Inhibition of Eukaryotic Translation by Analogues of Messenger RNA 5′-Cap: Chemical and Biological Consequences of 5′-Phosphate Modifications of 7-Methylguanosine 5′-Monophosphate†

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ABSTRACT: New analogues of 7-methylguanosine 5′-monophosphate (m^7GMP) were synthesized with modified 5′-phosphate moieties by replacement of –O with –H, $-CH_3$, or $-NH_2$. Additional analogues were synthesized with 8-methyl- or 8-aminoguanine base substitutions or ring-opened ribose (2′,3′-diol). These compounds were analyzed by ^{1}H and ^{31}P NMR for solution conformation. In addition, they were also analyzed for biological activity as analogues of mRNA 5′-caps by competition as inhibitors of translation in reticulocyte lysate. Substitution of oxygen on the 5′-monophosphate moiety by –H and $-CH_3$ diminished the activity of the cap analogue as a competitive inhibitor; however, replacement by $-NH_2$ did not diminish the activity of the analogue as an inhibitor. It was inferred from this result that cap binding proteins require a hydrogen bond acceptor as opposed to having an exclusive requirement for a second anionic group on the α-phosphate moiety. Inhibition results obtained with C8-substituted m^7GMP analogues indicated that the 8-amino derivative was a better inhibitor than the 8-methyl derivative of m^7GMP. The former is primarily anti whereas the latter is primarily syn with respect to glycosidic bond conformation. This result further supports the model that the anti conformation is the preferred form of the cap structure for interaction with cap binding proteins. The 2′,3′-diol derivative of m^7GMP was inactive as an inhibitor of translation.

Translation of eukaryotic mRNA is dependent on several aspects of mRNA structure. These macromolecules are for the most part monocistronic, possess 5′-termini of the form m^7G(5′)ppp(5′)N, and are polyadenylylated on the 3′-end. The 5′ modification is known as a cap and is necessary for optimum translation. It is recognized by at least three translation initiation factors, namely, eIF-4A,[1] eIF-4B, and eIF-4F (Grifo et al., 1983; Edery et al., 1983, 1985). These are also collectively termed cap binding proteins (CBPs; Shatkin, 1985). These factors bind at or near the 5′-cap of mRNA early in the initiation phase of protein biosynthesis and facilitate mRNA attachment to the 40S ribosomal subunit. Cap analogues inhibit this step of initiation both in complete translation assay (Hickey et al., 1977; Adams et al., 1978) and in partial reaction assays of initiation using purified initiation factors (Sonenberg et al., 1978; Grifo et al., 1983; Tahara et al., 1983; Darzynkiewicz et al., 1981, 1985). Thus there is a direct correlation between inhibition of overall translation and inhibition of CBP activity.

Cap analogues compete to varying degrees with mRNA 5′-ends for CBPs. As a result, differences in inhibition by analogues make it possible to infer required structural features of native mRNA caps. There are at least two structural requirements for ligand binding by CBPs to m^7GMP analogues; these are in addition to the known requirement for N7 alkyl or benzyl substitution (Adams et al., 1978; Furuichi et al., 1979). Replacement of ribose by (hydroxyethoxy)methyl ether affects inhibitory activity. By contrast, substitution of the ribose moiety of 7-methylguanosine 5′-monophosphate by other pentoses does not decrease inhibitory activity (Darzynkiewicz et al., 1985). This result suggests that the base and phosphate moieties must assume a particular spatial configuration. More significantly, derivatization of the α-phosphate moiety by O-methylation causes m^7GMP to become less effective as an inhibitor (Darzynkiewicz et al., 1981). The latter result suggests a need for an unhindered oxygen or dissociable second hydroxyl in the α-phosphate moiety. By comparison, O-methylation of the β-phosphate of m^7GDP or the γ-phosphate of m^7GTP does not diminish inhibitory activity.

As part of a continuing effort to elucidate the nature of the m^7G nucleotide binding sites of cytoplasmic CBPs, new compounds were synthesized to test the hypothesis that diminution of charge and/or added steric hindrance in the α-phosphate moiety would decrease the inhibitory effect of the analogue in translation. A new family of m^7GMP analogues was syn-

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[1] Abbreviations: m^7G, 7-methylguanosine; m^7GMP, 7-methylguanosine 5′-phosphate; m^7GMP-H, 7-methylguanosine 5′-phosphite; m^7GMP-NH_2, 7-methylguanosine 5′-phosphoramidate; m^7GMP-CH_3, 7-methylguanosine 5′-methylphosphonate; m^7GMP-OCH_3, 7-methylguanosine 5′-phosphate methyl ester; $m_2^{7,8}$GMP, 7,8-dimethylguanosine 5′-phosphate; $m^7$8-NH_2GMP, 7-methyl-8-aminoguanosine 5′-phosphate; $m_2^{7,8}$acycloGMP, 7,8-dimethyl-9-(4′-hydroxy-2′-oxabut-1′-yl)guanine 4′-phosphate; m^7acycloGMP-CH_3, 7-methyl-9-(4′-hydroxy-2′-oxabut-1′-yl)guanine 4′-methylphosphonate; m^7GMP-2′,3′-diol, 7-methyl-9-[1′,5′-dihydroxy-4′(*S*)-(hydroxymethyl)-3′-oxapent-2′(*R*)-yl]guanine 5′-phosphate; TEAB, triethylammonium bicarbonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; CBP, cap binding protein; eIF, eukaryotic initiation factor.

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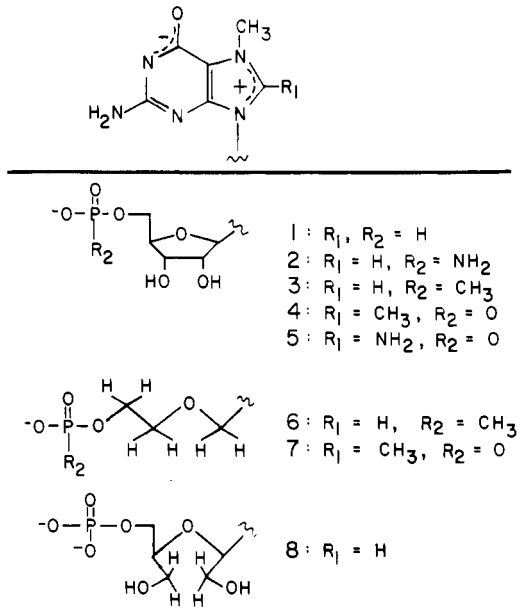

FIGURE 1: Structure of m^7G cap analogues.

thesized containing modified 5′-phosphates (**1–3**; Figure 1). Several other analogues were synthesized with C8 substituents (**4** and **5**), double modifications at the ribose and phosphate moieties (**6** and **7**), or modification at the ribose alone (**8**). **4** and **5** were synthesized to test the importance of syn vs. anti conformation as well as more subtle structural differences on cap analogue activity.

Experimental Procedures

Materials. Guanosine 5′-monophosphate, 2′,3′-*O*-isopropylideneguanosine, DEAE-Sephadex (A-25), and *N,N′*-dicyclohexylcarbodiimide (DCC) were from Sigma. Trimethyl phosphite, 1,3-di-*p*-tolylcarbodiimide (DTC), and phosphorous acid were from Aldrich. CH_3I was from POCH (Poland). 9-[(2-Hydroxyethoxy)methyl]guanine (acycloG) was the gift of Dr. Danny King of Burroughs-Wellcome (Research Triangle, NC). DE-52 was from Whatman; Dowex 50W-X8 (100–200 mesh), Na^+ form, Darco G-60 activated charcoal, and Celite 501 were from Serva. [^{35}S]Methionine (specific activity 1125 Ci/mmol) was obtained from New England Nuclear. Oligo(dT)–cellulose was from Collaborative Research. All other chemicals were of reagent grade or higher quality.

Methylphosphonic acid was obtained from the dimethyl ester by hydrolysis in concentrated hydrochloric acid/acetic acid (15:1). The dimethyl ester was prepared by reacting methyl iodide with an excess of trimethyl phosphite (Kosolapoff, 1950).

