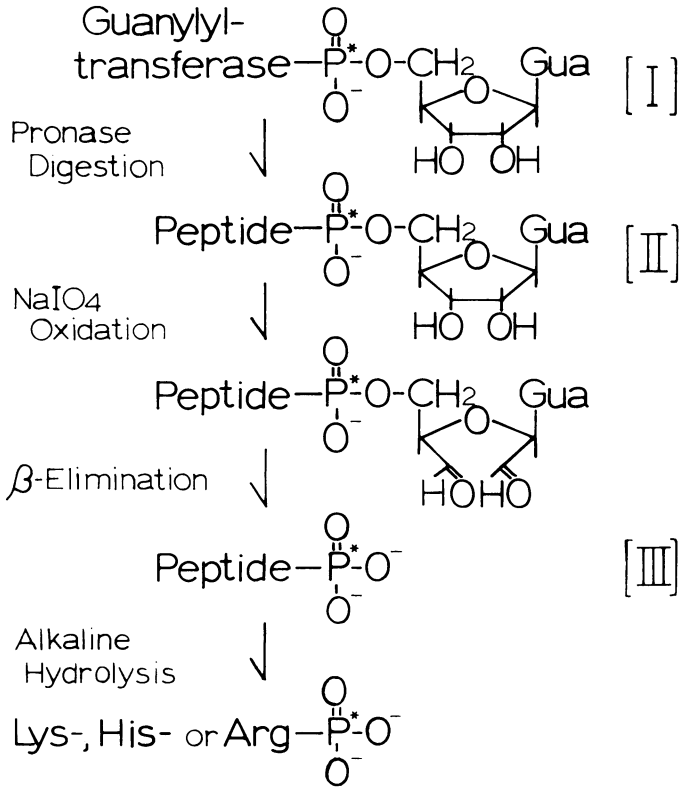

Fig. 1. Strategy for identification of the amino acid residue linked to GMP. * denotes ^{32}P labels.

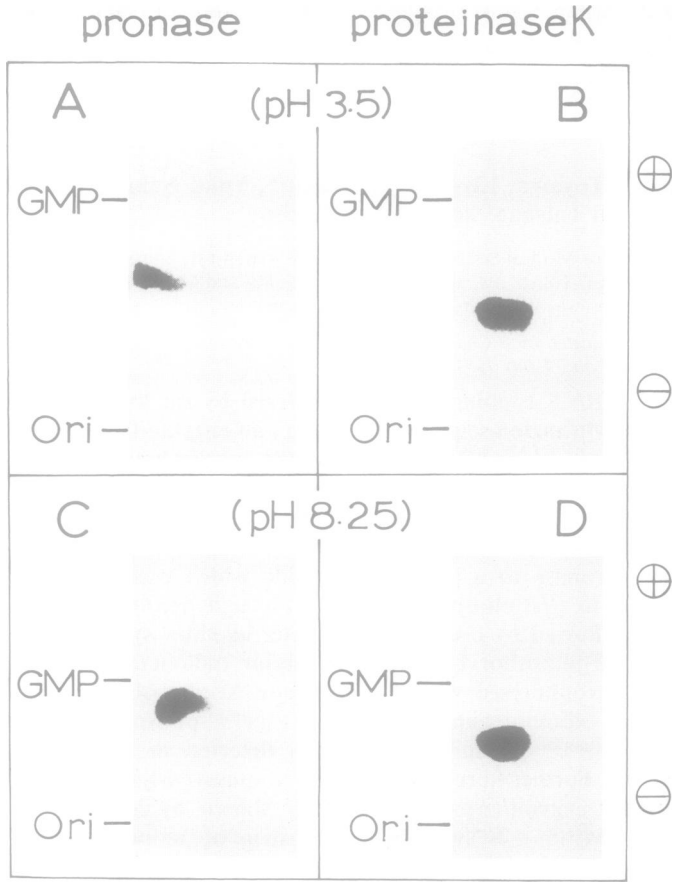

Fig. 2. Electrophoresis of proteolytic digests of enzyme-[^{32}P]GMP. The enzyme-[^{32}P]GMP complex was prepared as described in Materials and methods using rat liver guanylyltransferase purified up to the step of CM-Sephadex column chromatography (Mizumoto and Lipmann, 1979). The proteolytic digests of the complex with pronase (**A** and **C**) or proteinase K (**B** and **D**) were electrophoresed on Whatman DE81 paper at pH 3.5 (**A** and **B**) or 8.25 (**C** and **D**). Pronase digestion was carried out at a pronase/protein ratio of 1 (w/w) for 4 h at 37°C, and proteinase K digestion was for 1 h at 37°C at a proteinase K/protein ratio of 0.12. The buffer solutions used for electrophoresis were 10% (v/v) pyridine/1% (v/v) acetic acid (pH 3.5) or 1.67 M NH_4OAc/1 mM EDTA (pH 8.25 with NH_4OH).

estimation by gel-filtration on a Bio-Gel P2 column (data not shown).

