Chemical Synthesis and Characterization of 7-Methylguanosine Cap Analogues†

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ABSTRACT: Chemically synthesized nucleotide derivatives of 7-methylguanosine and guanosine were characterized with respect to their structural properties and functional effects on eukaryotic translation initiation. Derivatives of 7-methylguanosine 5′-phosphate (m^7GMP) that were modified in the ribose moiety by 2′-O- or 3′-O-methylation or by conversion to the 2′-deoxy or arabinosyl form retained cap analogue activity as determined by inhibition of reovirus mRNA binding to wheat germ ribosomes or chemical cross-linking of cap binding protein to the 5′-end of oxidized mRNA. Both reactions were decreased by 50% or more at a derivative concentration of 0.2 mM. The acyclo form of m^7GMP also had considerable inhibitory activity, consistent with recognition of elements of the cap other than the sugar ring during initiation complex formation. Analyses done with the methyl ester of acyclo m^7GMP, which is relatively inactive as a cap analogue, and the β-ester of m^7GDP and γ-ester of m^7GTP, which are both active, confirmed the importance for cap recognition of two dissociable hydroxyls on the phosphate group(s) adjacent to the positively charged m^7G.

Many different kinds of experiments have demonstrated that the 5′-terminal "cap", m^7G(5′)ppp(5′)N, influences eukaryotic mRNA maturation (Krainer et al., 1984), stability (Furuichi et al., 1977), and function (Shatkin, 1976). In particular, binding of mRNA to ribosomal small subunits during initiation of protein synthesis is enhanced by the presence of the cap. This effect on translation is apparently mediated by protein factors including cap binding proteins that promote attachment of ribosomes to the 5′-end of mRNA (Sonenberg et al., 1980; Grifo et al., 1983; Kozak, 1983).

One experimental approach that has provided some insight into the relationship between mRNA 5′-terminal structure and translational function has been the use of guanosine derivatives as cap analogues. For example, on the basis of NMR spectral analyses of 7-methylguanosine 5′-phosphate (m^7GMP),[1] it was proposed that the cap in mRNA adopts a rigid conformation that facilitates initiation of translation (Hickey et al., 1977). The importance of N7 substitution, but not cap methylation per se for initiation, was indicated by the finding that 7-methyl-, 7-ethyl-, and 7-benzylguanosine 5′-diphosphates all block attachment of wheat germ ribosomes to capped reovirus mRNA equally well and to the extent of ~80% at 0.1 mM (Adams et al., 1978).

Presumably, cap analogues inhibit protein synthesis by competing with the capped 5′-end of mRNA for the site(s) essential for initiation. Although the exact nature and location of these putative functional sites remain to be determined, it was observed previously that m^7GMP inhibits both attachment of purified cap binding protein to mRNA and the formation of 80S initiation complexes. Neither process was affected by the methyl ester of m^7GMP (Darzynkiewicz et al., 1981). Because the rigid conformation of m^7GMP was retained after methyl esterification, it is likely that other structural features of the cap in addition to a preferred conformation of m^7G are recognized during protein synthesis initiation. In an attempt to define them more clearly, we have synthesized and characterized a new series of cap analogues consisting of 7-methylguanosine nucleotides with modified sugar moieties and phosphate groups.

Experimental Procedures

Materials. Guanosine, 7-methylguanosine, guanosine 5′-phosphate, guanosine 5′-diphosphate, 2′-*O*-methylguanosine 5′-phosphate, 3′-*O*-methylguanosine 5′-phosphate, 7-methylguanosine 5′-phosphate, 7-methylguanosine 5′-diphosphate, and 7-methylguanosine 5′-triphosphate were all purchased from P-L Biochemicals. Guanosine 5′-triphosphate and 2′-deoxyguanosine 5′-phosphate were from Sigma Chemical Co. and Pharma-Waldhof GmbH, respectively. 9-(β-D-Arabinofuranosyl)guanine 5′-monophosphate was obtained from Calbiochem. 9-[(2-Hydroxyethoxy)methyl]guanine(acycloG) was the gift of Dr. G. B. Elion of Burroughs-Wellcome. *N*,*N*′-Dicyclohexylcarbodiimide (DCC) was purchased from Aldrich. Dimethyl sulfoxide (Me_2SO) and phosphoryl chloride were from Merck. Trimethyl phosphate was a product of Fluka, and methyl iodide was from POCH-Poland. Whatman DE-32 cellulose was precycled before equilibration as described by the manufacturer (Leaflet IL2).

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[1] Abbreviations: m^7G, 7-methylguanosine; m^7GMP, 7-methylguanosine 5′-phosphate; m^7dGMP, 7-methyl-2′-deoxyguanosine 5′-phosphate; $m_2^{2,7}$GMP, 7-methyl-2′-*O*-methylguanosine 5′-phosphate; $m_2^{3',7}$GMP, 7-methyl-3′-*O*-methylguanosine 5′-phosphate; m^7araGMP, 7-methyl-9-(β-D-arabinofuranosyl)guanine 5′-phosphate; acycloG, 9-[(2-hydroxyethoxy)methyl]guanine; m^7acycloGMP, 7-methyl-9-[(2-hydroxyethoxy)methyl]guanine 5′-phosphate; m^7acycloGMPme, methyl 7-methyl-9-[(2-hydroxyethoxy)methyl]-5′-guanyl phosphate; m^7GDPβme, P^2-methyl P^1-(7-methyl-5′-guanosyl) diphosphate; m^7GTPγme, P^3-methyl P^1-(7-methyl-5′-guanosyl) triphosphate; DCC, dicyclohexylcarbodiimide; TEAB, triethylammonium bicarbonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

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Synthesis of Cap Analogues. (*A*) *7-Methyl-2′-deoxyguanosine 5′-Phosphate* (*m^7dGMP*; **1**), *7-Methyl-2′-O-methylguanosine 5′-Phosphate* (*$m^{2'}_2{}^7$GMP*; **2**), *and 7-Methyl-3′-O-methylguanosine 5′-Phosphate* (*$m^{3'}_2{}^7$GMP*; **3**). These three compounds were prepared by the method of Hendler et al. (1970). Other methylguanosine analogues were obtained by methylation of the appropriate nucleotides according to procedures described by Adams et al. (1978) with some minor modifications. All the compounds synthesized were identified and characterized by paper chromatography, UV absorption and fluorescence spectra, and NMR spectroscopy.

