Characterization of the m7G(5')pppN-pyrophosphatase Activity from HeLa Cells

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The m7G(5')pppN-pyrophosphatase activity previously detected in HeLa cells has been further characterized. Results from DEAE-cellulose column chromatography and polyacrylamide gel electrophoresis under nondenaturing conditions revealed only one enzyme activity in HeLa cell extracts which was capable of selectively hydrolyzing m7G(5')pppN to yield m7pG + ppN (where N = 2'-O-methylated or unmethylated ribonucleosides or oligonucleotides of up to 8 to 10 nucleosides in length). The majority (~95%) of this activity was found in the cytoplasmic extract but appeared not to be associated with the lysosomal fraction. m7G(5')pppG was hydrolyzed by the partially purified enzyme in the absence of divalent cations at a pH optimum of 7.5 and a temperature optimum of 45°, with a Michaelis constant (Km) of 1.7 μM. Sedimentation analysis and gel filtration showed the molecular weight of the enzyme as approximately 81,000.

Inhibition studies testing the effect of a number of prospective substrates on the rate of m7G(5')pppG hydrolysis have confirmed the importance of the methyl moiety at the N' position of guanosine for enzyme-substrate interaction. Furthermore, the trimethylated guanosine-containing 5'-terminal structure derived from 1-2 RNA was found not to serve as substrate, and 7-methylguanosine, unlike 7-methylguanosine, was not an effective inhibitor of m7G(5')pppG hydrolysis. Thus, the 2-amino group of the 7-methylguanosine serves as substrate, and 1-methylinosine, unlike 7-methylguanosine, possesses the positive charge at the N' position of guanosine, which no longer possesses the positive charge at the N' position of guanosine, were not hydrolyzed by enzyme preparations which readily hydrolyzed m7G(5')pppG; (b) hydrolysis occurred only between the β and γ phosphate of m7G(5')pppG yielding m7pG and a 5'-diphosphate terminated molecule, ppN; (c) m7pG was not cleaved from capped RNA molecules larger than 8 to 10 nucleosides in length under the in vitro conditions investigated.

This report deals with the further characterization of the HeLa cell enzyme activity responsible for m7G(5')pppN hydrolysis. In addition, the possible biological role of this enzyme will be discussed with respect to mRNA turnover and the possible consequences that alterations in the metabolism of caps may pose for the cell.

EXPERIMENTAL PROCEDURES

Preparation of [3H]Methyl m7G(5')pppG—G (5')pppG, prepared chemically by an anhydride exchange reaction between pG and ppG according to the procedure of Michelson (9) and as used by Filipowicz et al. (10), was methylated by the reovirus-associated methyltransferase with S-adenosyl-L-methyl-HLmethionine (7.3 to 12.6 Ci/mmol) as the methyl donor (11). (1'H)m7G(5')pppG was purified by paper electrophoresis in pyridine acetate buffer (pH 3.5) as previously described (8). Nucleotide pyrophosphatase treatment of the purified, alkaline phosphatase-resistant compound released m7pG as the only radioactive product.

Assay for m7G(5')pppN-pyrophosphatase Activity—For rapid detection of m7G(5')pppN cleaving activity, [3H]methyl-labeled m7G(5')pppG at a concentration of 80 nM (3000 cpm/pmol) was incubated at 37° in a final volume of 25 μl containing 40 mM Tris·HCl (pH 7.5) and 0.2 unit of bacterial alkaline phosphatase (BAP) to hydrolyze m7pG to m7G. The reaction was terminated by the addition of 0.2 μmol of the competitive inhibitor m7pG, and the entire mixture was applied to 10-cm strips of DEAE-81 cellulose paper or polyethyleneimine-cellulose thin layer sheets and developed with water. The strips were then dried, cut into 1-cm fractions, and the radioactivity was eluted from each fraction prior to counting in aquasol scintillant by the addition of 0.5 ml of 2 M LiCl followed by 0.5 ml of H2O. This assay is referred to as the BAP-coupled assay system. Alternatively, in the absence of BAP, the reaction product [3H]m7pG was separated from the substrate [3H]m7G(5')pppG and...
quantitated by paper electrophoresis in pyridine acetate buffer (pH 3.5) (8). One unit of activity was defined as the amount of enzyme hydrolyzing 1 pmol of mG(5')pppG to mG and pppG in 5 min at 37°C and pH 7.0.