Isolation of N^{ϵ}-phospholysine. The peptide-[^{32}P]GMP complex obtained by pronase digestion was purified in a similar manner as that shown in Figure 2A, and was subjected to sodium periodate oxidation followed by β-elimination. As shown in Figure 3, >90% of the ^{32}P radioactivity was converted to the [^{32}P]phosphoryl-peptide which migrated faster than the peptide-[^{32}P]GMP complex on DEAE-cellulose paper electrophoresis (Figure 3). When a portion of [^{32}P]phosphoryl-peptide eluted from the electropherogram was treated with 0.1 N HCl for 5 min at 90°C, almost all the ^{32}P radioactivity was recovered as inorganic phosphate, indicating that the β-elimination was practically complete.

Further treatment of the [^{32}P]phosphoryl-peptide with proteinase K, pronase, carboxypeptidase Y, aminopeptidase M or subtilisin did not yield a smaller peptide. Since the phosphoamide bond is highly unstable to acid, we digested [^{32}P]phosphoryl-peptide with alkali to obtain [^{32}P]phosphoamino acid.

The [^{32}P]phosphoryl-peptide was hydrolyzed with 3 N KOH at 100°C for various times, and the hydrolysates were electrophoresed on DEAE-cellulose paper. As shown in Figure 4A, three radioactive spots were observed at an early stage of incubation (30 min). One of them exactly co-migrated with authentic N^{ϵ}-phospholysine. Another two spots (designated X and Y) migrated to the positions between phospholysine and phosphoryl-peptide but corresponded to neither N^{imid}-phosphohistidine nor $N^{guanido}$-phosphoarginine. On further incubation, the ^{32}P radioactivity in the spot of phospholysine gradually increased with a concomitant decrease of ^{32}P in spots X and Y. At 180 min, X and Y almost disappeared leaving phospholysine as the major reaction product. Spots X and Y are probably the intermediates of alkaline digestion, since both X and Y were converted to phospholysine by further incubation for 3 h with 3 M KOH (Figure 4B). The overall recovery of ^{32}P in phospholysine from the enzyme-GMP complex in Figure 4 was ~35%. In some experiments we observed a considerable amount of [^{32}P]Pi (for example, see Figure 4A, lanes 3 and 4). We do not know the exact reason for this Pi release but usually it is not time-dependent and seems to be largely due to the artefact formed during neutralization of the samples before electrophoresis.

Figure 5 compares the kinetics of degradation of guanylyltransferase-[^{32}P]GMP, [^{32}P]phosphoryl-peptide and [^{32}P]N^{ϵ}-phospholysine at two different pHs. Practically no degradation was observed when they were incubated in 0.1 N NaOH at 37°C whereas they were degraded in 0.1 N HCl at 37°C with almost identical first-order kinetics. These results suggest that there had not been any migration of the GMP moiety on the enzyme during proteolysis, oxidation, β-elimination and

isolation of phospholysine. They also eliminate the possibility that the Pi released in the experiments of Figure 4 is due to the presence of a different, alkali-labile type of bond between phosphate and amino acid which might have been formed during the course of phosphoryl-peptide preparation.

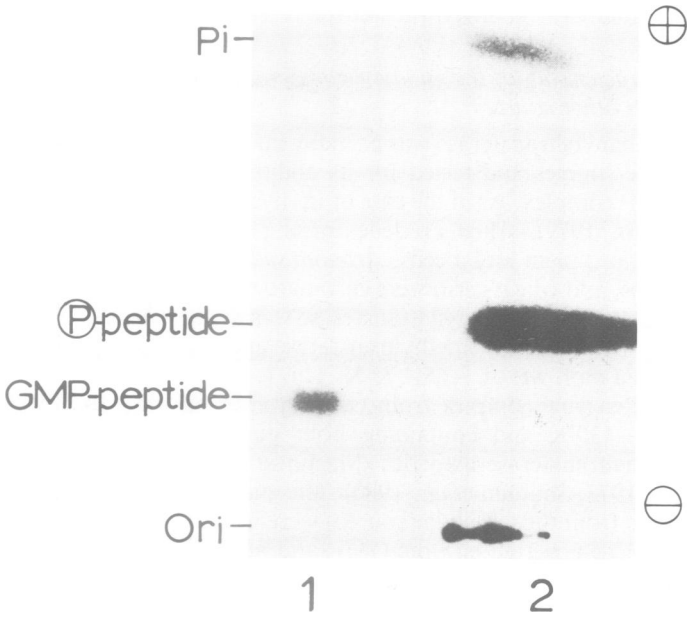

Fig. 3. Formation of [^{32}P]phosphoryl-peptide from peptide-[^{32}P]GMP by β-elimination. The peptide-[^{32}P]GMP formed by pronase digestion of the enzyme-[^{32}P]GMP complex was isolated and treated with sodium periodate and analine as described in Materials and methods. The reaction mixture was then electrophoresed on Whatman DE81 paper at pH 3.5. **Lanes 1** and **2** are before and after β-elimination, respectively.