Synthesis of Cap Analogues. Cap analogues were generally synthesized from the unmethylated guanosine nucleotide by reaction with methyl iodide in dimethyl sulfoxide (Me_2SO) (Darzynkiewicz et al., 1985). N7-Methylated nucleotides were isolated by column chromatography on DEAE-cellulose (Whatman DE-52; HCO_3^- form) or Sephadex A-25 (HCO_3^- form). Product(s) was (were) eluted with linear gradients of triethylammonium bicarbonate (TEAB) buffer (pH 7.4), 3 L total volume, unless indicated otherwise, and at the indicated beginning and ending concentrations.

(*A*) *7-Methylguanosine 5′-Phosphite* (*m^7GMP-H*, **1**). 2′,3′-*O*-Isopropylideneguanosine 5′-phosphite was synthesized by a general method for synthesis of nucleoside phosphites (Schofield & Todd, 1961). A mixture of crystalline phosphorous acid (63.4 mg, 0.77 mmol) and 2′,3′-*O*-isopropylideneguanosine (250 mg, 0.77 mmol) was thoroughly dried by three cycles of evaporation from dry pyridine. This mixture was dissolved in dry pyridine (20 mL), combined with 1,3-di-*p*-tolylcarbodiimide (DTC; 200 mg, 0.92 mmol), and allowed to react for 72 h at room temperature under anhydrous conditions. Thin-layer chromatography (TLC) of the final mixture on cellulose (2-propanol/concentrated NH_4OH/water, 7:1:2 v/v/v) showed it to contain ~50% 2′,3′-*O*-isopropylideneguanosine (R_f = 0.89) and ~50% of expected 2′,3′-*O*-isopropylideneguanosine 5′-phosphite (R_f = 0.65). Reactants were precipitated away from product and unmodified nucleoside by addition of 50 mL of water. Insoluble material was removed by filtration, and the collected filtrate was evaporated to dryness. The residue was dissolved in 10 mL of 20% acetic acid and heated for 10 h at 52 °C to hydrolyze the isopropylidene group. TLC on cellulose [1% $(NH_4)_2SO_4$/2-propanol, 1:2 v/v; system B) showed complete deprotection of 2′,3′-*O*-isopropylideneguanosine (R_f = 0.96) and 2′,3′-*O*-isopropylideneguanosine 5′-phosphite to guanosine (R_f = 0.52) and guanosine 5′-phosphite (R_f = 0.34), respectively. Guanosine 5′-phosphite and guanosine were separated by DE-52 column chromatography using a 0–0.4 M TEAB gradient. Guanosine was unbound and eluted from the column in the initial water wash; guanosine 5′-phosphite eluted at 0.17 M TEAB as monitored by absorbance at 260 nm. Fractions containing the latter were pooled and desalted by several evaporations with ethanol under reduced pressure. This material was converted from the triethylammonium (TEA) salt to the Na^+ salt by ion exchange using a small column of Dowex 50W-X8 (Na^+ form). The product was precipitated with ethanol and dried over P_2O_5 to give 77 mg (0.21 mmol) of a white powder of guanosine 5′-phosphite monosodium salt (**1a**) in 27% yield.

1a (sodium salt; 38.3 mg, 0.1 mmol) was dissolved in 0.3 mL of water and 2.5 mL of Me_2SO, to which was added 0.2 mL of CH_3I (3.2 mmol). The mixture was stirred at room temperature for 3 h. Thirty milliliters of water was added to dilute the reaction mixture prior to several extractions with diethyl ether. The aqueous phase was reduced by evaporation to 20 mL and chromatographed on DE-52 with a 0–0.25 M gradient of TEAB. 7-Methylguanosine 5′-phosphite eluted as a peak with 0.08 M TEAB. Pooled peak fractions were desalted, converted to the sodium salt by ion exchange, and lyophilized. The resulting amorphous powder (24.6 mg, 0.064 mmol) was 7-methylguanosine 5′-phosphite (**1**) in 64% yield.

(*B*) *7-Methylguanosine 5′-Phosphoramidate* (*m^7GMP-NH_2*, **2**). For the synthesis of guanosine 5′-phosphoramidate (**2a**), guanosine 5′-monophosphate, free acid (0.5 g, 1.38 mmol), was dissolved in 7.5 mL of 2.5 N ammonium hydroxide plus 5 mL of formamide (Chambers et al., 1960). DCC (3 g, 14.6 mmol) was dissolved in 20 mL of *tert*-butyl alcohol and added to the nucleotide solution. The heterogeneous mixture was heated to 80 °C in a water bath for 2 h until it became homogeneous; the mixture was kept at this temperature for another 15 h. Cyclohexylurea was precipitated from the mixture by addition of 50 mL of cold water and removed by filtration. The filtrate and washes were coevaporated several times with water under reduced pressure to remove ammonia. The residue was taken up in 30 mL of water and chromatographed on DE-52 by using a gradient of 0–0.4 M TEAB. The product eluted at 0.22 M TEAB; it was desalted by coevaporation with ethanol, converted to the Na^+ form, and dried over P_2O_5 under vacuum to afford 220 mg (0.61 mmol) of **2a** in 44.2% yield.

2a (100 mg, 0.24 mmol) was dissolved in 4 mL of Me_2SO to which was added 0.6 mL (9.6 mmol) of CH_3I. The reaction

mixture was stirred for 5 h at room temperature and then diluted with 50 mL of water, followed by several extractions with diethyl ether. The aqueous phase was evaporated to ~30 mL, applied to a column of DE-52, and eluted with a 0–0.3 M TEAB gradient. The desired product eluted at 0.11 M TEAB. Pooled fractions were desalted, converted to the sodium salt, and precipitated with ethanol. The final product (**2**; 19.6 mg, 0.049 mmol) was obtained in 20.4% yield.

(*C*) *7-Methylguanosine 5′-Methylphosphonate* (*m*^{7}*GMP-CH*$_3$, **3**). Guanosine 5′-methylphosphonate (**3a**) was prepared according to Myers et al. (1965) as adapted by Kusmierek and Shugar (1979). Methylphosphonic acid (527 mg, 5.48 mmol) and 2′,3′-*O*-isopropylideneguanosine (397 mg, 2.3 mmol) were dissolved in 75 mL of dry dimethylformamide (DMF) to which DCC (2.36 g, 11.6 mmol) was added. The reaction mixture was kept at 37 °C for 2 days followed by further reaction at 50 °C for 5 h. The mixture was diluted with cold water (400 mL) and kept at 4 °C overnight. Precipitated cyclohexylurea was collected by filtration and washed thoroughly with water; the combined washes and filtrate were evaporated to dryness. The residue was dissolved in 20% acetic acid (10 mL) and heated for 5 h at 60 °C to remove the isopropylidene group. Residual acetic acid was removed by several cycles of coevaporation with water. TLC (cellulose) in system B showed quantitative conversion of 2′,3′-*O*-isopropylideneguanosine and 2′,3′-*O*-isopropylideneguanosine 5′-methylphosphonate (R_f = 0.89 and 0.67, respectively) to guanosine and guanosine 5′-methylphosphonate (R_f = 0.52 and 0.36, respectively); the latter products were present in a 1:2 ratio. **3a** was purified from guanosine by chromatography on DE-52 (HCO_3^- form) using a gradient of 0–0.5 M TEAB. Guanosine eluted from the column in the initial water wash, and **3a** eluted from the column at 0.2 M TEAB; pooled fractions containing the latter were desalted and converted to the Na^+ salt. After precipitation with ethanol, 233.4 mg (0.61 mmol; 49.6% yield) of amorphous powder of the Na^+ salt of **3a** was obtained.