Cell Fractionation Procedure—All operations were performed at 0-4°C. HeLa cells (2 x 10^9), grown in suspension culture in Eagle’s Minimal Essential Medium supplemented with 5% fetal calf serum were harvested by centrifugation, washed twice with 125 ml NaCl, 17 mm NaN3, 2.6 mm KH2PO4, and 7.5 mm potassium phosphate buffer (pH 7.5), and 0.5 mm dithiothreitol. After 10 min, the cells were disrupted with 15 to 20 strokes in a Dounce homogenizer and the nuclei were removed by centrifugation at 1000 x g for 10 min. The washed nuclear pellet was resuspended in 50 ml of 0.5 mm MgCl2, 5 mm potassium phosphate buffer (pH 7.5), and 0.5 mm dithiothreitol and disrupted by sonication.

The cytoplasmic supernatant was further fractionated by centrifugation at 30,000 x g for 30 min to remove lysosomes and mitochondria. The resulting pellet was resuspended in 5 ml potassium phosphate buffer (pH 7.5), 0.5 mm dithiothreitol, and 5% glycerol while the ribosomal pellet was prepared by further centrifugation of the postmitochondrial supernatant at 105,000 x g for 4 h in a SW41 rotor. The ribosomal pellet was rinsed with 20 ml Hapes (pH 7.6), 70 mm KH2PO4, 0.1 mm EDTA, 1 mm MgCl2, 0.5 mm dithiothreitol, and 5% glycerol and stirred in this buffer for 2.5 h at 4°C. The low salt buffer-washed ribosome fraction was then resedimented at 116,000 x g for 12 h and the pellet was rinsed with high salt buffer containing 200 mm Hapes (pH 7.6), 500 mm KCl, 5 mm MgAc2, 0.1 mm EDTA, 2 mm dithiothreitol, and 5% glycerol, and stirred in this buffer for 3 h followed by centrifugation for 4.5 h at 116,000 x g. The resulting pellet was finally resuspended in the high salt buffer.

**DEAE cellulose Chromatography and Polyacrylamide Gel Electrophoresis—For preparation of the partially purified enzyme, HeLa cell nuclei (2 x 10^9) were suspended in 300 ml of 10 mm Tris/HCl, 10 mm Tris/HCl (pH 7.5), and 1 mm MgCl2. After 30 min at 4°C, the cell suspension was disrupted with 15 to 20 strokes in a Dounce homogenizer and the nuclei were removed by centrifugation at 1000 x g for 10 min. The postribosomal supernatant prepared by centrifugation of the cytoplasmic supernatant at 88,700 x g for 4 h, was adsorbed onto a 150-ml DE52 cellulose column previously equilibrated with 20 mm Tris/HCl (pH 7.6), 10 mm NaCl, and 1 mm MgCl2 (Buffer A). The column was then washed with 300 ml of Buffer A and the activity was eluted with a 1000 ml linear gradient of Buffer A against Buffer B (pH 7.6, 0.4 M NaCl (Fig. 1). Seven-milliliter fractions were collected and assayed for mG(5')pppG cleaving activity. The pooled active fraction was concentrated by ammonium sulfate precipitation (66%), resuspended in 20 mm Tris/HCl (pH 7.5) and 5% glycerol, and dialyzed against this buffer. Enzyme preparations in this buffer were stable for five weeks when stored at -20°C.

**Polyacrylamide gel electrophoresis was performed under non-denaturing conditions as described by Davis (12) using a 10-cm, 7% separating gel and a 1.5-cm stacking gel. After electrophoresis, the gel was stained for the presence of thioerythric acid (0.1 ml/liter of reservoir buffer) for approximately 6 h at 2 mA/gel, the gels were fractionated into 2-mm slices by a Gilson automatic gel crusher and the enzyme activity was eluted overnight in the cold with a 20 mm Tris/HCl buffer (pH 7.5) containing 5% glycerol. The cleaved fractions were then tested for mG(5')pppG cleaving activity in the absence of BAP and the reaction mixtures were analyzed by paper electrophoresis.