Isolation of GMP(5′→N$^{\epsilon}$)lysine from the rat liver enzyme-GMP complex

The above results strongly support the proposal that GMP is linked to a lysine residue of the liver enzyme. This was further confirmed by directly isolating the lysine (ϵ-amino)-linked guanosine monophosphoramidate (GMP(5′→N$^{\epsilon}$)-lysine) from the enzyme-GMP complex (Figure 6). When peptide-[^{32}P]GMP (compound II in Figure 1) was hydrolyzed in 3 N KOH, >80% of the ^{32}P co-migrated exactly with authentic GMP(5′→N$^{\epsilon}$)lysine by paper electrophoresis (Figure 6, A, lane 2) or t.l.c. (Figure 6, B, lane 1). Furthermore, an acid treatment of the alkaline hydrolysis product converted almost all the ^{32}P to GMP (Figure 6, B, lane 2) indicating that there had been no deamination of guanine base during the alkaline hydrolysis.

Isolation of N$^{\epsilon}$-phospholysine from vaccinia virus capping enzyme-GMP complex

Since a phosphoamide-type linkage has also been suggested for the vaccinia virus enzyme-GMP complex (Shuman and Hurwitz, 1981), we examined whether GMP is also linked to the ϵ-amino group of lysine in the case of the viral capping enzyme. The vaccinia virus guanylyltransferase-[^{32}P]GMP complex was isolated and degraded to the phosphoamino acid by a procedure similar to that described for the rat liver enzyme. The electrophoretic pattern of the alkaline hydrolysate is shown in Figure 7. A major radioactive spot which exactly co-migrated with the phospholysine marker was obtained in addition to minor radioactive spots moving more slowly than