3a (sodium salt; 113 mg, 0.3 mmol) was dissolved in 0.4 mL of water plus 3 mL of Me_2SO (a drop of 1 M HCl was added to facilitate dissolution). CH_3I (0.45 mL, 7.2 mmol) was added, and the mixture was stirred for 3 h at room temperature. Water (40 mL) was added to the reaction mixture, and the aqueous phase was extracted several times with diethyl ether. The aqueous phase was concentrated to ~30 mL and applied to a column of DE-52. The column was washed with water and eluted with a linear gradient of 0–0.25 M TEAB. The peak of 260-nm absorbance eluted at 0.08 M TEAB and was desalted and converted to the Na^+ salt to give 29.7 mg (0.075 mmol) of **3** as an amorphous powder (25% yield).

(*D*) *7,8-Dimethylguanosine 5′-Monophosphate* (*m*$_2^{7,8}$*GMP*, **4**). 8-Methylguanosine 5′-monophosphate (**4a**) (Lassota et al., 1984) sodium salt (27 mg, 0.064 mmol) was dissolved in 1 mL of Me_2SO and 0.05 mL of water. CH_3I (0.35 mL; 5.6 mmol) was added, and the reaction mixture was stirred at room temperature for 3.5 h. The reaction mixture was diluted with 30 mL of water and extracted twice with 20-mL portions of diethyl ether. The aqueous phase was concentrated to 20 mL and chromatographed on a DE-52 column with a 0–0.3 M TEAB gradient (2 L). The peak of 260-nm absorbance eluting at 0.12 M TEAB was pooled, desalted, and converted to the Na^+ salt. After a final precipitation with ethanol, 14.6 mg (0.035 mmol) of **4a** sodium salt was obtained (55% yield).

(*E*) *7-Methyl-8-aminoguanosine 5′-Monophosphate* (*m*^{7}*8-NH*$_2$*GMP*, **5**). 8-Amino-5′-GMP (**5a**) (19.3 mg, 0.046 mmol) (Lassota et al., 1984) was dissolved in 0.2 mL of water plus 4 mL of Me_2SO, and to this was added 0.7 mL of CH_3I (11.2 mmol). The reaction was stirred at room temperature for 3 h. 50 mL of water was added, and the reaction mixture was extracted several times with diethyl ether. The aqueous phase was concentrated to ~30 mL by evaporation and applied to a column of DE-52. The column was washed with water and eluted with a 0–0.3 M linear gradient of TEAB. The peak of material, as measured by absorbance at 260 nm, eluted at 0.13 M TEAB and was pooled and desalted by several cycles of evaporation with ethanol. The product was converted to the Na^+ salt and given a final precipitation with ethanol. A total of 10 mg (0.023 mmol) of **5** as an amorphous white powder was obtained (50% yield).

(*F*) *7-Methyl-9-(4′-hydroxy-2′-oxabut-1′-yl)guanine 4′-Methylphosphonate* (*m*^{7}*acycloGMP-CH*$_3$, **6**). Acycloguanosine 5′-methylphosphonate (**6a**) was synthesized as described for 2′,3′-*O*-isopropylideneguanosine methylphosphonate. Acycloguanosine (acycloG; 300 mg, 1.33 mmol) was dissolved in 50 mL of DMF containing 400 mg of methylphosphonic acid (4.17 mmol) and 1.5 g (7.27 mmol) of DCC. The mixture was allowed to react for 24 h at 37 °C. Cyclohexylurea was precipitated with water as above, and the filtrate plus washes were applied to a column of Sephadex A-25 and chromatographed. The column was washed with water and eluted with a linear gradient of 0–0.5 M TEAB (2 L total). The product peak eluted at 0.25 M TEAB and was pooled; half of it was converted to the Na^+ salt of **6a** to give 147.2 mg (0.46 mmol; 68% overall yield).

The N7-methylated derivative (**6**) was synthesized from 5143 A_{260} units of the TEA salt of **6a** (0.45 mmol). It was dissolved in 6 mL of Me_2SO, and to this was added 0.9 mL of CH_3I (14.4 mmol). The mixture was stirred for 2 h, diluted with 50 mL of water, and extracted several times with diethyl ether. The aqueous phase was reduced to 30 mL by evaporation and applied to a column of Sephadex A-25. The column was washed with water and eluted with a linear gradient of 0–0.3 M TEAB (2 L). The peak of 260 nm absorbing material eluting at 0.15 M TEAB was desalted and converted to the Na^+ salt to afford 81.8 mg (0.24 mmol; 53% yield) of **6**.

(*G*) *7,8-Dimethyl-9-(4′-hydroxy-2′-oxabut-1′-yl)guanine 4′-Phosphate* (*m*$_2^{7,8}$*acycloGMP*, **7**). 8-Methylacycloguanosine was prepared from acycloG essentially as described earlier (Kawazoe et al., 1972). A total of 500 mg of acycloG (2.21 mmol) was dissolved in 100 mL of 0.2 N H_2SO_4 containing 2 g of $FeSO_4 \cdot 7H_2O$ (7.2 mmol) at room temperature. To the mechanically stirred solution was added dropwise over 1 h *tert*-butyl hydroperoxide [0.7 mL of 80% *tert*-butyl hydroperoxide (5.5 mmol) in 30 mL of water], and the solution was stirred for another 30 min. The product was desalted on a column of activated charcoal/Celite (7.5 g of Darco G60 + 7.5 g of Celite 501). After sample application, the column was washed with ~250 mL of water until the effluent was pH ~5 and gave a negative test with NaSCN. Product was eluted from the column with water/ethanol/25% NH_4OH (10:10:1). The eluate was evaporated to dryness, and the residue was dissolved in ~15 mL of hot water and alkalinized with added 25% NH_4OH. This solution was left to crystallize at room temperature for 72 h. The resulting crystals were filtered, washed thoroughly with water, and dried in vacuo over P_2O_5. This gave 350 mg (1.46 mmol, 66% yield) of 8-methylacycloguanosine as light yellow crystals. The latter was shown to be homogeneous by TLC (cellulose F_{254}, Merck; R_f = 0.34) with *n*-butanyl alcohol/water/ethanol (80:25:10).

8-Methyl-9-(4′-hydroxy-2′-oxabut-1′-yl)guanine 4′-Phosphate (*m*^{8}*acycloGMP*, **7a**). 8-Methylacycloguanosine was 5′-phosphorylated as for synthesis of acycloGMP from acycloG

(Darzynkiewicz et al., 1985) by adaptation of a general method (Pal et al., 1978). 8-Methylacycloguanosine (300 mg, 1.25 mmol) was added to a solution of freshly distilled phosphoryl chloride (0.45 mL, 4.95 mmol) in trimethyl phosphate (9 mL) and stirred at 0 °C for 1 h. The conversion of 8-methylacycloguanosine (R_f = 0.74) to the 5'-phosphorylated product (R_f = 0.41) was monitored by cellulose TLC in 2-propanol/concentrated NH_4OH/water (4:4:2). Upon completion, the mixture was neutralized with cold 5% $NaHCO_3$, diluted to 60 mL with water, and applied to a column of Sephadex A-25. The column was washed with water (300 mL) and eluted with a linear gradient of 0–0.8 M TEAB (2-L volume). The phosphorylated product eluted at 0.5 M TEAB and was desalted as above. Half of the product was converted to the Na^+ salt to yield 195.6 mg (0.54 mmol; 86% overall yield). The remaining TEA salt (**7a**) was taken for methylation.

7 was synthesized from m^{8}acycloGMP (**7a**). The TEA salt of **7a** (7700 A_{260} units, 0.54 mmol) was dissolved in 0.4 mL of water plus 10 mL of Me_2SO. CH_3I (1 mL, 16 mmol) was added, and the mixture was stirred for 6 h at room temperature. The reaction mixture was diluted with 60 mL of water and extracted several times with diethyl ether. The aqueous material was chromatographed on a column of Sephadex A-25 with a linear gradient of 0–0.5 M TEAB (2 L). The peak of 260-nm absorbance eluted with 0.25 M TEAB and was desalted and converted to the Na^+ salt. This afforded 149.8 mg (0.4 mmol; 73% yield) of **7** Na^+ salt.