**Sucrose Gradient Sedimentation and Gel Filtration—Sucrose gradient sedimentation was performed on a 12-ml 5 to 20% linear sucrose gradient in 20 mm Tris/HCl (pH 7.5), 0.3 mm NaCl, 2 mm dithiothreitol, and 1 mm EDTA. The samples were applied in 0.2-ml volumes and the gradients were centrifuged in a SW41 rotor for 26.5 h at 164,000 x g. Fractions of 0.4 ml were collected from the bottom of the tubes and mG(5')pppG cleaving activity was detected by the BAP-coupled assay system. Three marker proteins were included in parallel tubes: horse heart alcohol dehydrogenase (4.88 S), yeast alcohol dehydrogenase (7.4 S), and catalase (11.3 S). Assays for this marker enzymes were performed as described for the alcohol dehydrogenase by Valle and Hoch (13) and Beers and Sizer (14) for catalase.

**For gel filtration studies, a portion of the pooled active fractions (0.6 ml) were assayed for mG(5')pppG cleaving activity. Markers with known Stokes radii used for calibration of the column were catalase (3.22), yeast alcohol dehydrogenase (4.55), and bovine serum albumin (3.5). The excluded and included volumes were determined by using blue dextran and phenol red, respectively. The location of the respective marker proteins were determined by the procedure of Beers and Sizer (14), Valle and Hoch (13), and by measuring absorbance of 235 nm.

**Results—**

**Assay Conditions—**HeLa cell cytoplasmic extracts which have been purified free of contaminating cellular phosphatase activity convert [3H]methyl-labeled mG(5')pppG to ppG and [3H]mG (8). In the presence of an excess concentration of bacterial alkaline phosphatase (8 units/ml), the reaction products are G, F, and [3H]mG, with cleavage of the pyrophosphate linkage being the rate-limiting step. The reaction can be terminated either by boiling or by addition of an excess of mG or mG pppG, both competitive inhibitors of the pyrophosphatase reaction as is discussed later. The reaction mixture can then be analyzed by chromatography on strips of DEAE-sil paper or polyacrylamide cellulose thin layer plates with H2O as the solvent. Because of its negative charge, mG migrates with the solvent front, while the negatively charged alkaline phosphatase-resistant unhydrolyzed mG(5')pppG remains at the origin (Fig. 1). This assay is rapid, accurate, and can accommodate a large number of reaction samples. Alternatively, the extent of hydrolysis can be determined in the absence of BAP by paper electrophoretic analysis in pyridine acetate buffer (pH 3.5) (8). The latter method is very accurate but can accommodate only a limited number of samples in a reasonable time period.

In a series of experiments to determine suitable assay conditions using crude cytoplasmic extracts, it was found that the cleavage of mG(5')pppG proceeded in the absence of divergent cations, i.e. in the presence of EDTA. Furthermore, phosphate, pyrophosphate, dithiothreitol, mercaptoethanol, or NaCl (up to 0.4 M) had essentially no effect on the hydrolysis of mG(5')pppG. As previously reported (8), the unmethylated compound G(5')pppG was stable to hydrolysis by crude cytoplasmic extracts under all conditions tested.

**Distribution of mG(5')pppG Cleaving Activity upon Subcellular Fractionation—**Prior to attempts at purification of the mG(5')pppG cleaving activity, HeLa cells were fractionated as described under "Experimental Procedures" to determine which subcellular fraction contained the majority of the mG(5')pppG pyrophosphatase activity. As indicated in Table I, roughly 6% of the total activity was found in the nuclear wash and nuclear pellet fractions combined. After centrifugation of the crude cytoplasmic extract to remove mitochondria and lysosomes, over 95% of the recovered activity remained in the supernatant. Following centrifugation of the 30,000 x g supernatant at 105,000 x g, between 60 and 80% of the recovered activity was found in the supernatant fraction while 20 to 40% (20% in Table I) was found in the low salt ribosome.
HeLa m7G(5')pppN-pyrophosphatase

Fig. 1 (left). Alkaline phosphatase-coupled assay of m7G(5')pppN-pyrophosphatase activity. Reaction mixtures (25 μl) containing 2 pmol of [3H]methyl-m7G(5')pppG, 40 mM Tris/HCl (pH 7.5), and 0.2 unit of bacterial alkaline phosphatase were incubated at 37°C for 10 min with or without 0.8 μg of the m7G(5')pppN-pyrophosphatase fraction recovered from DEAE-cellulose chromatography. The reaction mixture was spotted onto a polyethyleneimine cellulose sheet sectioned into lanes (10 x 1 cm) with 7-methylguanosine as a marker. The polyethyleneimine cellulose sheet was developed with H2O as a solvent, dried, cut into 1-cm fractions, and counted.