(*B*) *7-Methyl-9-(β-D-arabinofuranosyl)guanine 5′-Phosphate* (*m^7araGMP*; **4**). araGMP monoammonium salt dihydrate (5 mg, 0.012 mmol) was dissolved in 0.3 mL of Me_2SO. Methyl iodide was added (50 μL, 0.8 mmol), and the solution was stirred at room temperature for 3 h before addition of 2 mL of cold acetone. The resulting suspension was kept at 0–4 °C overnight and then centrifuged. The pelleted solid was dissolved in 2 mL of H_2O and applied to DE-32 cellulose (HCO_3^- form, 33 × 2 cm column). The column was washed with H_2O (570 mL), and a gradient of NH_4HCO_3 was applied (0–0.2 M, 0.5 L each). To avoid decomposition, all chromatography procedures were at 3–5 °C. Fractions of 15-mL were collected at a rate of 120 mL/h; the same size column, fraction volume, and flow rate were used for purification of other derivatives. Fractions 46–51 were pooled (30 A_{256} units) and desalted by repeated evaporation under reduced pressure (bath temperature 25 °C) to obtain 1.3 mg (0.003 mmol) of solid m^7araGMP ammonium salt (**4**), yield 25%.

(*C*) *7-Methyl-9-[(2-hydroxyethoxy)methyl]guanine 5′-Phosphate* (*m^7acycloGMP*; **5**). 9-[(2-hydroxyethoxy)-methyl]guanine 5′-phosphate was obtained from acycloG by a published procedure (Pal et al., 1978). AcycloG (266.7 mg, 1.18 mmol) was added to a solution of freshly distilled phosphoryl chloride (0.4 mL, 4.4 mmol) in trimethyl phosphate (8 mL), and the mixture was stirred at 0 °C for 1.5 h. To monitor the reaction, samples were removed and analyzed by TLC on cellulose F in 2-propanol–concentrated NH_4OH–H_2O, 7:1:2 (v/v/v); the phosphorylated product remained at the origin in this solvent system. To stop the reaction, the mixture was neutralized by addition of cold 5% $NaHCO_3$ and diluted to 60 mL with H_2O. Samples were applied to DEAE-Sephadex A-25 (HCO_3^- form, 33 × 2.5 cm column). After being washed with H_2O (225 mL), samples were eluted with a linear gradient (0.005–0.6 M) of triethylammonium bicarbonate (TEAB, pH 7.6, 1.5 L each). Fractions of 17 mL collected at a flow rate of 130 mL/h were typical conditions for this column in all separations. Fractions 87–99 were pooled (8360 A_{256} units), desalted by evaporation to dryness in vacuo with several additions of ethanol, and dried over P_2O_5, affording 446 mg (0.88 mmol) of acycloGMP triethylammonium salt (75% yield). AcycloGMP triethylammonium salt (100 mL, 0.2 mmol) was converted to the ammonium salt (61 mg, 0.18 mmol) by cation exchange on Dowex 50W-X8 (NH_4^+ form).

m^7acycloGMP (**5**) was synthesized by dissolving acycloGMP NH_4^+ salt (32.7 mg, 0.096 mmol) in 0.4 mL of Me_2SO and adding 75 μL (1.2 mmol) of methyl iodide. The reaction mixture was stirred for 12 h at room temperature, treated with 2 mL of acetone, left at −12 °C overnight, and centrifuged. The solid was dissolved in 2 mL of H_2O and applied to DE-32 cellulose (HCO_3^- form). The column was washed with water (150 mL), and a linear gradient of NH_4HCO_3 was applied (0–0.25 M, 1 L each). Peak fractions (36–41) were pooled, desalted, and acetone precipitated as for m^7araGMP to obtain the ammonium salt of m^7acycloGMP (**5**; 7.3 mg, 0.02 mmol, 20.8% yield).

(*D*) *Methyl 7-Methyl-9-[(2-hydroxyethoxy)methyl]-5′-guanyl Phosphate* (*m^7acycloGMPme*; **6**). AcycloGMP methyl ester (acycloGMPme) was prepared by reaction of acycloGMP with methanol in the presence of dicyclohexylcarbodiimide (Khorana, 1959), exactly as for the preparation of GMP methyl ester (Darzynkiewicz et al., 1981). AcycloGMP triethylammonium salt (340 mg, 0.67 mmol) was dissolved in 26 mL of distilled methanol, and DCC (1 g, 4.85 mmol) was added. After 10 h at 37 °C the reaction mixture was poured into 80 mL of cold H_2O, and the cyclourea that precipitated during 16–18 h at 4 °C was removed by filtration. The filtrate was evaporated to about 40 mL and applied to DEAE-Sephadex A-25 (HCO_3^- form). The column was washed with 750 mL of H_2O and eluted with a linear gradient (0–0.5 M) of TEAB (pH 7.6, 1.5 L each). A sharp peak (fractions 65–73; 6800 A_{256} units) was pooled and desalted by evaporation with several additions of ethanol. The triethylammonium salt of acycloGMPme was converted to the sodium salt by passage through a column (13.5 × 1.3 cm) of Dowex 50W-X8, 100/200 mesh (Na^+ form). The effluent and wash were concentrated by evaporation and drying over P_2O_5; 193 mg (0.57 mmol) of acycloGMPme sodium salt was obtained (85% yield).