(H) 7-Methyl-9-[1',5'-dihydroxy-4'(S)-(hydroxymethyl)-3'-oxapent-2'(R)-yl]guanine 5'-Phosphate (m^{7}GMP-2',3'-diol, **8**). 5'-GMP 2',3'-dialcohol (**8a**) was obtained by a modification (R. Stolarski, Z. Kazimierczuk, P. Lassota, and D. Shugar, personal communication) of the method of Rosenthal et al. (1982). GMP·Na_2 (2.4 g, 6 mmol) was dissolved in 20 mL of water, to which was added 1.7 g HIO_4 (7 mmol). The pH was adjusted to ~7 with triethylamine, and the solution was stirred for 1 h at room temperature. Ethylene glycol (0.5 mL) was added to react with remaining IO_4^-, and the 2',3'-dialdehyde was reduced by addition of $NaBH_4$ (2.5 g, 66 mmol) in small portions over a 3-h period. After the last addition the mixture was stirred for an additional 3 h. The reaction mixture was filtered to remove inorganic salts, and the filtrate was divided into three equal portions and chromatographed separately on Sephadex A-25. Each batch column was eluted with a linear gradient of 0–0.8 M TEAB (2-L total volume). The 2',3'-dialcohol derivative of GMP (**8a**) eluted at 0.65 M TEAB. Half of the pooled peak fractions was converted to the Na^+ salt; this gave, after ethanol precipitation, 0.8 g (1.96 mmol) of the disodium salt of **8a** (65% overall yield).

A total of 7000 A_{260} units (0.52 mmol) of **8a** TEA salt was dissolved in 10 mL of Me_2SO, to which was added 0.9 mL (14.4 mmol) of CH_3I. The mixture was stirred for 4 h at room temperature, diluted with 50 mL of water, and extracted several times with diethyl ether. The aqueous phase was concentrated to ~30 mL by evaporation and applied to a column of Sephadex A-25. The column was washed with water and then eluted with a 0–0.45 M TEAB linear gradient (2 L). **8** eluted from the column at 0.26 M TEAB and was converted into the Na^+ salt to obtain 192.3 mg (45 mmol, 87% yield).

Spectroscopy. Proton NMR spectra were obtained with Bruker WP-80, AM-200, and AM-500 instruments. ^{31}P NMR spectra were obtained with a Bruker CXP-300 spectrometer. Samples were dissolved in D_2O (99.96% D; Aldrich), and

Table I: R_f Values of Synthetic Cap Analogues[a]

compd	no.	system A	system B
m^{7}GMP[b]		0.70	0.18
GMP[b]		0.53	0.28
m^{7}GMP-H[c]	**1**	0.51	0.28
GMP-H	**1a**	0.30	0.37
m^{7}GMP-NH_2[c]	**2**	0.57	0.14
GMP-NH_2	**2a**	0.33	0.19
m^{7}GMP-CH_3[c]	**3**	0.58	0.29
GMP-CH_3	**3a**	0.36	0.35
m$_2^{7,8}$GMP[c]	**4**	0.70	0.14
m^{8}GMP	**4a**	0.39	0.26
m^{7}8-NH_2GMP	**5**	0.53	0.13
8-NH_2GMP	**5a**	0.31	0.14
m^{7}acycloGMP-CH_3[c]	**6**	0.65	0.32
acycloGMP-CH_3	**6a**	0.41	0.39
m$_2^{7,8}$acycloGMP[c]	**7**	0.70	0.19
m^{8}acycloGMP	**7a**	0.40	0.22
m^{7}GMP-2',3'-diol[c]	**8**	0.78	0.19
GMP-2',3'-diol	**8a**	0.64	0.27

[a] Samples were analyzed by chromatography on cellulose F_{254} (Merck DC-Plastikfolien) in solvent systems A [saturated $(NH_4)_2SO_4$/2-propanol/0.1 M potassium phosphate (pH 7.4) (79:2:19 v/v/v)] and B [1% $(NH_4)_2SO_4$/2-propanol (1:2 v/v)]. Compounds were detected by absorbance under UV light unless otherwise noted. [b] Values taken from Darzynkiewicz et al. (1985). [c] Compounds were detected by fluorescence.

NMR spectra were obtained at ambient temperature and indicated pH. Proton chemical shifts were measured relative to internal 3-(trimethylsilyl)propanesulfonic acid (TSP) sodium salt. Phosphorus chemical shifts were reported relative to 85% H_3PO_4. However, the primary standard was (2-aminoethyl)phosphonic acid in a coaxial capillary. These data were normalized to phosphoric acid ($\Delta\delta$ = 11.39 ppm). Conformational analysis of the cap derivatives was determined as described earlier (Darzynkiewicz et al., 1981, 1985). All spectra used for conformational analyses were simulated by using the standard Nicolet software. UV spectra were obtained with a Zeiss (Jena, GDR) VSU-2P spectrophotometer.

mRNA Isolation. Rabbit reticulocytes were prepared as previously described from phenylhydrazine-treated animals (Tahara et al., 1981). Globin mRNA was purified from salt-washed rabbit reticulocyte ribosomes by using oligo(dT)–cellulose as per the procedure of Krystosek et al. (1975). Aqueous solutions of mRNA were stored routinely at the vapor temperature of liquid nitrogen.

In Vitro Translation. Translation assays were performed in micrococcal nuclease treated rabbit reticulocyte lysate prepared essentially as described (Pelham & Jackson, 1976). Reaction mixtures contained the following in a 0.025-mL volume: 19 mM Hepes (pH 7.5), 114 mM potassium acetate, 0.48 mM magnesium acetate, 0.048 mM each of 19 amino acids (minus Met), 1.9 mM dithiothreitol (DTT), 7.6 mM creatine phosphate, 95 μM spermidine, 25 μM hemin, 100 μg/mL creatine phosphokinase, 9 μCi [^{35}S]methionine (specific activity 1125 Ci/mmol), and 12.4 μL of micrococcal-treated lysate. Globin mRNA (100 ng) was added to each reaction mixture, and incubation was at 30 °C for 15 min. Aliquots (0.015 mL) were taken from each incubation mixture and spotted onto Whatman 3MM filter paper squares. Incorporation of radiolabel into hot trichloroacetic acid (TCA) precipitable material was measured by liquid scintillation spectrometry using a toluene-based scintillation fluid.

RESULTS

Characterization of Cap Analogues. All cap analogues were analyzed by thin-layer chromatography (Table I) and UV spectroscopy (Table II). The putative cap analogues showed

Table II: Ultraviolet Absorption Spectra (in nm) of Cap Analogues

compd	no.	pH 2		pH 7		pH 12	
		λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}	λ_{min}
m^7GMP[a]		257	230	258, 280	236, 271	268	244
m^7GMP-H	**1**	258	230	259, 281	236, 274	267	247
m^7GMP-NH_2	**2**	260	233	259, 278	236, 270	266	246
m^7GMP-CH_3	**3**	260	233	259, 279	238, 271	266	246
$m_2^{7,8}GMP$	**4**	260	233	262	235	267	247
m^78-NH_2GMP	**5**	252, 291	225, 276	253, 287	230, 273	260, 294	239, 274
m^7acycloGMP-CH_3	**6**	257	230	258, 281	229, 271	262	247
$m_2^{7,8}$acycloGMP	**7**	257	225	258	233	264	246
m^7GMP-2′,3′-diol	**8**	257	230	257, 278	234, 270	263	245

[a] Values taken from Hendler et al. (1970); pH 7 values were actually measured at pH 7.4.