Fig. 2 (right). DEAE-cellulose chromatography of the m7G(5')pppN-pyrophosphatase. The postribosomal supernatant was prepared and applied to a DE52 cellulose column as described under "Experimental Procedures." m7G(5')pppN-pyrophosphatase activity (○-○) was detected with the alkaline phosphatase coupled assay system. Also presented is the A280 (OD280) (○-○) and NaCl concentration (C- - - C) of the column fractions. Recovery of m7G(5')pppN-pyrophosphatase activity after electrophoresis of the DEAE-cellulose active fraction on polyacrylamide gels under non-denaturing conditions is shown in the inset.

Table 1

<table>
<thead>
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<th>Subcellular fraction</th>
<th>Total activity</th>
</tr>
</thead>
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<td>Crude (whole cell)</td>
<td>70,947</td>
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<tr>
<td>Nuclear wash</td>
<td>3,077</td>
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<tr>
<td>Nuclear pellet</td>
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<tr>
<td>Cytoplasmic extract</td>
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<td>30,000 x g supernatant</td>
<td>60,606</td>
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<td>30,000 x g pellet</td>
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<td>105,000 x g supernatant</td>
<td>38,500</td>
</tr>
<tr>
<td>Low salt ribosomal wash</td>
<td>10,000</td>
</tr>
<tr>
<td>High salt ribosomal wash</td>
<td>2,075</td>
</tr>
</tbody>
</table>

Subcellular distribution of m7G(5')pppG cleaving activity

Fractionation of 2 x 10⁶ HeLa cells was performed as described under "Experimental Procedures." The crude extract, containing intact nuclei as well as cytoplasmin, was briefly sonicated to lyse the nuclei prior to testing for m7G(5')pppG hydrolysis. The reaction mixtures were analyzed by paper electrophoresis at pH 3.5 to determine the relative amounts of m7G, m7pG, and m7G(5')pppG.

washed. A high salt wash of the low salt-washed ribosomes resulted in the removal of all activity from the ribosome fraction.

m7G(5')pppN cleaving activity was also detected in crude reticulocyte initiation factor preparations but was not found in significant amounts in the purified reticulocyte initiation factors M₁, MP, M₃, or M₄ (provided by Dr. W. C. Merrick and Dr. W. F. Anderson, National Institutes of Health, Bethesda, MD) or associated with reticulocyte native 40 S subunits (obtained from Dr. G. Blobel, Rockefeller University).

DEAE-cellulose Chromatography and Polyacrylamide Gel Electrophoresis of the m7G(5')pppN-pyrophosphatase Activity—Attempts at the purification of the HeLa cell m7G(5')pppN-pyrophosphatase revealed that the activity was adsorbed onto DEAE-cellulose and hydroxylapatite but not to phosphocellulose at pH 7.5. Shown in Fig. 2 is the elution profile of the activity following DEAE-cellulose chromatography. One discrete peak of activity was consistently observed eluting between 0.13 and 0.16 M NaCl with a usual recovery of 50 to 60%. No additional activity was recovered when the column was stripped with a 0.5 M NaCl wash. When the active fraction recovered from DEAE-cellulose was electrophoresed on polyacrylamide gels under non-denaturing conditions, again only one very sharp peak of activity was eluted with up to 70% recovery (Fig. 2, inset). These results are consistent with the possibility that HeLa cells contain only one enzyme which can specifically hydrolyze m7G(5')pppN. However, the existence of other m7G(5')pppN cleaving activities not detectable under the present conditions of fractionation and assay cannot be ruled out.