A solution of 49.1 mg (0.14 mmol) of acycloGMPme sodium salt in 1.4 mL of Me_2SO was treated with 0.2 mL (3.2 mmol) of methyl iodide. The reaction mixture was stirred for 2.5 h at room temperature, precipitated with 5 mL of cold acetone, and centrifuged. The solid was dissolved in 4 mL of H_2O (1328 A_{256} units) and applied to DE-32 cellulose (HCO_3^- form). Elution was with 450 mL of H_2O followed by a linear gradient of 0–0.1 M TEAB (pH 7.4, 1 L each). Fractions 28–35 (729 A_{256} units) were pooled, desalted by repeated evaporations with ethanol, and loaded in ~40 mL of aqueous solution onto a column of Dowex 50W-X8, 100/200 mesh (Na^+ form). The combined effluent and wash were concentrated to 3 mL and precipitated with cold ethanol to obtain 29.2 mg (0.082 mmol) of the sodium salt of m^7acycloGMPme (**6**; 59% yield).

(*E*) *P^2-Methyl P^1-(7-Methyl-5′-guanosyl) Diphosphate* (*m^7GDPβme*; **7**). β-Methyl ester of GDP (GDPβme) was prepared as described in the preceding section for acycloGMPme. Guanosine 5′-diphosphate dihydrate sodium salt (210.5 mg, 0.42 mmol) was converted to its triethylammonium salt by passage through Dowex 50W-X8, 100/200 mesh ($TEA-H^+$ form). Effluent and wash were evaporated to dryness, and the dry solid was dissolved in 30 mL of distilled methanol. DCC (1.5 g, 7.27 mmol) was added, and the reaction was allowed to proceed at 37 °C for 2.5 h before the mixture was poured into 100 mL of cold H_2O and kept overnight in the cold. The precipitated cyclourea was removed by filtration, and the filtrate was condensed to about 40 mL and loaded onto DEAE-Sephadex A-25 (HCO_3^- form). After being washed with 525 mL of H_2O, a linear gradient (0–0.6 M) of TEAB (pH 7.6, 1 L each) was applied. A sharp peak (fractions 77–85, 3780 A_{256} units) was pooled, desalted by evaporation, and converted to GDPβme sodium salt by chromatography on Dowex 50W-X8, 100/200 mesh (Na^+ form). A final precipitation with ethanol gave 141.4 mg (0.29 mmol) of GDPβme sodium salt (68% yield).

GDPβme sodium salt (64.5 mg, 0.13 mmol) was dissolved in 0.4 mL of H_2O and diluted to 10 mL with Me_2SO. Methyl iodide (2 mL, 32 mmol) was added, and the solution was

stirred for 2 h at room temperature. The reaction mixture was then diluted with 150 mL of H_2O and extracted twice with 40-mL portions of ethyl ether. The aqueous phase was concentrated by evaporation to 30 mL and applied to DEAE-32 (HCO_3^- form). The column was washed with 450 mL of H_2O and eluted with a linear gradient (0–0.15 M) of TEAB (pH 7.4, 1 L each). Fractions 59–68 were pooled (412 A_{256} units), desalted by evaporation with ethanol, and converted to the sodium salt as above. Precipitation with ethanol gave 30.2 mg (0.06 mmol) of sodium salt of m^{7}GDPβme (**7**) as a white powder (46% yield).

*(F) P^3-Methyl P^1-(7-Methyl-5′-guanosyl) Triphosphate (m^{7}GTPγme; **8**).* γ-Methyl ester of GTP (GTPγme) was prepared in the same manner as GDPβme. Anhydrous GTP disodium salt (225 mg, 0.4 mmol) was converted to its triethylammonium salt and condensed with 50 mL of distilled methanol in the presence of 2.5 g DCC (12.1 mmol) at 37 °C for 2 h. After removing cyclourea as described above, purification was by column chromatography on DEAE-Sephadex A-25 (HCO_3^- form) with a 695-mL H_2O wash followed by a linear gradient of 0–0.8 M TEAB (pH 7.4, 1.5 L each). Fractions 107–120 (2773 A_{256} units), after desalting, were passed through Dowex 50W-X8, 100/200 mesh (Na^+ form), and precipitated with ethanol to obtain 135.3 mg (0.24 mmol) of GTPγme sodium salt (60% yield).

GTPγme sodium salt (51.2 mg, 0.092 mmol) dissolved in 0.25 mL of H_2O was methylated at the N7 position by stirring with 1.5 mL (24 mmol) of methyl iodide and 8 mL of Me_2SO for 10 h at room temperature. The reaction mixture was diluted with 150 mL of H_2O and extracted twice with 60-mL portions of ether. The aqueous phase was evaporated to 40 mL and applied to DE-32 (HCO_3^- form). The column was washed with H_2O (495 mL) and eluted with a gradient (0–0.25 M) of TEAB (pH 7.4, 1.5 L each). Fractions 95–103 were pooled (250 A_{256} units), desalted, and, as for **7**, converted to the sodium salt of m^{7}GTPγme (**8**; 14.1 mg, 0.025 mmol, 27% yield).

NMR Spectroscopy. For ^{31}P NMR spectra, samples were dissolved in 1.2 mL of D_2O (3–15 mM) and analyzed in 10-mm tubes with sodium 3-(trimethylsilyl)propionate as an internal standard. A volume of 0.5 mL containing 10–30 mM sample (3 mM for m^{7}araGMP) and 5-mm tubes were used for ^{1}H NMR. All spectra were recorded at ambient temperature (28–30 °C). pH was determined before and after each measurement, and the average is given in the tables. ^{1}H NMR spectra were recorded on Bruker WP80 and Bruker 270 HX instruments; for ^{31}P NMR, a Bruker CXP-300 spectrometer was used (121.5 MHz). Conditions were ~45° pulses with 11–14-μs 90° pulses and 2-s recycling time.

UV Spectroscopy. Absorption spectra were obtained in a Zeiss (Jena, GDR) VSV-2P instrument. Fluorescence spectra were determined in an Aminco-Bowman Model SPF spectrofluorometer fitted with Hanovia 901 cXe lamp and Hamamatsu 1P 28 photomultiplier.