Table III: ^1H NMR Chemical Shifts of Cap Analogues (in ppm vs. Internal TSP)

compd	no.	pH	1′	2′	3′	4′	5′	5″	N–CH_3	8-CH_3	8-H	other
m^7GMP[a]		7.0	5.99	4.68	4.37	4.27	3.94	3.83	4.07			
GMP[a]		8.0	5.92	4.79	4.49	4.31	4.00	3.97			8.20	
m^7GMP-H	**1**	2.8	6.06	4.68	4.45	4.38	4.23	4.12	4.10			P–H: 6.78
GMP-H	**1a**	4.3	5.92	4.77	4.47	4.32	4.11	4.10			8.05	P–H: 6.73
m^7GMP-NH_2	**2**	6.9	6.05	4.61	4.44	4.37	4.19	4.08	4.08			
GMP-NH_2	**2a**	5.7	5.91	4.74	4.46	4.32	4.07	4.04			8.08	
m^7GMP-CH_3	**3**	7.3	6.07	4.65	4.45	4.40	4.21	4.10	4.11			P–CH_3: 1.32
GMP-CH_3	**3a**	8.2	5.93	4.76	4.49	4.34	4.08	4.08			8.06	P–CH_3: 1.38
$m_2^{7,8}GMP$	**4**	6.4	6.02	5.08	4.57	4.30	4.10	4.07	4.03			
m^8GMP	**4a**	6.2	5.84	5.13	4.53	4.25	4.12	4.07		2.52		
m^78-NH_2GMP	**5**	6.5	6.08	4.70	4.45	4.38	4.08	4.04	3.69			
8-NH_2GMP	**5a**	6.3	5.91	4.80	4.45	4.30	4.08	4.04				
m^7acycloGMP-CH_3	**6**	6.6	5.69			3.85	3.95	3.95	4.11			P–CH_3: 1.24
acycloGMP-CH_3	**6a**	2.5	5.58			3.79	3.95	3.95			8.23	P–CH_3: 1.23
$m_2^{7,8}$acycloGMP	**7**	6.5	5.69			3.81	3.91	3.91	4.03	2.78		
m^8acycloGMP	**7a**	6.7	5.47			3.86	3.90	3.90		2.49		
m^7GMP-2′,3′-diol	**8**	6.8	6.07	3.93, 4.02	3.87, 3.76	3.90	3.81	3.79	4.12			
GMP-2′,3′-diol	**8a**	6.7	5.86	3.99, 4.02	3.70–3.78	3.87	3.70–3.78				8.06	

[a] Values from Kim and Sarma (1978). Data were recalculated for relative chemical shifts from tetramethylammonium chloride to TSP internal standards by using $\Delta\delta$ of 3.18 ppm.

Table IV: Vicinal Proton Coupling Constants for Selected Analogues (Hz)

compd	no.	pH	J(1′,2′)	J(1′,2″)	J(2′,3′)	J(3′,4′)	J(3″,4′)	J(4′,5′)	J(4′,5″)	J(5′,P)	J(5″,P)	J(4′,P)
m^7GMP[a]		7.0	3.6		4.9	5.1		2.3	2.0	4.5	4.5	1.7
GMP[a]		8.0	6.1		5.2	3.4		4.3	2.9	4.7	4.7	1.1
m^7GMP-H	**1**	2.8	3.6		4.9	5.7		2.6	2.6	6.0	6.9	
GMP-H	**1a**	4.3	5.8		5.2	3.8		3.4	3.4	6.8[b]	6.8[b]	
m^7GMP-NH_2	**2**	6.9	3.5		4.7	6.0		2.6	2.5	4.3	5.5	
GMP-NH_2	**2a**	5.7	5.8		5.2	3.7		3.3	3.3	5.0	5.5	
m^7GMP-CH_3	**3**	7.3	3.6		4.8	5.5		2.6	2.8	4.7	5.8	1.8
GMP-CH_3	**3a**	8.2	5.6		5.2	4.1		3.4[b]	3.4[b]	5.4[b]	5.4[b]	1.3
$m_2^{7,8}GMP$	**4**	6.4	6.2		5.6	3.7		4.0	5.0	5.1	5.2	
m^8GMP	**4a**	6.2	6.7		5.5	3.4		4.9	5.2	5.2	5.6	
m^78-NH_2GMP	**5**	6.5	7.7		5.5	2.1		1.6	2.4	5.0	2.3	
8-NH_2GMP	**5a**	6.3	7.8		5.7	2.4		2.5	2.5	5.2	3.2	
m^7GMP-2′,3′-diol	**8**	6.8	3.9	4.2		3.9	5.0	7.3	4.0	6.1	5.6	
GMP-2′,3′-diol	**8a**	6.7	5.1	5.8		*c*	*c*	*c*	*c*	*c*	*c*	

[a] Values from Kim and Sarma (1978). [b] Averaged values. [c] Values not available due to very tight coupling of spin system.

single spots by TLC in two solvent systems and were fluorescent under UV light as expected for 7-methylated guanine moieties. The only exception was found to be m^78-NH_2GMP (**5**), which did not fluoresce apparently due to the electronic effects of the 8-amino moiety on the guanine base. The latter modification was also found to affect the NMR spectra of these compounds as discussed further below.

Confirmation of Cap Analogue Identity by NMR Spectroscopy. N7-Methylation of guanine nucleotides was confirmed by the appearance of a sharp ^1H signal at 4.03–4.12 ppm (Table III). This was true for all of the cap analogues with the exception of the 8-NH_2 derivative (**5**) for which the N–CH_3 signal was shifted to 3.69 ppm. The H8 signal for the N7-methylated derivatives disappeared because of exchange with D_2O. For most of the compounds, the conformation of the furanose ring was found to differ, as monitored by changes in J(1′,2′) from ~5.5 to ~3.2 Hz (Table IV).

Identity of the 5′-phosphate derivatives (**1–3**) was verified by both ^1H and ^{31}P NMR. Guanosine 5′-phosphite (**1a**) and its 7-methylated derivative (**1**) both gave doublets in their ^1H NMR spectra for P–H at 6.73–6.78 ppm (Table III) with ^1J(H,P) = 640 Hz in the ^{31}P NMR spectra (Table V). For **3** and **3a**, a doublet was observed in the proton NMR spectra for P–CH_3 at 1.23–1.32 ppm (Table III) with $^2J(CH_3,P)$ = 16 Hz in the ^{31}P NMR spectra (Table V). ^{31}P NMR resonance showed a quartet for P–CH_3 at 27.60–27.78 ppm (Table V).

Conformational Properties of 5′-Phosphate Derivatives of m^7GMP. Populations of sugar ring conformers (assuming a typical N ⇌ S equilibrium) and rotamers about the C4′–C5′ and C5′–O5′ bonds were calculated as described earlier (Darzynkiewicz et al., 1981); these data are presented in Table

Table V: ^{31}P NMR of m^7GMP Phosphate Derivatives

compd	no.	pH	chemical shift (ppm)[a]	coupling constant (Hz)
m^7GMP[b]		8.9	3.2	
GMP[b]		8.9	3.3	
m^7GMP-H	**1**	6.6	7.18	$^1J_{H,P}$ = 641 Hz; $^3J_{P,5'H}{}^{av}$ = 6.3 Hz
GMP-H	**1a**	6.8	7.32	$^1J_{H,P}$ = 640 Hz; $^3J_{P,5'H}{}^{av}$ = 6.3 Hz
m^7GMP-NH_2	**2**	6.8	9.98	
GMP-NH_2	**2a**	5.7	10.01	
m^7GMP-CH_3	**3**	6.7	27.60	$^2J_{CH_3,P}$ = 16 Hz
GMP-CH_3	**3a**	6.0	27.67	
m^7acycloGMP-CH_3	**6**	6.8	27.68	
acycloGMP-CH_3	**6a**	6.8	27.78	
m^7GMP-2′,3′-diol	**8**	7.4	3.96	
GMP-2′,3′-diol	**8a**	7.0	3.18	

[a] Chemical shifts were measured relative to (aminoethyl)phosphonic acid external standard and corrected to chemical shift relative to 80% phosphoric acid. [b] Values from Kim and Sarma (1978). Data were corrected to 80% phosphoric acid from trimethyl phosphate internal standard by using a $\Delta\delta$ of 2.4 ppm.