Physical Properties of m7G(5')pppN-pyrophosphatase—The active fraction obtained from DEAE-cellulose chromatography contained no phosphatase or previously reported 7-methylguanosine modifying activity (8). Since analysis of this fraction by non-denaturing polyacrylamide gel electrophoresis indicated the presence of only one active molecular species of
pyrophosphatase which could hydrolyze m'G(5')pppN, this fraction was used to determine a number of physical properties of the enzyme. As shown in Fig. 3, the pyrophosphatase exhibited activity over a rather broad pH range from pH 4.5 to 10 with an optimum at pH 7.5. The optimum temperature for m'G(5')pppG hydrolysis was between 40 and 50 °C with no activity observed below 4 °C or above 65-70 °C (Fig. 3).

A sedimentation coefficient of approximately 4.9 S was obtained for the m'G(5')pppN pyrophosphatase by sucrose gradient sedimentation using catalase (11.3 S), yeast alcohol dehydrogenase (7.4 S), and horse liver alcohol dehydrogenase (4.88 S) as markers (16). The pyrophosphatase activity co-sedimented with horse liver alcohol dehydrogenase (Fig. 4). Gel filtration studies on Sephadex G-200 (17) revealed a Stokes radius of 3.9 nm (data not shown). From the sedimentation coefficient and Stokes radius, a value of 81,000 was calculated (18) as the approximate molecular weight.

The influence of substrate concentration on m'G(5')pppN cleavage is shown in Fig. 5 (inset). Lineweaver-Burk analysis gave a Michaelis constant (K_m) of 1.7 μM. This low K_m suggests that the enzyme possesses a high level of affinity to select substrate of low concentration in the cell.

**Substrate Specificity** — It was previously reported that G(5')pppG and the ring-opened derivative of m'G(5')pppGm (2-amino-4-hydroxyl-5-(N-methylcarboxamide-6-ribosylamino-pyrimidine-5'-(2'-O-methyl)guanosine) were stable in HeLa cell cytoplasmic extracts which catalyzed the hydrolysis of m'G(5')pppGm to m'pG and ppGm (8). These results suggested that the enzyme responsible for m'G(5')pppG hydrolysis recognized the methyl group, or at least the partial positive charge, at the N7 position of the guanosine moiety of m'G(5')pppN.

In an effort to further define the substrate specificity of the HeLa pyrophosphatase, a number of potential substrates were tested as inhibitors of the m'G(5')pppG hydrolysis reaction using the DEAE-cellulose fraction as the enzyme source. Inhibition curves exhibited by a number of unmethylated compounds are presented in Fig. 6, Panel A. Guanosine mono- and...
FIG. 6. Inhibition of m7G(5')pppG hydrolysis by potential substrates. The reaction mixtures containing 80 nM [3H]methyl-
m7G(5')pppG, 40 mM Tris/HCl (pH 7.5), and 0.8 µg of enzyme (DEAE-cellulose fraction) in a 25-µl final volume, were incubated
with various concentrations of prospective substrates for 5 min at 37°C

diphosphate, inorganic pyrophosphate, and NAD, which also contains a 5'-5' pyrophosphate linkage, each inhibited the
m7G(5')pppG cleavage reaction only at very high concentrations with an IC₅₀ (inhibition of 50%) above 8 mM, i.e. a concentration over 200,000-fold in excess of the concentration of m7G(5')pppG in the mixture. Guanosine triphosphate and the
5'-5'-linked guanosine structures G(5')pppG and G(5')ppppG were slightly more inhibitory than pG and ppG having an IC₅₀ of approximately 200 to 800 µM or from 5,000 to 20,000-fold in excess of m7G(5')pppG.

In contrast, compounds methylated at the N7 position were very effective inhibitors of m7G(5')pppG hydrolysis at concentrations in the nanomolar range (Fig. 6, Panel B). m7ppG and m7pppG inhibited the m7G(5')pppG cleavage reaction by 50% at concentrations equal to that of m7G(5')pppG in the reaction mixture, i.e. they competed with m7G(5')pppG for the pyrophosphatase on an equal concentration basis. It is evident that in the case of the methylated guanosine-containing compounds, the level of inhibitory activity increases with the number of 5'-phosphates in the compound up to the diphosphate level (compare the inhibition by m7G in Fig. 6 with that exhibited by m7pG, m7ppG and m7pppG in Fig. 6, Panel B). These inhibition studies, in addition to the previous results which showed that G(5')pppG and ring-opened m7G(5')pppGm are not substrates for the m7G(5')pppN cleaving enzyme, demonstrate the importance of the N7 position of m7G(5')pppN for enzyme-substrate interaction by this specific pyrophosphatase.