Ribosome Binding. Reovirus mRNA radiolabeled in the 5′-terminal m^{7}GpppGm cap at a specific activity of (1.3–2.6) $\times 10^5$ cpm/μg was synthesized by incubating viral cores in a transcription reaction mixture containing [^{3}H]methyl-*S*-adenosylmethionine (Amersham, sp act. 62 or 73 Ci/mmol). Wheat germ S_{23} translation extract was incubated with 20 000–50 000 cpm of purified viral mRNA under conditions of 80S initiation complex formation, i.e., in the presence of 0.16 mM sparsomycin. Samples were assayed by glycerol gradient centrifugation, and the extent of ribosome binding to mRNA was estimated on the basis of the proportion of the total recovered radioactivity that sedimented in the 80S position. In the absence of cap analogue inhibitors this value was in the range of 52–80%. The details of these procedures have been reported previously (Muthukrishnan et al., 1976; Darzynkiewicz et al., 1981).

Table I: R_f Values of 7-Methylguanosine and Guanine Derivatives[a]

compd	no.	A	B
m^{7}G*		0.64	0.27
GMP		0.53	0.28
m^{7}GMP*		0.70	0.18
dGMP	**1a**	0.45	0.33
m^{7}dGMP*	**1**	0.63	0.24
m$^{2'}$GMP	**2a**	0.51	0.32
m$_2^{2',7}$GMP*	**2**	0.58	0.25
m$^{3'}$GMP	**3a**	0.45	0.30
m$_2^{3',7}$GMP*	**3**	0.62	0.25
araGMP	**4a**	0.53	0.28
m^{7}araGMP*	**4**	0.69	0.20
acycloGMP	**5a**	0.52	0.26
m^{7}acycloGMP*	**5**	0.79	0.22
acycloGMPme	**6a**	0.40	0.46
m^{7}acycloGMPme*	**6**	0.67	0.38
GMPme		0.36	0.41
m^{7}GMPme*		0.59	0.32
GDP		0.57	0.12
GDPβme	**7a**	0.47	0.26
m^{7}GDP*		0.76	0.09
m^{7}GDPβme*	**7**	0.66	0.23
GTP		0.65	0.07
m^{7}GTP*		0.82	0.05
GTPγme	**8a**	0.56	0.18
m^{7}GTPγme*	**8**	0.73	0.15

[a] Samples were analyzed by chromatography on Whatman 3MM paper in solvent systems A [saturated $(NH_4)_2SO_4$ solution/2-propanol/0.1 M potassium phosphate buffer, pH 7.4 (79:2:19 v/v/v)] and B [1% $(NH_4)_2SO_4$ solution/2-propanol (1:2 v/v)]. Compounds were detected in both systems by fluorescence (*) or absorbance under UV light.

Chemical Cross-Linking of Cap Binding Protein to Oxidized mRNA. Reovirus mRNA containing [^{3}H]methyl-labeled, 5′-terminal m^{7}GpppGm was periodate oxidized and repurified by gel filtration (Sonenberg et al., 1978). Rabbit reticulocyte initiation factor eIF-4F, which contains the 24 000-dalton cap binding protein, was purified as described (Grifo et al., 1983). Reaction mixtures (25 μL) consisting of 20 mM Hepes buffer, pH 7.5, 1 mM magnesium acetate, 1 mM ATP, 1 mM dithiothreitol, 0.1 M KCl, 0.2 μg of oxidized mRNA (57 000 cpm), 1.6 μg of eIF-4F, and the indicated cap analogue were incubated at 30 °C for 10 min. Incubation mixtures were chilled and, after addition of $NaBH_3CN$ to a final concentration of 20 mM, kept on ice for 12 h. Samples were then digested for 30 min at 37 °C with RNase A (5 mg/mL) and RNase T1 (250 units/mL) before analysis by electrophoresis in 10% polyacrylamide–SDS gels (Grifo et al., 1983). Proteins that were radiolabeled by cross-linking of ^{3}H-labeled mRNA 5′-fragment were detected by fluorography (3-day exposure). For quantitation, X-ray films were traced in a Cliniscan densitometer.

Results

Characterization of Derivatives. Compounds were analyzed by several procedures including paper chromatography in two different solvents–a high ionic strength, aqueous system (A) and a low salt, hydrophobic mixture (B). The fluorescent 7-methylguanosine derivatives migrated faster in system A and slower in system B than the corresponding non-7-substituted parental structures (Table I).

Ultraviolet absorption spectra of the 7-methylguanosine derivatives shown diagrammatically in Figure 1 were determined in acidic, neutral, and basic solutions. Maximum and

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

cap analogue no.	compd	pH 2		pH 7		pH 12	
		λ_{max}	λ_{min}	λ_{max}	λ_{min}	λ_{max}	λ_{min}
1	m^7dGMP	256	230	255, 280	234, 271	269	243
2	$m_2^{2',7}$GMP	259	230	259	236	267	243
3	$m_2^{3',7}$GMP	257	230	257	234	267	243
4	m^7araGMP	259	232	260, 274	235	265	243
5	m^7acycloGMP	257	229	257, 280	235, 273	268	243, 263
6	m^7acycloGMPme	257	229	257, 281	237, 272	266	244
7	m^7GDPβme	258	233	258	236	267	244
8	m^7GTPγme	258	231	257	235	268	244

Table III: ^{1}H NMR Chemical Shifts and Coupling Constants of Guanine Derivatives[a]

compd	no.	pH	δ (H-8)	δ (H-1′)	J(1′,2′)	δ (O-CH_3)	^3J(P,CH_3)	δ (N-CH_3)
m^7dGMP	**1**	5.6	8.02[b]	6.42	5.5			4.101
$m^{2'}$GMP	**2a**	5.0	8.20	5.985	5.5	3.40		
$m_2^{2',7}$GMP	**2**	4.5		6.16	3.5	3.595		4.105
$m^{3'}$GMP	**3a**	4.1	8.11	5.91	5.8	3.52		
$m_2^{3',7}$GMP	**3**	5.5		6.06	3.6	3.48		4.100
araGMP	**4a**	6.0	8.08	6.24	5.7			
m^7araGMP	**4**			6.37	5.6			4.126
acycloGMP	**5a**	5.5	7.958	5.549[c]				
m^7acycloGMP	**5**	5.8		5.718[c]				4.126
acycloGMPme	**6a**	7.0	7.955	5.549[c]		3.525	10.6	
m^7acycloGMPme	**6**	7.1		5.531[c]		3.529	10.8	4.124
GDP		7.3	8.127	5.935	5.5			
GDPβme	**7a**	6.8	8.106	5.939	6.1	3.645	11.2	
m^7GDPβme	**7**	6.8		6.082	3.7	3.660	11.0	4.129
GTP		5.6	8.118	5.933	6.2			
GTPγme	**8a**	7.0	8.117	5.938	6.3	3.667	11.5	
m^7GTPγme	**8**	6.8		6.081	3.5	3.667	11.4	4.138