Table VI: Calculated Conformational Parameters

compd	no.	pH	N state (%)	g^+ (%)	g'^+ (%)
m^7GMP-H	**1**	2.8	60	78	62
GMP-H	**1a**	4.3	40	62	58
m^7GMP-NH_2	**2**	6.9	63	79	79
GMP-NH_2	**2a**	5.7	39	64	75
m^7GMP-CH_3	**3**	7.3	58	76	75
GMP-CH_3	**3a**	8.2	43	62	73
$m_2^{7,8}GMP$	**4**	6.4	39	40	76
m^8GMP	**4a**	6.2	36	29	73
m^78-NH_2GMP	**5**	6.5	22	90	93
8-NH_2GMP	**5a**	6.3	25	80	87
8-NH_2AMP[a]		8.2	22	91	90
5′-GMP[b]		0.9	62	79	77
		8.0	36	58	81
m^7GMP[b]		5.0	60	81	80
		7.0	54	87	83

[a] From Evans and Kaplan (1976). [b] From Darzynkiewicz et al. (1981).

VI. Substitution of the 5′-phosphate group did not significantly influence furanose ring conformation. The population of the N state was 39–43% in compounds with a nonalkylated N7 and increased to 58–63% after N7-methylation. Also, populations of the g^+ rotamer were similar to those observed for GMP and m^7GMP. As might be expected, phosphate group conformation was most sensitive to the 5′-P substituents: for both GMP-H (**1a**) and m^7GMP-H (**1**), the g'^+ populations were reduced by 20% in comparison with GMP and m^7GMP. A similar but lesser tendency (ca. 5%) was also observed for GMP-NH_2 (**2a**), m^7GMP-CH_3 (**3**), and GMP-CH_3 (**3a**). However, the 5′-phosphate-derivatized analogues of m^7GMP did not differ significantly from each other in the conformation of the ribose or the 5′-phosphate. Thus any observed differences in biological activity would be due to the replacement of oxygen on phosphate by other atoms or functional groups.

Conformational Properties of 8-Substituted Analogues. (*A*) *Sugar Moiety.* Populations of the N and g^+ states in 8-NH_2GMP (**5a**) and m^8GMP (**4a**) were similar to those previously reported (Lassota et al., 1984). The 8-methyl group did not significantly change the conformation of the sugar ring in m^8GMP (**4a**) compared to that in 5′-GMP, but it did decrease the population of the N state of $m_2^{7,8}GMP$ (**4**) by 15% relative to that of m^7GMP. Similar to GMP and its 7-methylated homologue, there was no difference in the conformation of the sugar ring between m^8GMP and $m_2^{7,8}GMP$. The 8-methyl substituent did reduce the population of the g^+ rotamer of the exocyclic hydroxymethylene group of **4a** by 30% compared to 5′-GMP and by 47% in **4** compared to m^7GMP. The 8-methyl group had little effect on the conformation of the 5′-phosphate groups of **4** and **4a** relative to their corresponding non-8-methylated homologues.

The 8-amino substituent influenced the conformation of the nucleotide in a very different way relative to the 8-methyl analogue. For both 8-NH_2GMP (**5a**) and m^78-NH_2GMP (**5**), the S state was strongly preferred together with the g^+ and g'^+ conformers. **5** has a much more rigid conformation than m^7GMP and a completely different ribose conformation as well. Conformational states similar to those observed for 8-NH_2AMP (Evans & Kaplan, 1976) were also observed for 8-NH_2GMP (**5a**) and m^78-NH_2GMP (**5**). The 8-amino group of 8-NH_2GMP and 8-NH_2AMP stabilizes the S, g^+, g'^+ conformation, perhaps by forming an intramolecular hydrogen bond with the phosphate group. Values shown in Table VI indicated that the 7-methyl group exerted small but consistent changes in the conformation of **5**: the population of the S state increased as well as g'^+. This suggested that the 7-methyl substituent actually facilitated formation of an intramolecular hydrogen bond between the 8-amino and 5′-phosphate moieties.

(*B*) *Conformation about the Glycosidic Bond.* All analyses performed in this study monitored changes in the chemical shift of H2′. Interpretation of chemical shifts in terms of relative populations of conformers was based on earlier, more detailed studies of the syn–anti equilibrium in purine nucleotides (Lassota et al., 1984; Evans & Kaplan, 1976). The chemical shifts of H2′ in m^8GMP (**4a**) and $m_2^{7,8}GMP$ (**4**) were very similar (Table III). On the basis of earlier analyses (Lassota et al., 1984), both compounds were therefore predominantly in the syn conformation (80% syn). 8-NH_2GMP (**5a**) and m^78-NH_2GMP (**5**) also had very similar values of δ(H2′), which were in turn similar to that for GMP. This indicated a similar population of syn and anti states for **5** and **5a** (about 30% syn). Small differences (0.1 ppm) between H2′ proton shifts among the 8-substituted compounds may indicate a small increase in the anti population in the 7-methylated analogue, but it can also be a consequence of the charge redistribution within the purine caused by N7-methylation.

Globin Translation in the Presence of Cap Analogues. Translation in reticulocyte lysate was used as the basis for comparison of the inhibitory activity of the various cap analogues. Lysate was treated with micrococcal nuclease and assayed with exogenous rabbit globin mRNA as translation template. This was done to ensure that only new initiation events were being measured. The cap analogues with 5′-phosphate modifications were analyzed for inhibition of translation in vitro. The relative effectiveness of the analogues as inhibitors can be ascertained by comparing the concentrations required for 50% inhibition of translation (legend to Figure 2). As shown in Figure 2A, m^7GMP-H (**1**; 50% inhibition at >0.4 mM) and m^7GMP-CH_3 (**3**; 50% inhibition at 0.22 mM) were relatively ineffective as inhibitors compared to m^7GMP (50% inhibition at 0.02 mM). Unlike the others, m^7GMP-NH_2 (**2**; 50% inhibition at 0.02 mM) was essentially as effective as m^7GMP. This result was surprising since it was anticipated that the amino moiety would reduce efficient competition of the analogue and native caps for CBPs, similar to the effect exhibited by **3**. Analogue **2** was checked for stability in reticulocyte lysate. Incubation of **2** in reticulocyte lysate for up to 30 min under translation assay conditions