The 5'-terminal structure of the low molecular weight nuclear RNA from rat hepatoma cells, U-2 RNA, is similar to m7G(5')pppN- except that it is also dimethylated at the 2-amino group of the terminal guanosine residue (19-21). Therefore, the 5'-terminal cap derived from U-2 RNA (generously provided by Dr. Ro-Choi and Dr. Harris Busch of Baylor College of Medicine), was tested as substrate for the HeLa m7G(5')pppN-pyrophosphatase. As shown in Fig. 7, under conditions where m7G(5')pppG was readily hydrolyzed, the cap from U-2 RNA was completely stable even at enzyme concentrations 10-fold in excess of that required to hydrolyze m7G(5')pppG by greater than 90%.

To further investigate the importance of the 2-amino position for substrate recognition, guanosine, inosine, 7-methyl-
guanosine, and 7-methylinosine were tested as inhibitors of the m7G(5')pppG hydrolysis reaction. As shown in Fig. 8, neither guanosine nor inosine was inhibitory at concentra-

tions up to 10 mM, while 7-methylguanosine was considerably more inhibitory than guanosine but less inhibitory than m7pG (compare Fig. 6, Panel B with Fig. 8), having an IC₅₀ of approximately 25 µM. By comparison with the inhibition obtained with m7G, 7-methylinosine, which also possesses a partial positive charge at the N7 position but lacks the 2-amino group, was not inhibitory. Thus, the 2-amino position of 7-methyl-
guanosine is also important for interaction with the HeLa m7G(5')pppN-pyrophosphatase.

The specificity of hydrolysis and of enzyme-substrate interaction exhibited by the m7G(5')pppN-pyrophosphatase is sum-
marized diagrammatically in Fig. 9. The pyrophosphatase

Fig. 7. The 5'-terminal structure derived from U-2 low molecular
weight nuclear RNA as a possible substrate for the m7G(5')pppN-
pyrophosphatase. [3H]Methyl m7G(5')pppG (—) and [32P]U-2 cap
(----) were tested under standard reaction conditions for hydroly-
sis. The reaction was for 5 min at 37°C in the absence or the presence of 0.8 µg of enzyme for m7G(5')pppG hydrolysis or 10 µg of enzyme for the U-2 cap. The reaction mixtures were analyzed by paper electrophoresis at pH 3.5.
rapid degradation of the remaining molecule, then caps and mRNA molecule (23, 33, 34). Provided that hydrolysis of the production of capped mRNA degradation products in the cyto-

The 5' cap structure is resistant to various ribonucleases which cleave internal regions of messenger RNA. Venom phosphodiesterase and nucleotide pyrophosphatase which are both secreted enzymes in snake venom and two recently described nucleases from tobacco (31) and potato (32) are the only enzymes in addition to the HeLa m7G(5')pppN-pyrophosphatase, so far reported to cleave the 5'5' pyrophosphate linkage, such as NAD (Fig. 6) or FAD, appears unlikely.

It became evident in the course of this study that there are some intriguing parallels between the effects of a number of cap analogs on both mG(5')pppG hydrolysis and in vitro protein synthesis. In a recent report, Hickey et al. (35) have compared the effect of a number of 7-methylguanosine analogs on in vitro protein synthesis and attempted to establish the relationship between the inhibitory effect of these molecules and their conformation in solution. The similarities between the inhibition of in vitro protein synthesis and of mG(5')pppG hydrolysis are as follows.

1. The di- and triphosphate derivatives of 7-methylguanosine were found to inhibit protein synthesis at lower concentrations than 7-methylguanosine 5'-monophosphate and, particularly, 7-methylguanosine (35). Similar results are shown in Fig. 6, Panel B, and Fig. 8 for mG(5')pppG hydrolysis. The greater inhibitory effect of the 5' phosphate derivatives of 7-methylguanosine on protein synthesis was found to correlate with a decreased conformational freedom of the backbone of these molecules, i.e. a conformation in which the C4'-C5' and C5'-O5' bonds are locked in a "w" arrangement (35).