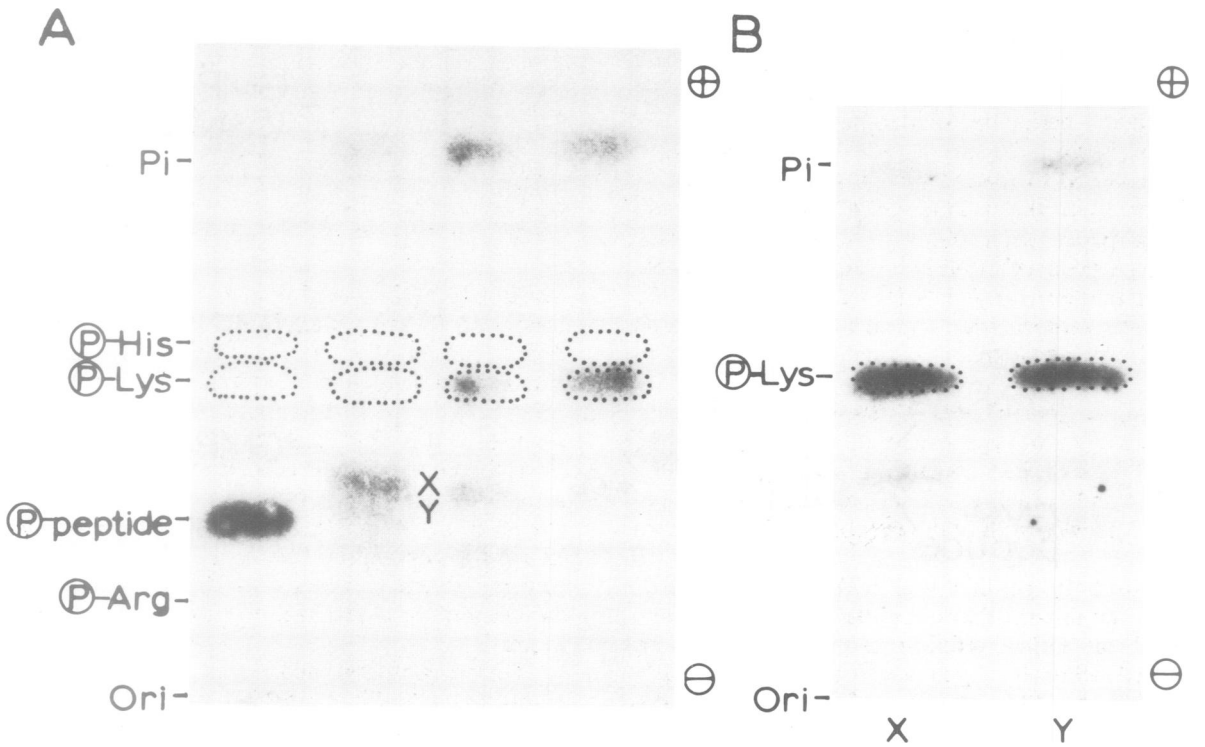

Fig. 4. Alkaline hydrolysis of [^{32}P]phosphoryl-peptide obtained from rat liver guanylyltransferase-[^{32}P]GMP. **(A)** [^{32}P]phosphoryl-peptide isolated as described in Figure 3 was subjected to alkaline hydrolysis in 15 μl of 3 N KOH for 0, 30, 90 and 180 min (**lanes 1, 2, 3** and **4**, respectively) at 100°C. After neutralization with perchloric acid, the samples were electrophoresed on Whatman DE81 paper at pH 3.5 together with authentic N-phosphoamino acids. The standard N-phosphoamino acids were located by spraying ninhydrin reagent. X and Y denote alkaline-hydrolysis intermediates. **(B)** Spots X and Y obtained in **A** were separately eluted from the electropherogram with 1 M triethylamine-HCO_3^- buffer (pH 7.5), lyophilized, and further hydrolysed in 3 N KOH for 3 h at 100°C. The samples were electrophoresed as in **A**.

phospholysine. Neither ^{32}P-labeled N^{guanido}-phosphoarginine nor N^{imid}-phosphohistidine was detected in the hydrolysate. On prolonged alkaline hydrolysis, the slowly-moving spots disappeared with a concomitant increase of ^{32}P in the phospholysine spot. Isolation of [^{32}P]phospholysine as the only N-phosphoamino acid indicates that GMP(5′ → N$^{\epsilon}$)-lysine linkage is also involved in the covalent catalysis by the vaccinia virus guanylyltransferase.

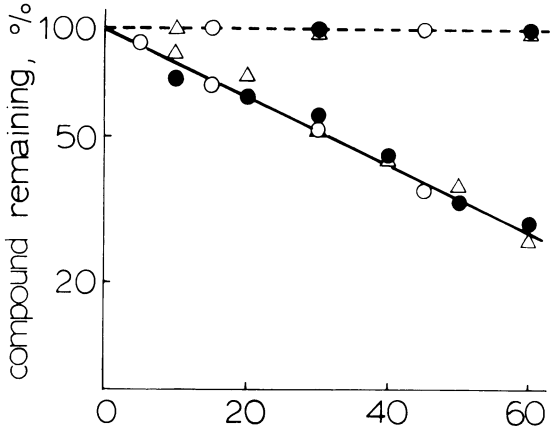

Fig. 5. Stability of enzyme-[^{32}P]GMP, [^{32}P]phosphoryl-peptide and [^{32}P]N$^{\epsilon}$-phospholysine obtained from rat liver guanylyltransferase. Enzyme-[^{32}P]GMP, [^{32}P]phosphoryl-peptide and [^{32}P]N$^{\epsilon}$-phospholysine were prepared as described in Materials and methods. Aliquots of each sample were incubated in 0.1 N NaOH (- - -) or 0.1 N HCl (——) for the indicated time at 37°C. At each time point, the residual amount of each compound was measured and its logarithm was plotted against time. For determination of the enzyme-[^{32}P]GMP, cold TCA-precipitable ^{32}P was measured (○), while for [^{32}P]phosphoryl-peptide (●) and [^{32}P]phospholysine (△), compounds were separated by electrophoresis on Whatman DE81 paper at pH 7.6 and the radioactivity counted.

Characteristics of the guanylyltransferase-GMP intermediates from other sources

Guanylyltransferase was partially purified from various enzyme sources and tested for its ability to form an enzyme-GMP complex. As in the case of rat liver (Mizumoto and Lipmann, 1979), when crude extracts (100 000 *g* supernatant fraction) from HeLa cells, *Artemia salina* (brine shrimp) embryos, and wheat germ were chromatographed on a Sephadex G-200 column, guanylyltransferase was easily separated from mRNA(guanine-7-)methyltransferase and eluted at a position with a mol. wt. of ~65 – 75 K. This is in contrast to the capping enzyme complex from vaccinia virus having a mol. wt. of ~130 K and containing both guanylyltransferase and methyltransferase activities (Martin *et al.*, 1975; Monroy *et al.*, 1978; Shuman *et al.*, 1980). The guanylyltransferase fractions from those cellular sources were incubated with [α-^{32}P]-GTP to generate enzyme-GMP and analyzed on SDS-polyacrylamide gel along with the enzyme-GMP complexes of rat liver (69 K) (Mizumoto *et al.*, 1982) and vaccinia virus (95 K) (Shuman and Hurwitz, 1981) (Figure 8).