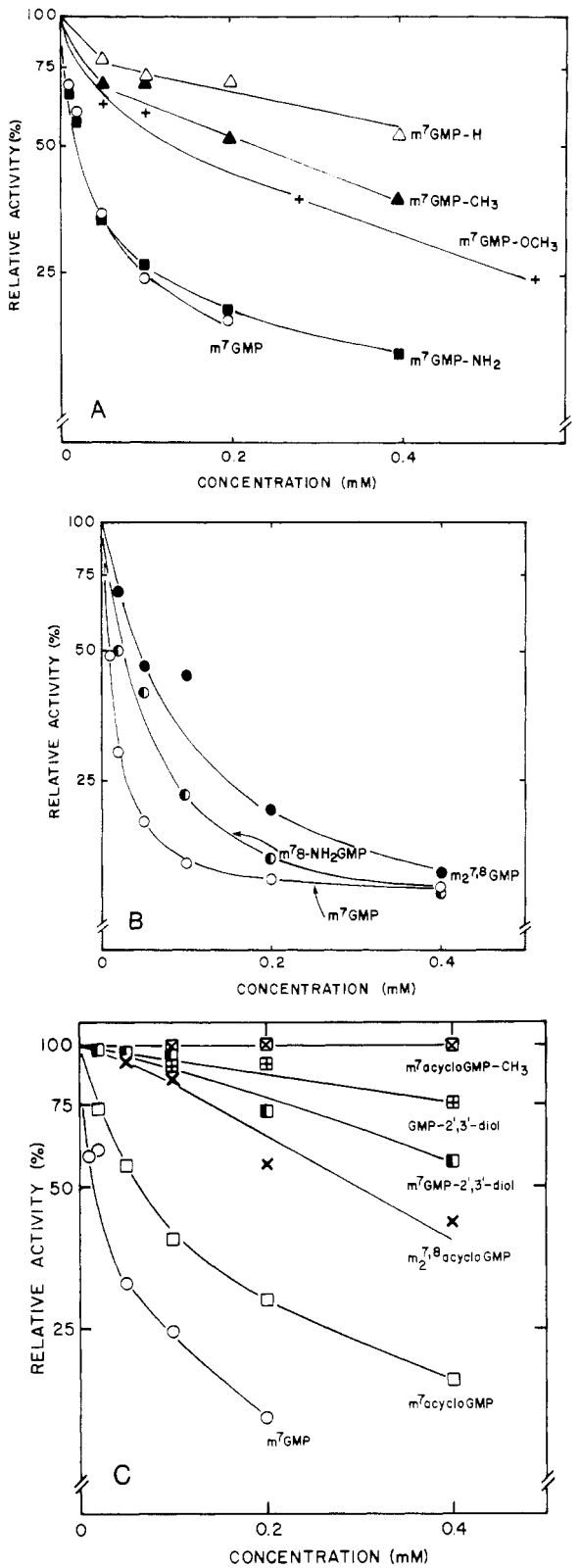

FIGURE 2: Comparison of cap analogues in inhibition of globin translation. Protein synthesis in reticulocyte lysate was assayed in the presence of cap analogues at the indicated concentrations with exogenous globin mRNA as template. Data were normalized relative to [^{35}S]Met incorporation in the absence of analogue. Analogue concentrations resulting in 50% inhibition of translation are given in parentheses. Panel A: m^7GMP (0.02 mM; ○); m^7GMP-H (<0.4 mM; △); m^7GMP-NH_2 (0.02 mM; ■); m^7GMP-CH_3 (0.22 mM; ▲); m^7GMP-OCH_3 (0.14 mM; +). Panel B: m^7GMP (0.015 mM; ○); $m_2^{7,8}GMP$ (0.045 mM; ●); m^78-NH_2GMP (0.025 mM; ◐). Panel C: m^7GMP (0.015 mM; ○); $m_2^{7,8}$acycloGMP (0.3 mM; ×); m^7GMP-2′,3′-diol (>0.4 mM; ◧); GMP-2′,3′-diol (>0.4 mM; ⊞); m^7acycloGMP (0.065 mM; □); m^7acycloGMP-CH_3 (≫0.4 mM; ⊠).

resulted in no degradation as determined by TLC in two solvent systems (data not shown). Thus the inhibitory activity observed was not due to conversion of **2** to m^7GMP or some other inhibitory degradation product. A possibility that must now be considered is that the phosphoramidate moiety of **2** is still able to act as a hydrogen bond acceptor, unlike **1** and **3**. For comparison, m^7GMP *O*-methyl ester was also tested for activity (Figure 2A). This analogue was slightly more inhibitory than **1** and **3**. We previously reported that this modification inactivated m^7GMP as an inhibitor of reovirus mRNA binding to ribosomes (Darzynkiewicz et al., 1981). However, inhibition of globin translation was not tested at that time. The intermediate activity of the *O*-methyl phosphate ester analogue may be accountable in that the methylated oxygen is still able to act as a hydrogen bond acceptor although it does this poorly due to the presence of the methyl moiety. Analogue **2** was compared to m^7GMP as an inhibitor of mRNA–ribosome association. As expected, **2** was found to inhibit mRNA–ribosome association, thus further supporting its activity as a competitive inhibitor of capped mRNA binding to cap binding proteins (data not shown).

8-Substituted m^7GMP analogues were moderately effective as cap analogues compared to m^7GMP (Figure 2B). m^78-NH_2GMP (**5**; 50% inhibition at 0.03 mM) was more effective than $m_2^{7,8}GMP$ (**4**; 50% inhibition at 0.045 mM) as an inhibitor of globin translation. On the basis of our NMR studies, the glycosidic bond of **5** was predominantly (70%) in the anti conformation, whereas **4** was predominantly (80%) syn.

The cap analogue with a ring-opened ribose (**8**) was ineffective as a translation inhibitor (Figure 2C; 50% inhibition at >0.4 mM). The inhibition exhibited by **8** was deemed to be slight since the unmethylated precursor showed some nonspecific inhibition as well (Figure 2C). **6** was totally inactive as an inhibitor. The inhibitory activity of **8** was similar to the trend shown by **4**; the addition of 8-methyl to m^7GMP or to m^7acycloGMP further decreased the activity of the analogue.

Discussion

The 5′-cap of eukaryotic mRNAs is a unique structure. The major features that distinguish it from the remainder of the polyribonucleotide chain include the following: (1) the N7-methyl moiety; (2) the 5′-to-5′ triphosphate linkage between ribose moieties; and (3) the presence of additional base and ribose modifications in the penultimate and antepenultimate nucleotide units (Banerjee, 1980). Our efforts have been directed toward understanding the recognition of structure by cap binding proteins for the $m^7G(5')ppp$ portion of native caps. The analogues that we synthesized were designed to test the relative weightings of the base, ribose, and phosphate moieties in terms of their importance for recognition and binding by CBPs.

We have tested the α-phosphate and the ribose moieties for their relative contributions to activity. O-Methylation of the β-phosphate of m^7GDP or the γ-phosphate of m^7GTP does not diminish inhibitory activity of these compounds (Darzynkiewicz et al., 1985), whereas *O*-methylation of the α-phosphate of m^7GMP reduces its activity as a cap analogue (Darzynkiewicz et al., 1981). We concluded that, as a minimum, two dissociable hydroxyls of m^7GMP and the di- and triphosphate analogues were required for binding to CBPs. For inhibition of translation by m^7GMP we inferred that an intact α-phosphate contributed significantly to binding. This was more clearly shown with m^7acycloGMP analogues. 7-Methyl-acycloGMP was about 4-fold less active than m^7GMP on the basis of concentration required for 50% inhibition of

translation (0.065 vs. 0.015 mM). Similarly, **4** was more inhibitory than **7** (50% inhibition at 0.045 and 0.3 mM, respectively). Thus replacement of ribose by (hydroxyethoxy)methyl ether diminished but did not totally negate the activity of m^7acycloGMP as a cap analogue. However, methyl esterification of the 5′-phosphate of m^7acycloGMP renders the analogue noninhibitory to mRNA–ribosome binding (Darzynkiewicz et al., 1985) as did replacement of oxygen by $-CH_3$ for translation (this study, **6**). We conclude that a functionally intact α-phosphate is more important than an intact ribose for binding to CBPs. This statement is further modified in light of the results obtained with the derivatized phosphate analogs.

Replacement of one oxygen of the phosphate moiety of m^7GMP with –H (**1**) or $-CH_3$ (**3**) greatly decreased the activity of the compound as an inhibitor (50% inhibition of translation at >0.4 and 0.22 mM, respectively). These results supported the earlier hypothesis requiring at least two dissociable hydroxyls. By contrast, results obtained with the 5′-phosphoramidate of m^7GMP (**2**) required modification of the hypothesis. For in vitro translation, **2** was as effective as m^7GMP as an inhibitor. The chemical form of the phosphoramidate moiety, under physiological conditions, is not apparent since several canonical resonance forms are possible. However, because of its ability to inhibit translation, it must resemble m^7GMP chemically. A titration of **2** by ^{31}P NMR indicated that there was no p*K* between pH 3 and pH 12 (I. Ekiel and E. Darzynkiewicz, unpublished results). Therefore, at neutral pH the amino group is unprotonated and the 5′-phosphoramidate moiety will have but a single negative charge. This does not fit the earlier hypothesis requiring a minimum of two dissociable hydroxyls. Our explanation for the inhibitory activity of **2** is that there is need for a hydrogen bond acceptor in the α-phosphate position of the nucleotide. This would actually correspond to the oxygen in the phosphoanhydride bond linking the α- and β-phosphates of the native cap structure. As a corollary, we predict a hydrogen bond donor must be present within the nucleotide binding site of CBPs. The activity of m^7GMP-OCH_3 (Figure 2A; 50% inhibition at 0.14 mM) tends to support this, since $-OCH_3$ is a possible hydrogen bond acceptor. It also cannot be excluded that a metal ion is complexed to the phosphate chain of the cap structure; an analogue might be rendered inactive by the inability to form a metal–nucleotide complex. A more conclusive test of this hypothesis will await synthesis and assay of m^7GTP and m^7GDP analogue derivatized at the α-phosphate and with substitutions of the oxygen between the α- and β-phosphates.