[a] Values for δ given in ppm and for J in hertz. [b] Very broad signal. [c] For acycloGMP derivatives, H-1′ and H-1″ chemical shifts are observed together.

minimum wavelengths obtained at the three pH values are reported in Table II. Fluorescence spectra of the same 7-methylated compounds in 0.1 M phosphate buffer, pH 7, yielded in each case maximum emission at λ 380 nm for λ_{ex} at 260 nm. The shape and position of the emission band were independent of λ_{ex} over a wide range.

Confirmation of the Structure of Derivatives by NMR Spectroscopy. In ^{1}H NMR spectra, the N7 methyl group on each of the cap analogues appeared as a singlet with chemical shifts of 4.10–4.14 ppm (Table III). The presence of the *N*-methyl substituent caused fast exchange of H-8 with ^{2}H from ^2H_2O. This is reflected in the absence of an H-8 signal with these compounds. ^{1}H NMR spectra also provided evidence for the presence of an O-CH_3 group on the phosphate moiety of acycloGMPme and m^7acycloGMPme. The signal at 3.5 ppm due to the phosphate O-methyl gave a distinctive doublet with coupling constant $^3J(P,CH_3)$ = 10.6–10.8 Hz (Table III). More direct information about the presence of the phosphate O-methyl group could be obtained from ^{31}P NMR. In spectra of acycloGMPme and m^7acycloGMPme, the phosphorus signal appeared as a broad multiplet due to coupling with protons from the O-CH_3 and changed to a singlet after proton decoupling (data not shown).

Verification of the O-CH_3 location in GDPβme and GTPγme and in the corresponding 7-methylated derivatives was based mainly on ^{31}P NMR spectroscopy. The chemical shifts of P_α, P_β, and P_γ in GDP and GTP were assigned according to published work (Labotka et al., 1976; O'Neil & Richards, 1980) (Table IV). The P_α signal had a chemical shift typical of P_α in nucleoside triphosphates; without decoupling of protons, the signals were doublets that broadened to two triplets because of coupling with protons 5′ and 5″; J(P,H) = 5–6 Hz and $J(P_\alpha,P_\beta)$ = 18–22 Hz. In the GTP analogues, a typical triplet structure was obtained for P_β due to coupling with the two neighboring phosphate groups; J-

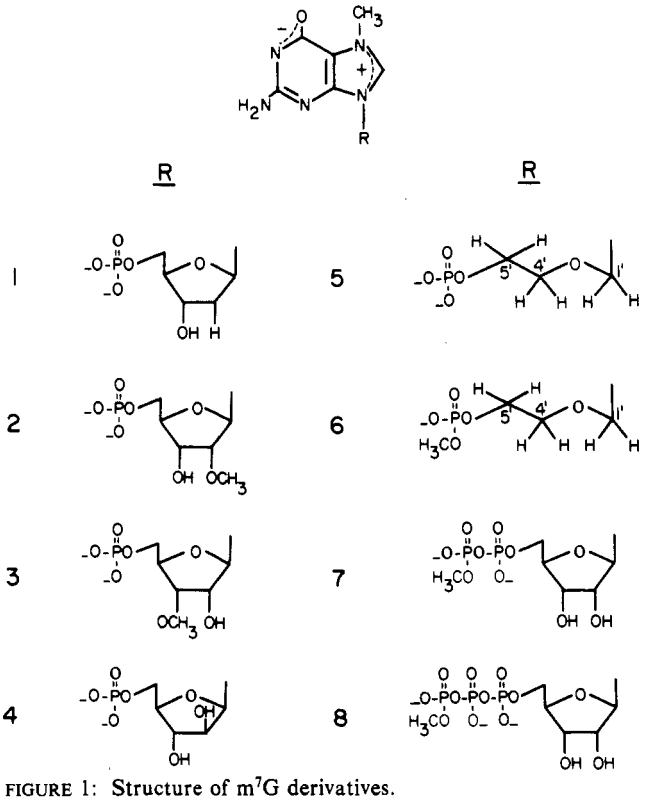

FIGURE 1: Structure of m^7G derivatives.

$(P_\alpha,P_\beta) = J(P_\beta,P_\gamma)$ = 18–22 Hz. In the case of the methyl esters, proton decoupling resulted in no signal change; obviously, a methyl group was not located on P_β.

The P_β and P_γ signals in the non-methyl-esterified GDP and GTP compounds, respectively, appeared as doublets. In the methyl-esterified derivatives these P signals adopted a broad multiplet shape as a result of coupling with the three protons

Table IV: ^{31}P NMR Chemical Shifts of GDP and GTP Derivatives[a]

compd	no.	pH	P_α	P_β	P_γ
GDP		4	−11.0	−10.50	
		6.5	−10.86	−9.16	
		7.4	−10.66	−6.85	
GDPβme	**7a**	4.1	−11.13	−9.35	
		4.9	−11.10	−9.33	
		7.5	−11.09	−9.19	
m^7GDPβme	**7**	4.1	−11.14	−9.22	
		5.5	−11.16	−9.23	
		6.9	−11.18	−9.26	
GTP		4.4	−11.06	−22.83	−10.51
		7.9	−10.88	−21.80	−5.83
GTPγme	**8a**	4.6	−11.26	−22.89	−9.46
		7.5	−11.31	−22.97	−9.51
m^7GTPγme	**8**	4.3	−11.31	−22.62	−9.51
		5.9	−11.21	−22.45	−9.38
		7.0	−11.24	−22.50	−9.48