The guanylyltransferases from HeLa cells, *A. salina*, wheat germ and yeast each gave a single ^{32}P-labeled protein band

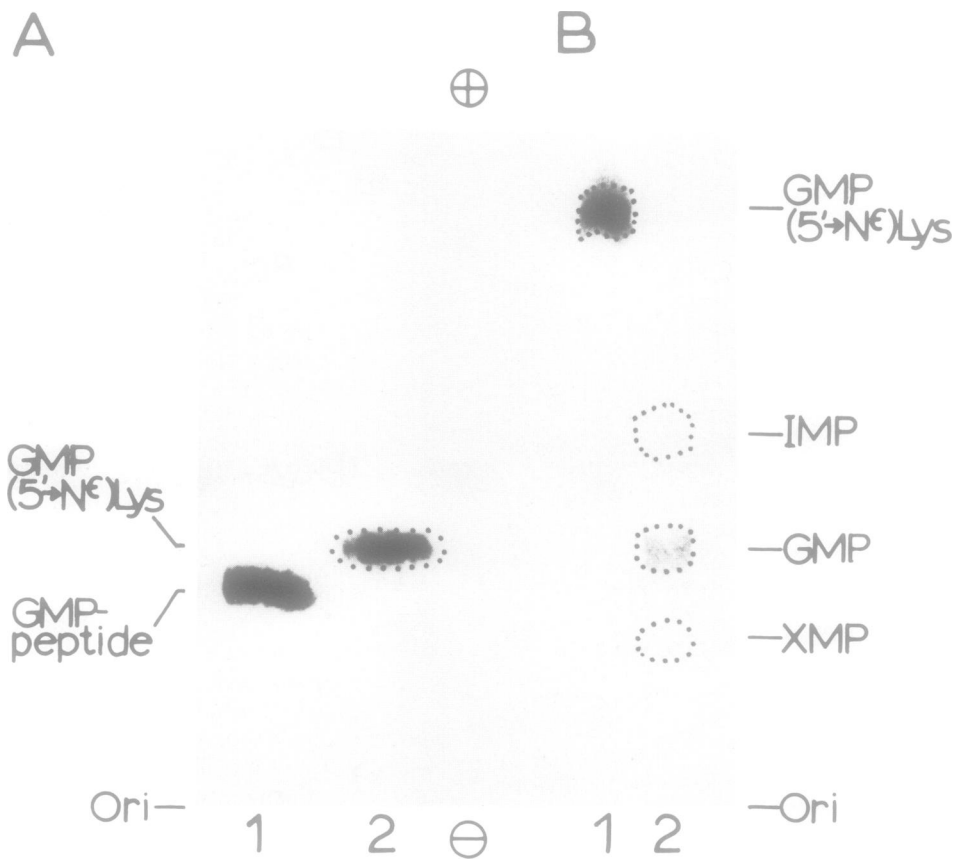

Fig. 6. Isolation of GMP(5′ → N$^{\epsilon}$)lysine from rat liver guanylyltransferase-GMP complex. The peptide-[^{32}P]GMP (compound II) purified as in Figure 2A was dissolved in 3 N KOH and incubated under the conditions specified below. The samples were then neutralized with perchloric acid, and subjected to electrophoresis on Whatman DE81 paper at pH 3.5 **(A)** or t.l.c. on a polyethyleneimine-cellulose plate with 1 M LiCl **(B)**. The incubation was at 0°C for 30 min **(A, lane 1)** or at 100°C for 3 h **(A, lane 2**; and **B, lane 1)**. In experiment **B, lane 2**, the alkaline hydrolysate was acidified with perchloric acid to 0.1 N and incubated further for 5 min at 85°C.