The observation that m^7acycloGMP was moderately active as an inhibitor led us to believe this would be true with the 2′,3′-diol derivative (**8**) as well. By analogy, periodate oxidation of capped mRNAs, which causes ribose ring opening to the 2′,3′-dialdehyde at the 5′-cap, does not inactivate the cap. Oxidized mRNAs are still 69–80% as effective as translation templates when compared to intact capped mRNAs (Muthukrishnan et al., 1976). Thus this modification does not significantly affect the ability of cap binding proteins to interact with oxidized mRNA. The failure of **8** to act as an inhibitor was therefore somewhat surprising. Instead, these results indicated other conformational aspects of **8** must be taken into account in order to explain its lack of inhibitory activity.

Conceptually, the absence of a C2′–C3′ bond should allow greater degrees of freedom for the hydroxyl moieties of **8**. Thus the analogue could assume a conformation that mimicked the intact nucleotide. Conversely, and apparently more likely, the analogue could also assume a stable conformation unlike the intact nucleotide. As a result, the relative positions of the phosphate, base, and hydroxyl groups of **8** could be quite different from those of intact m^7GMP. The latter possibility was supported by studies which indicated that the 3′-CH_2OH and the 5′-CH_2OP moieties were not as hindered in rotation about the C3′–C4′ and C4′–C5′ bonds as compared to ribose (I. Ekiel and E. Darzynkiewicz, unpublished results). With respect to the 3′-CH_2OH moiety, all three rotamers were found to be present in significant populations (g^+ was 41%); however, for 5′-CH_2OP the population of g^+ was only 17%, indicating that either the g^- or *t* rotamer was significantly preferred over the other. It was not possible to determine the predominant conformation due to uncertainty in assigning the multiplets in the ^{1}H NMR spectra. Other NMR studies in progress suggest that the C1′–O and O–C4′ bonds of **8** differ from those of the intact nucleotide. **8** apparently has an extended structure in solution unlike the structure of the intact ribose of m^7GMP (R. Stolarski and E. Darzynkiewicz, unpublished results). Therefore, we must conclude that **8** is inactive as a competitive inhibitor of translation due to an inappropriate conformation.

Our results indicated that glycosidic bond conformation affected the ability of cap analogues to bind to cap binding proteins. From the conformational analysis of analogues **4** and **5** we know that 80% of $m_2^{7,8}$GMP (**4**) exists as the syn conformer, whereas for $m^7$8-NH_2GMP (**5**), 30% of the population is syn. Inhibition could be due to this difference in syn–anti conformation; however, other factors such as hydrophobicity of the methyl and amino moieties must also be considered since the latter may be more readily hydrated than the former. It is clear from the spectroscopy data that the 8-NH_2 group has different electronic effects on the purine ring as compared to the 8-CH_3 derivative. The consequences of this on nucleotide binding by CBPs are not known. Other aspects of conformation may be significant; for example, $m_2^{7,8}$GMP is 40% in the g^+ form whereas the 8-amino-substituted analogue is 90% in the g^+ state. These data are generally consistent with the idea that the cap structure binds preferentially in the anti conformation (Hickey et al., 1977).

The activity of analogues **4**, **5**, and **7** indicated that substituents added to the imidazole portion of the base do not totally prevent binding of analogues to CBPs. As further evidence, synthetic analogues containing N7-ethyl (Furuichi et al., 1979), -benzyl, or -carboxymethyl (Adams et al., 1978) are also active as inhibitors. The delocalized positive charge on the purine ring is the most important feature of the base, not necessarily the nature of the N7 substituent. The purine binding domains of CBPs must be therefore relatively large and able to accommodate bulky substituents, and/or the N7 and C8 positions of the purine are oriented toward the solvent phase. It is thus likely that CBPs interact with the delocalized positive charge on the imidazole portion from one or both sides of the plane of the purine ring. However, preliminary results obtained by us indicate that there is a size threshold for the N7 substituent for binding to cap binding proteins, contrary to results published earlier (E. Darzynkiewicz and S. M. Tahara, unpublished results). Until it is rigorously proven that the size of the N7 substituent is important, we must conclude that these positions play a minor role in determining contact points between ligand and protein.

The slight inhibitory activity of **7** (50% inhibition at 0.3 mM) further supports the notion that the ribose is secondarily important for inhibitory activity of cap analogues (compare activities of **4** vs. **7**, Figure 2). 8-Methyl addition to m^7G

destabilizes binding of both **4** and **7** to CBPs. Due to higher flexibility of the (hydroxyethoxy)methyl ether moiety, the effect of the 8-methyl group may not be to shift "glycosidic bond" conformation in **7** as in **4**. However, it is not apparent if the same phenomenon is operative for decreasing the activity of these analogues compared with the non-8-methylated counterparts of **4** and **7**.

A comparison of X-ray diffraction structural data for guanine nucleotide binding proteins indicates that there is conservation of the nucleotide binding site of bacterial EF-Tu with other eukaryotic GTP/GDP binding proteins (Bourne, 1986; McCormick et al., 1985). The similarity of the m^7G binding region of CBPs to other guanine nucleotide binding proteins has yet to be demonstrated; however, this comparison is a useful starting point. For bacterial EF-Tu, GDP is specifically bound by a combination of electrostatic and hydrogen-bonding interactions (Jurnak, 1985). Several key interactions appear to be conserved. The 2-amino and 6-keto moieites of GDP interact with aspartyl and asparaginyl residues, respectively, on the binding proteins. The importance of this "edge" of the purine ring in binding is consistent with observations that the size and nature of C8 substituents appear to have minor steric effects on ligand binding to CBPs, as discussed above. With eukaryotic EF-2, modification of the ribose moiety of GTP leads to a reduction in affinity for the ligand, although stable protein–GTP complexes still form. This is similar to our observations that ribose modifications do not greatly affect the activity of cap analogues (Darzynkiewicz et al., 1985). Therefore, on the basis of data obtained to date it appears that cap binding proteins generally possess ligand binding requirements similar to those of other guanine nucleotide binding proteins, but these requirements have been further modified to accommodate the unique structure of the 5′-cap of eukaryotic mRNA.

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Registry No. **1**, 108592-28-5; **1**·Na, 108592-47-8; **1a**, 106549-73-9; **1a**·Na, 108592-35-4; **2**, 108592-29-6; **2**·Na, 108592-48-9; **2a**, 15483-93-9; **2a**·Na, 108592-36-5; **3**, 108592-30-9; **3**·Na, 108592-49-0; **3a**, 29740-94-1; **3a**·Na, 108592-37-6; **4**, 72051-09-3; **4**·2Na, 108592-50-3; **4a**, 39023-79-5; **5**, 108592-31-0; **5**·2Na, 108592-51-4; **5a**, 57460-97-6; **6**, 108592-32-1; **6**·Na, 108592-52-5; **6a**, 108592-38-7; **6a**·Na, 108592-43-4; **6a**·TEA, 108592-44-5; **7**, 108592-33-2; **7**·2Na, 108592-53-6; **7a**·TEA, 108592-40-1; **8**, 108592-34-3; **8**·2Na, 108592-34-3; **8a**, 52114-73-5; **8a**·2Na, 108592-45-6; **8a**·TEA, 108592-46-7; GMP·2Na, 5550-12-9; m^7GMP, 10162-58-0; GMP, 85-32-5; 2′,3′-*O*-isopropylideneguanosine 5′-phosphite, 108592-41-2; 2′,3′-*O*-isopropylideneguanosine, 362-76-5; guanosine, 118-00-3; methylphosphonic acid, 993-13-5; 2′,3′-*O*-isopropylideneguanosine 5′-methylphosphonate, 108592-42-3; acycloguanosine, 59277-89-3; 8-methylacycloguanosine, 91897-97-1.

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