[a] Chemical shifts in ppm were determined relative to the capillary containing (2-aminoethyl)phosphonic acid and recalculated to 85% H_3PO_4. $J(P_\alpha,P_\beta)$ = 18–22 Hz; $J(P_\alpha$,H-5′,H-5″) = 5–6 Hz; $J(P_\beta,CH_3)$ = $J(P_\gamma,CH_3)$ = 10–11 Hz.

from *O*-CH_3 and P_α for the diphosphates and P_β for the 5′-triphosphates. After proton decoupling the signals returned to the doublet forms observed for non-methyl-esterified GDP and GTP derivatives; the ^{31}P NMR spectra of GDPβme and m^7GDPβme, with and without decoupling of protons, are shown in Figure 2.

Additional support for the position assignments of the phosphate *O*-CH_3 groups was obtained by ^{31}P NMR spectral analyses at different pH values. For GDP and GTP, the ^{31}P chemical shifts change drastically at pHs close to the p*K* of the phosphate group (Moon & Richards, 1973). This sensitivity is demonstrable as a change of 3.7–4.7 ppm between acidic and neutral pHs for P_β and P_γ in the respective guanosine di- and triphosphates (Table IV). By contrast, the ^{31}P chemical shifts of the methyl esters of either GDP and GTP or the corresponding N7-methylated cap analogues showed little or no dependence on pH between ca. 4.1 and 7.5 (Table IV). This is in accord with the methyl esters containing no phosphate groups having a p*K* between 3 and 8.5, i.e., with *O*-methylation of the marginal phosphate. The results agree with the observed coupling of the marginal phosphate with the three protons of the methyl group (Figure 2). Note also the similar ^{31}P chemical shift values for the marginal phosphate in the *O*-methylated compounds and the nonesterified analogues protonated at acidic pHs (Table IV).

Conformation. Vicinal coupling constants between protons within the furanose ring and between protons and phosphorus were used to follow conformations of the compounds studied. Interpretation of coupling constants in terms of the conformational equilibria of the ring has been reviewed in detail (Davies, 1978). In all the ribose-containing derivatives, ^3J(1′,2′) was between 5.5 and 6.3 Hz for compounds without a substituent on the N7 position. The range was reduced to 3.5–3.7 Hz after N7-methylation (Table III). Methylation of the N7 position of dGMP also shifted the conformational equilibrium toward the N-type state but to a smaller extent (~10%) than in the ribose compounds. These results support the previously found rule (Darzynkiewicz et al., 1981) that a methyl group on N7 shifts the conformational equilibrium of the furanose ring and increases the population of the N-type state (3′-endo). Phosphate group esterification had negligible influence on the conformation of the furanose ring, i.e., <10% change in the populations of the N and S states.

Conformation of the phosphate group in the various analogues was also assessed. For compounds containing a furanose ring, the average value of ^3J(H-5′,H-5″;P) = 5.5 ± 1 Hz, which is very similar to that found in GMP (Tran-Dinh & Guschlbauer, 1975). Coupling constants with phosphate group and conformation of the phosphate group do not depend on the protonation of the base; they also do not change after methylation of N7. In the acyclo derivatives, ^3J(H-5′,H-5″;P) = 6.5 Hz, suggesting that the population of the rotamer about the C5′–O bond with phosphorus trans to C4′ is decreased by about 10% (to 57%) in comparison with other compounds studied. In the case of acycloGMPme, ^3J(P,C4′) = 7.5 Hz, which is smaller than 8.5 Hz usually found in 5′-nucleotides (Davies, 1978), again suggesting reduction of the population of the rotamer with P trans to C4′ (to 66%). This result may reflect the greater conformational flexibility of the acyclo derivatives. Further studies are necessary to determine conformation of the "sugar" moiety of the acyclo compounds.

From these considerations, it is concluded that the important influence of the methyl group at N7 on conformation is the shift in conformational equilibrium of the furanose ring (Darzynkiewicz et al., 1981).

Effect of Cap Analogues on the Formation of Translation Initiation Complexes. Reovirus mRNA attaches to ribosomes to form 80S complexes in wheat germ translating extracts incubated in the presence of sparsomycin to block polypeptide chain elongation. Removal of the 5′-terminal m^7G from reovirus mRNA and other capped messages diminishes ribosome binding, and addition of cap analogues such as m^7GMP

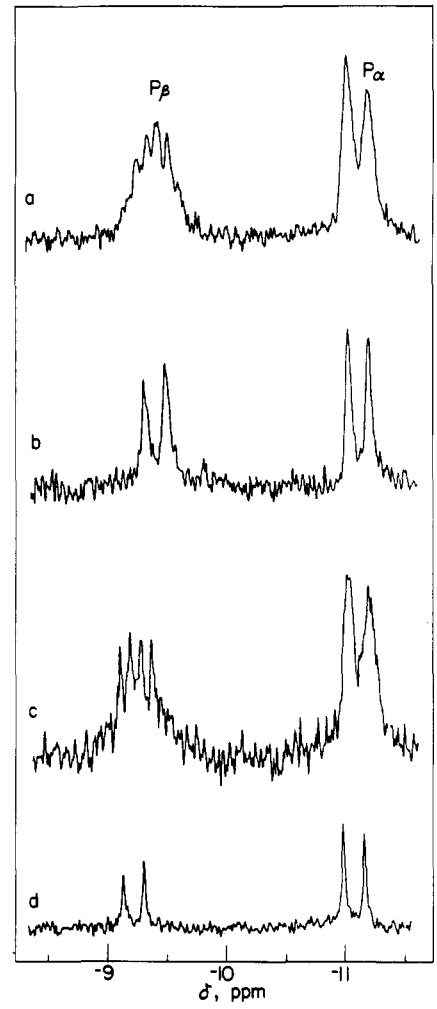

FIGURE 2: ^{31}P NMR spectra of guanine nucleotide β-methyl esters. GDPβme at pH 4.9 without (a) and with (b) decoupling from ^{1}H and m^7GDPβme at pH 5.5 without (c) and with (d) decoupling. All at 121.5 MHz.