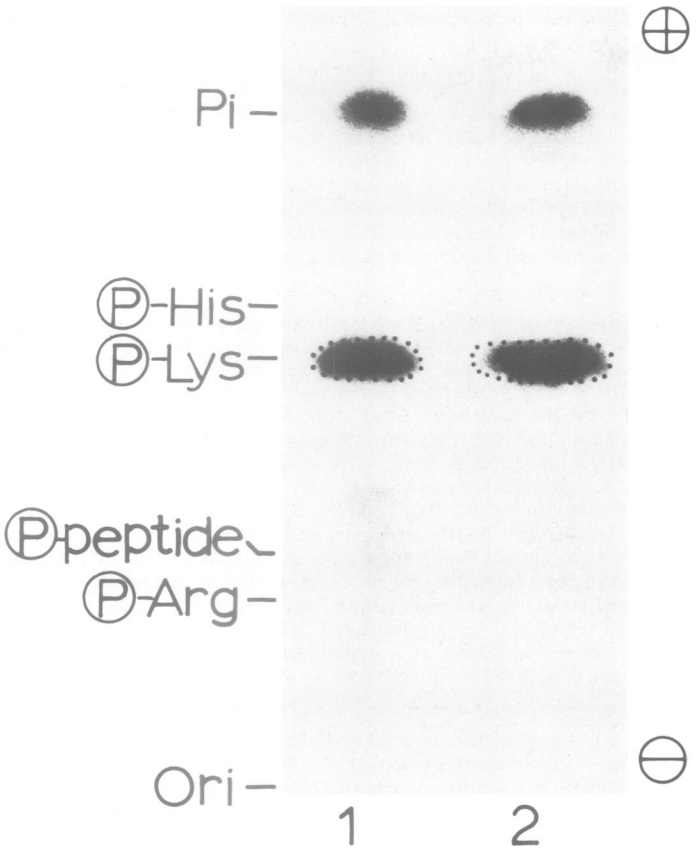

Fig. 7. Isolation of N'-phospholysine from vaccinia virus capping enzyme-GMP. The enzyme-[^{32}P]GMP complex was prepared from vaccinia virus capping enzyme under the conditions described in Materials and methods for rat liver enzyme. Enzyme-[^{32}P]GMP was subjected to pronase digestion, β-elimination and alkaline hydrolysis. Alkaline hydrolysis was for 90 min **(lane 1)** and 180 min **(lane 2)** at 100°C in 3 N KOH. The hydrolysate was neutralized and electrophoresed as described in the legend to Figure 4.

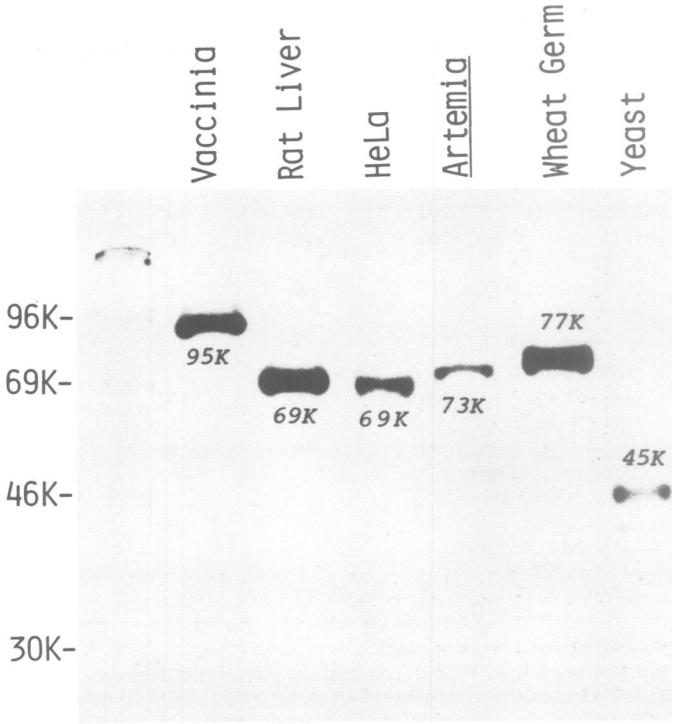

Fig. 8. SDS-polyacrylamide gel electrophoresis of the guanylyltransferase-GMP intermediates. Guanylyltransferase was partially purified from various enzyme sources as described in Materials and methods. Enzyme-[^{32}P]GMP was formed, acid-precipitated and electrophoresed on SDS/10% acrylamide gel as described by Weber and Osborn (1969). The leftmost lane contained [^{14}C]methylated-protein markers (CFA 626, Amersham).

with respective mol. wts. of 69 K, 73 K, 77 K and 45 K. The mol. wt. of the enzyme-GMP complex from HeLa cells (69 K) agreed with the value reported previously by Shuman (1982) and Wang *et al.* (1982). It should be noted that the mol. wt. of the yeast enzyme-GMP complex was smaller (45 K) than those from other sources, while the mol. wt. of the undenatured form of the yeast enzyme was found to be 130 K (see also Itoh *et al.*, 1983). In all cases, the three activities, i.e., cap formation, GTP-PPi exchange, and formation of an enzyme-GMP complex, co-chromatographed on the Sephadex G-200 column or, by further purification, on ion-exchange (CM- and DEAE-) Sephadex columns (data not shown).

To characterize the linkage between GMP and the enzyme in these complexes, their stability against acid, alkali and hydroxylamine was tested. The enzyme-GMP complexes formed with guanylyltransferase from *A. salina*, wheat germ and yeast, as well as that with rat liver enzyme (Mizumoto *et al.*, 1982), revealed strikingly similar behavior towards these reagents (Table I), namely the alkali-stable, acid-labile nature and the susceptibility to cleavage by acidic hydroxylamine, that are typical properties of phosphoamide bonds (Shabarova, 1970).

Discussion

Evidence has been presented that the ϵ-amino group of a lysine residue of the enzyme is directly involved in the formation of a phosphoamide linkage in the rat liver and vaccinia virus guanylyltransferase-GMP intermediate. This suggests that an initial step of mRNA capping catalyzed by guanylyltransferase occurs by nucleophilic attack of the ϵ-amino group of lysine on the α-phosphate of GTP. The above conclusion is based on the isolation of [^{32}P]N$^{\epsilon}$-phospholysine from the enzyme-[^{32}P]GMP complex using a modified procedure which was originally developed by Yang and Frey (1979) to isolate N^{imid}-phosphohistidine from the enzyme-UMP intermediate of *Escherichia coli* galactose-1-phosphate uridylyltransferase. Our procedure includes pronase digestion of the enzyme-[^{32}P]GMP, sodium periodate oxidation followed by β-elimination and alkaline hydrolysis. The overall recovery of ^{32}P in N$^{\epsilon}$-phospholysine starting from the enzyme-[^{32}P]GMP complex was estimated to be ~35% in the experiment described in Figure 4. Essentially the same result was obtained without pronase digestion. Furthermore, [^{32}P]GMP(5'→N$^{\epsilon}$)lysine was isolated when the enzyme-[^{32}P]GMP complex was degraded directly with alkali without prior periodate oxidation and β-elimination.