2. The unmethylated dinucleotide G(5')pppA was not an effective inhibitor of protein synthesis and the methyl moiety at the 2'-O-position found in many cap structures had no apparent effect on the inhibition of protein synthesis (35). Similarly G(5')pppG was found to inhibit mG(5')pppG hydrolysis only at quite high concentration (Fig. 6, Panel A) and was not a substrate for cleavage by the mG(5')pppN-pyrophosphatase (8). Furthermore, the methyl group at the 2'-O-position of the penultimate base of mG(5')pppN is not required for substrate recognition by the pyrophosphatase (8).

3. 7-Methylinosine was ineffective as an inhibitor of protein synthesis suggesting that the 2'-amino position of 7-methylguanosine is important in mRNA-ribosome complex formation (35). As shown in Fig. 8, 7-methylguanosine was also ineffective as an inhibitor of mG(5')pppG hydrolysis. Moreover, the 5'-terminal structure derived from U-2 low molecular weight nuclear RNA which is dimethylated at the 2-amino position of the 7-methylguanosine moiety (19, 20), was not a substrate for cleavage by the HeLa mG(5')pppN-pyrophosphatase (Fig. 7).

The similarities in the effect of cap analogues on both in vitro protein synthesis and mG(5')pppG hydrolysis and the possible loose association of the mG(5')pppN-pyrophosphatase activity with the ribosome fraction (Table I) suggest the need for further studies to determine possible relationships between this enzyme activity and protein synthesis. It is not unreasonable to expect cap metabolism to occur in association with these structures (polyribosomes) actively involved in mRNA translation.

**DISCUSSION**

Although considerable progress has been made concerning the kinetics and mechanism of formation of the 5' terminus of eukaryotic cellular and viral mRNA (11, 22-30), little information is available with respect to the decay of the cap moiety. The 5' cap structure is resistant to various ribonucleases which cleave internal regions of messenger RNA. Venom phosphodiesterase and nucleotide pyrophosphatase which are both secreted enzymes in snake venom and two recently described nucleases from tobacco (31) and potato (32) are the only enzymes in addition to the HeLa mG(5')pppN-pyrophosphatase, so far reported to cleave the 5'-5' pyrophosphate linkage in caps. However, only the HeLa enzyme exhibits any specificity for the 7-methylguanosine-containing structure.

At present no evidence exists indicating that the 5'-terminal cap has a turnover rate different from that for the rest of the mRNA molecule (23, 33, 34). Provided that hydrolysis of the cap is not the primary event in mRNA decay, followed by a rapid degradation of the remaining molecule, then caps and capped oligonucleotides would be expected intermediates of mRNA turnover. Under steady state conditions, the rate of production of capped mRNA degradation products in the cyto-

![Fig. 8. Inhibition of mG(5')pppG hydrolysis by inosine and guanosine derivatives. The reaction conditions were as described in Fig. 6.](http://www.jbc.org/content/252/28/2820/F1.large.jpg)
The results presented in Fig. 2 suggest that HeLa cells may contain only one enzyme activity which can specifically hydrolyze the cap structure. m\(^7\)G(5')pppN cleaving activity has also been detected in cell-free extracts prepared from wheat embryos, Artemia salina, mouse lymphocytes, and rabbit reticulocytes. Furthermore, in experiments designed to compare the stability of m\(^7\)G(5')pppG and G(5')pppG in extracts from Escherichia coli and HeLa cells, it was observed that both compounds were hydrolyzed at equal rates by the E. coli extracts, while only m\(^7\)G(5')pppG but not G(5')pppG was readily hydrolyzed by the HeLa cytoplasmic extracts. The widespread distribution of the m\(^7\)G(5')pppN-specific pyrophosphatase in eukaryotic cells which have 5'-m\(^7\)G(5')pppN-terminated mRNAs suggests an important functional role for this enzyme in the metabolism of capped mRNA in eukaryotic cells. However, attempts at removing m\(^7\)pG from a number of capped polynucleotides including both intact viral and cellular mRNA and capped poly(U) (4) under a wide variety of conditions have been unsuccessful. Although the HeLa pyrophosphatase may cleave m\(^7\)pG from intact mRNA in vivo as they have been reported to do in vitro, then the enzyme responsible for cap metabolism constitutes a potential control point in the regulation of the overall rate of mRNA translation in eukaryotic cells.

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