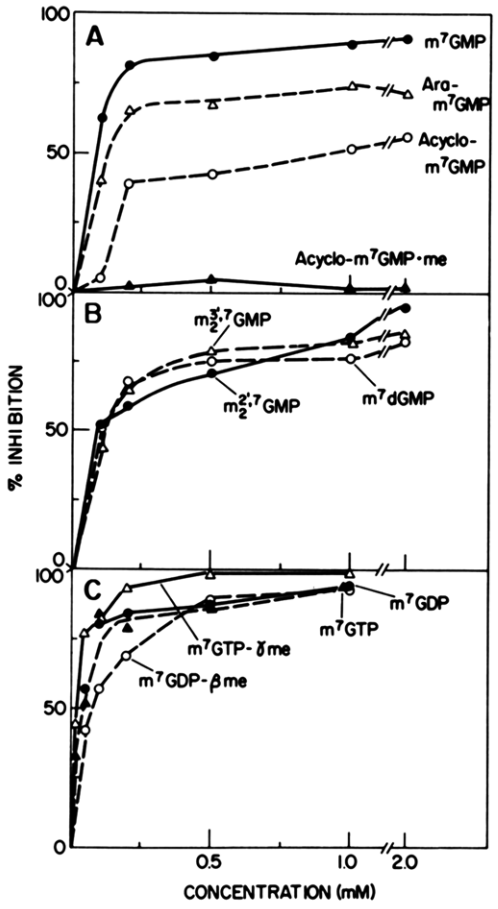

FIGURE 3: Effect of 7-methylguanosine derivatives on formation of 80S initiation complexes. [^{3}H]Methyl-labeled reovirus mRNA binding to wheat germ ribosomes was assayed in the presence of increasing concentrations of the indicated compounds as described under Experimental Procedures.

block stable initiation complex formation (Shatkin, 1976; Hickey et al., 1977). At 0.2 mM, m^{7}GMP decreased ribosome binding of [^{3}H]methyl-labeled reovirus mRNA by 81%, and higher concentrations were only slightly more inhibitory (Figure 3A). The importance of N7 substitution for cap analogue activity was confirmed for m^{7}GMP and the other nucleotide derivatives. For example, 0.5 mM araGMP diminished ribosome binding by only 10%, and similar values were obtained with the other non-7-methylated compounds (data not shown). Alteration of the ribose moiety, by 2′-O- or 3′-O-methylation or replacement with arabinose or deoxyribose, resulted in a small (~20%) but consistent decrease in cap analogue activity (Figure 3A,B). However, conversion to the acyclo form was somewhat more deleterious, decreasing the inhibitory activity by 2-fold (Figure 3A).

In contrast to the relatively small effect obtained by changing the ribose, an unmodified 5′-phosphate group is apparently essential for cap recognition. As observed previously for m^{7}GMPme (Darzynkiewicz et al., 1981), the ~50% residual activity of m^{7}acycloGMP was abolished by methyl esterification of the phosphate group (Figure 3A). The distinctive functional significance of the α-phosphate is also indicated by the retention of cap analogue activity following methylation of the β- and γ-phosphate groups of m^{7}GDP and m^{7}GTP, respectively (Figure 3C). Inhibition by m^{7}GDPβme and m^{7}GTPγme was not via conversion to other derivatives, for example, pyrophosphate cleavage to form m^{7}GMP and m^{7}GDP, since the methyl-esterified compounds comigrated with authentic compounds by thin-layer chromatography after incubation in wheat germ extract (data not shown).

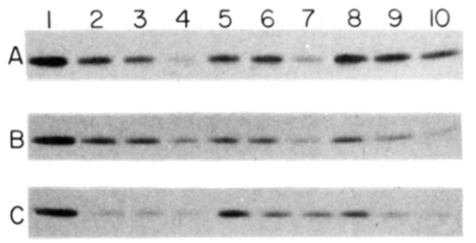

FIGURE 4: Chemical cross-linking of cap binding protein to oxidized mRNA. Reactions between eIF-4F and oxidized, [^{3}H]methyl-labeled reovirus mRNA were carried out as detailed under Experimental Procedures. Fluorograms of that portion of polyacrylamide gels containing the 24000-dalton cap binding protein, radiolabeled by covalent attachment to the mRNA 5′-cap, are shown. Cross-linking without analogue added (lane 1 in each panel) or in the presence of 0.1, 0.2, and 0.5 mM, respectively, of (panel A) m^{7}araGMP (lanes 2–4), m^{7}acycloGMP (lanes 5–7), and m^{7}acycloGMPme (lanes 8–10), (panel B) m^{7}dGMP (lanes 2–4), m$_2^{2',7}$GMP (lanes 5–7), and m$_2^{3',7}$GMP (lanes 8–10), and (panel C) m^{7}GMP (lanes 2–4), m^{7}GDPme (lanes 5–7), and m^{7}GTPme (lanes 8–10). Relative cross-linking as determined by densitometry of lanes 1–10: (panel A) 1.0, 0.55, 0.40, 0.11, 0.45, 0.43, 0.17, 0.49, 0.46, 0.45; (panel B) 1.0, 0.52, 0.48, 0.27, 0.44, 0.34, 0.18, 0.40, 0.28, 0.08; (panel C) 1.0, 0.14, 0.17, 0.11, 0.57, 0.30, 0.26, 0.34, 0.13, 0.12.