In the case of T4 DNA ligase, a similar enzyme-[^{32}P]AMP intermediate has been isolated, and Gumport and Lehman (1971) demonstrated that AMP is linked to the ϵ-amino group of a lysine residue through isolation of IMP(5'→N$^{\epsilon}$)lysine after proteolytic digestion of the enzyme-AMP complex followed by the enzymatic deamination of AMP to IMP. We have also prepared T4 DNA ligase-[^{32}P]AMP with purified enzyme from T4 *lig* lysogen (Tait *et al.*, 1980) and subjected it to periodate oxidation and β-elimination as described above. After alkaline hydrolysis, only N$^{\epsilon}$-phospholysine was detected as N-phosphoamino acid (data not shown). Therefore, the

Table I. Stability of the guanylyltransferase-GMP complex isolated from various sources

Additions and incubations	Enzyme sources [E-pG remaining (%)]			
	A. salina	Wheat germ	Yeast	Rat liver
1. Acid and alkali				
Control[a]	100	100	100	100
H_2O, 5 min at 90°C	100	85	87	103
0.1 N NaOH, 5 min at 90°C	117	102	88	94
0.1 N HCl, 5 min at 90°C	13	12	7	13
2. Hydroxylamine				
Control[a]	100	100	100	100
H_2O, 25 min at 37°C	84	100	92	89
0.2 M NH_2OH (pH 7.5), 25 min at 37°C	86	77	102	96
3.8 M NH_2OH (pH 4.8), 25 min at 37°C	2	11	1	8

[a]No additions and no incubation.
Guanylyltransferase-[^{32}P]GMP complex was prepared as described in Materials and methods. Aliquots (5 μl) of the enzyme-GMP complex (1000 – 3000 c.p.m.) received 0.1 ml of various reagents listed in the Table and were incubated as indicated. After incubation, the proteins were precipitated with cold 5% TCA and the radioactivity in the pellets was measured by Cerenkov counting.

procedure to identify the nucleotidyl-enzyme linkage as phosphoamino acids also proved to be successful in this case.

Recently, we have shown that both rat liver (Mizumoto *et al.*, 1982) and yeast (Itoh *et al.*, 1983) guanylyltransferase-GMP complexes isolated in a native form can transfer their GMP moiety to ppG-terminated RNA to form GpppG-RNA. We have also isolated similar enzyme-GMP complexes obtained with wheat germ and *A. salina* guanylyltransferase of which the activities of capping, GTP-PPi exchange and the enzyme-GMP formation were co-chromatographed on Sephadex G-200 or ion-exchange (CM- and DEAE-) Sephadex columns (data not shown).

Furthermore, the linkage between GMP and the enzyme was suggested to be a phosphoamide type in all cases. The transfer of GMP from the enzyme-GMP complex of vaccinia virus (Shuman and Hurwitz, 1981) and HeLa cells (Venkatesan and Moss, 1982; Wang *et al.*, 1982) has also been demonstrated recently. Thus, the covalent catalysis via a gunaylylated capping enzyme seems to be the universal mechanism of mRNA capping in eukaryotes.

Materials and methods

Materials

[α-^{32}P]GMP (410 Ci/mmol) was purchased from Amersham, Japan. Pronase P, proteinase K, carboxypeptidase Y, and subtilisin were obtained from Kaken Kagaku (Tokyo), Boehringer Mannheim, Oriental Yeast (Tokyo), and Sigma Chemical Co., respectively. N-phosphoarginine was a commercial product of Sigma.

N$^{\epsilon}$-phospholysine was synthesized according to the method of Zetterqvist and Engström (1967) and purified by Dowex-I column chromatography with a linear gradient of 0.02 – 0.4 M triethylamine-HCO_3 (pH 9.8). The synthesis of N^{imid}-phosphohistidine was carried out essentially as for N$^{\epsilon}$-phospholysine synthesis except that L-histidine was used instead of L-lysine. N^{imid}-phosphohistidine was purified through a Dowex-I column using a linear gradient of 0.2 – 0.8 M triethylamine-HCO_3 (pH 8.1). The column fractions were analyzed for amino acid and phosphate by the ninhydrin method and by the method of Bartlett (1959), respectively. N$^{\epsilon}$-phospholysine and N^{imid}-phosphohistidine were eluted at 0.2 M and 0.3 M triethylamine-HCO_3 buffer, respectively.