Effect of Cap Analogues on Interaction of mRNA with Cap Binding Protein. Protein synthesis initiation factor preparations from rabbit reticulocytes and other mammalian cells contain a ~24000-dalton "cap binding" protein that stimulates cell-free translation of capped mRNAs (Sonenberg et al., 1980). This polypeptide apparently interacts with the 5′-end of eukaryotic mRNAs and can be chemically cross-linked to the cap of oxidized mRNA (Sonenberg et al., 1978). Like ribosome binding, cross-linking is inhibited by m^{7}GMP, suggesting that the interaction between mRNA and cap binding protein is a specific one that is related to translation initiation. The 24000-dalton polypeptide is also present as a component of a high molecular weight protein complex recently termed eIF-4F on the basis of its requirement for maximal synthesis of rabbit globin in a reconstituted reticulocyte translation system (Grifo et al., 1983).

The effect of the cap analogues on cross-linking of eIF-4F to the cap of oxidized reovirus mRNA is shown in Figure 4. Since 83–100% of the specifically cross-linked radiolabel (as determined by densitometry) was associated with a single band corresponding to the 24000-dalton polypeptide, only this region of the gels is shown. Visual inspection of the band intensities and quantitation by comparative densitometry indicate a correlation between inhibition of mRNA cross-linking and ribosome binding. For example, as observed for ribosome binding, m^{7}GMP and m^{7}GTPme were among the most effective analogues; at 0.2 mM each inhibited cross-linking by more than 80% (Figure 4C). Correspondingly, m^{7}acycloGMPme was the least active analogue (Figure 4A); although cross-linking was decreased by 2-fold at all three concentrations in this experiment, another sample of m^{7}acycloGMPme was without effect at 0.1–0.5 mM. The combined results suggest that analogues of the 5′-terminal cap inhibit initiation complex formation by competing with mRNA for specific protein factor(s).

Discussion

A 5′-terminal cap, m^{7}GpppN, is characteristic of cellular and most viral eukaryotic mRNAs. In addition to enhancing mRNA stability (Furuichi et al., 1977; Green et al., 1983) and translation (Shatkin, 1976) and the initiation of influenza virus transcription (Krug, 1981), recent studies indicate that the presence of a cap is correlated with an increase in both the accuracy and efficiency of mRNA splicing (Krainer et al.,

1984; M. Konarska and P. Sharp, personal communication). With the exception of mRNA stability, enhancement of these processes is dependent on alkylation of the guanosine; i.e., 5′-terminal GpppN is apparently insufficient to obtain the effects.

The N7-substituted, positively-charged guanosine at the 5′-end of mRNA apparently constitutes part of a recognition site for cap binding proteins. Although such proteins were isolated originally from the cytoplasm of eukaryotic cells (Sonenberg et al., 1979; Tahara et al., 1981; Grifo et al., 1983), cap binding activity has also been attributed to the structural protein PB2 in influenza virus (Ulmanen et al., 1983). Nuclear cap binding proteins have also been detected by using a ^{32}P-labeled photoreactive benzophenone derivative of m^{7}GTP as an affinity probe (Patzelt et al., 1983). Presumably, the nuclear proteins interact with the capped 5′-ends of pre-mRNAs. Together with related polypeptides, they may function in nuclei to assemble RNA polymerase II transcripts and $m_3^{2,2,7}$ G-capped U1 small nuclear RNA into larger RNP complexes that mediate mRNA splicing (Mount et al., 1983). Cap binding proteins may also be involved in nucleocytoplasmic transport of mRNA.

Elements of the mRNA 5′-cap that contribute to recognition during formation of translation initiation complexes have been identified by using chemically synthesized analogues of m^{7}GMP. They include, for example, the positively charged imidazole ring resulting from modification of the 5′-terminal guanosine (Adams et al., 1978). In accord with the importance of N7 substitution (rather than methylation specifically), it was shown subsequently that reovirus transcripts containing 5′-terminal 7-ethylguanosine effectively direct viral protein synthesis (Furuichi et al., 1979).

The present studies involving several new derivatives of guanosine and 7-methylguanosine nucleotides confirm and extend the previous results. Guanosine derivatives that were not 7-methylated had little or no effect on translation initiation by capped reovirus mRNAs as compared to the extensive inhibition of ribosome binding observed at comparable concentrations (0.1–0.5 mM) of m^{7}GMP. Inhibitory activity was retained after the ribose moiety of m^{7}GMP was altered by 2′- or 3′-O-methylation or by conversion to the 2′-deoxyribose or arabinose compounds. These results and others indicate that the *cis*-diol structure of the sugar moiety is not essential for cap recognition (Muthukrishnan et al., 1976; Rose & Lodish, 1976; Sonenberg et al., 1978). Accordingly, the acyclo form of m^{7}GMP was about half as inhibitory as m^{7}GMP. However, methyl esterification of m^{7}acycloGMP, like conversion of m^{7}GMP to m^{7}GMPme (Darzynkiewicz et al., 1981), essentially eliminated inhibitory activity. Furthermore and in contrast to the monophosphate methyl esters, the β- and γ-methyl derivatives of m^{7}GDP and m^{7}GTP, respectively, were highly active cap analogues. This suggests that a minimum of two dissociable hydroxyls on the phosphate group(s) is necessary for cap analogue activity. Elimination of the second hydroxyl rather than steric effects of the added methyl group on m^{7}GMPme and m^{7}acycloGMPme may account for the loss of inhibition by these compounds. Taken together, these results point to the positively charged m^{7}G and the vicinal phosphate group(s), but not the connecting ribose moiety, as key features for cap recognition during initiation of translation. The m^{7}G cap analogues and unmethylated guanosine derivatives described in this paper should be useful for probing the possible involvement of cap binding proteins in mRNA splicing.

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Registry No. **1**, 22164-18-7; **2**, 94889-79-9; **3**, 94889-80-2; **4**, 94889-81-3; **5**, 94889-82-4; **6**, 94889-83-5; **7**, 94889-84-6; **8**, 94889-85-7; acycloG, 59277-89-3; araGMP, 59981-79-2; acycloGMP, 66341-16-0; acycloGMP methyl ester, 94889-86-8; GDPβMe, 57817-66-0; GDP, 146-91-8; GTPγMe, 57817-58-0; GTP, 86-01-1; methyl iodide, 74-88-4; methanol, 67-56-1.

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