GMP(5′ → N$^{\epsilon}$)lysine was synthesized by a modification of the procedure of Moffatt and Khorana (1961) for the synthesis of nucleoside5′-phosphoromorpholidates, using N-α-acetyl-L-lysine benzyl ester in place of morpholine. After deblocking with alkali (and hydrogenation with palladium on charcoal), GMP(5′ → N$^{\epsilon}$)lysine was purified by DEAE-Sephadex column chromatography with a linear gradient of 0.05 – 0.5 M triethylamine-HCO_3 (pH 7.6). The product was alkali-stable, while mild acid treatment (0.2 N HCl for 30 min at 37°C) released GMP quantitatively. It showed a positive ninhydrin reaction and the structure was confirmed by n.m.r.

Capping enzymes

Rat liver guanylyltransferase was purified from the isolated nuclei up to the step of CM-Sephadex column chromatography as described previously (Mizumoto and Lipmann, 1979). Crude capping enzyme of vaccinia virus (first DEAE-cellulose fraction) was prepared as described by Martin *et al.* (1975) from the particles of Lister strain which were kindly supplied by T.Urushibara (Kitasato University, Tokyo). Crude extracts were prepared from HeLa cells (Manley *et al.*, 1980), wheat germ (Marcu and Dudock, 1974), *A. salina* (Zasloff and Ochoa, 1971) and yeast (*Saccharomyces cerevisiae* A364A) (Itoh *et al.*, 1983) according to the reported procedures. Partially purified preparations of guanylyltransferase from these cells were obtained by chromatography of the 100 000 *g* supernatant (S-100 fraction) of crude extracts on a Sephadex G-200 column. Detailed purification procedures will be presented elsewhere. The guanylyltransferase activity was assayed by three different procedures; i.e. cap formation, GTP-PPi exchange, and enzyme-GMP complex formation as described (Mizumoto and Lipmann, 1979; Mizumoto *et al.*, 1982).

Isolation of the guanylyltransferase-[^{32}P]GMP intermediate

The covalent guanylyltransferase-GMP complex was formed and isolated essentially as described (Mizumoto *et al.*, 1982). The standard reaction mixture contained in 50 μl: 60 mM Tris-HCl (pH 7.9), 5 mM $Mg(OAc)_2$, 20 mM dithiothreitol, 0.1 μg yeast inorganic pyrophosphatase, 20 μg of bovine serum albumin, 1 μM [α-^{32}P]GTP (10 – 40 x 10^4 c.p.m./pmol) and the capping enzyme. Incubation was for 15 min at 30°C. The reaction was terminated by the addition of 10 mM EDTA, and the reaction mixture was immediately passed through a Sephadex G-50 column (0.6 x 18 cm) pre-equilibrated with 50 mM $NaHCO_3$. The void volume fractions were combined and proteins were precipitated with cold 5% trichloroacetic acid (TCA). The precipitates were washed once with 5% TCA, three times with ether, and dissolved in 15 – 50 μl of 10 mM Tris-HCl (pH 7.9) containing 1% SDS.

Preparation of the peptide-[^{32}P]GMP

The enzyme-[^{32}P]GMP complex was digested with pronase for 4 h at 37°C at a pronase/protein ratio of 1. After electrophoresis on Whatman DE81 paper with pyridine-acetic acid buffer (pH 3.5) for 2 – 3 h at 20 V/cm, peptide-[^{32}P]GMP was eluted with 1 M triethylamine-HCO_3^- (pH 7.5). The eluate was lyophilized and the residue was dissolved in 10 – 20 μl of 50 mM Na_2CO_3.

Sodium periodate oxidation and β-elimination of the peptide-[^{32}P]GMP

To the purified peptide-[^{32}P]GMP, 0.5 mM sodium periodate was added to cleave the ribose ring. After incubation for 60 min at room temperature in the dark, a 5-fold molar excess of ethylene glycol relative to periodate was added to terminate the oxidation. For β-elimination, the peptide-[^{32}P]GMP_{ox} was adjusted to pH 11 with 1 N NaOH and incubated for 60 min at 50°C, or was treated with 0.3 M aniline (pH 5.3) for 3 h at room temperature. The [^{32}P]-phosphoryl-peptide thus formed was purified by paper electrophoresis and was eluted as above.

Alkaline hydrolysis of the [^{32}P]phosphoryl-peptide

The [^{32}P]phosphoryl-peptide was hydrolyzed with 10 – 20 μl of 3 N KOH for indicated times at 100°C in sealed capillary tubes. The hydrolysates were analyzed by paper electrophoresis on Whatman DE81 at pH 3.5 with appropriate N-phosphoamino acid standards. Before electrophoresis, the alkaline hydrolyzates were neutralized with 3 M perchloric acid, and the precipitates of potassium perchlorate were removed by centrifugation.

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