(1984) J. Biol. Chem. 259, 5698–5704.
Nesheim, M. E., Taswell, J. B., & Mann, K. G. (1979) J. Biol. Chem. 254, 10952–10962.
Nesheim, M. E., Kettner, C., Shaw, E., & Mann, K. G. (1981) J. Biol. Chem. 256, 6537–6540.
Rosing, J., Tans, G., Govers-Reimslag, J. W. P., Zwaal, R. F. A., & Hemker, H. C. (1980) J. Biol. Chem. 255, 274–283.
Stryer, L. (1965) J. Mol. Biol. 13, 482–495.
Suzuki, K., Dahlback, B., & Stenflo, J. (1982) J. Biol. Chem. 257, 6556–6564.
van de Waart, P., Hemker, H. C., & Lindhout, T. (1984) Biochemistry 23, 2838–2842.
Weber, G., & Laurence, D. J. R. (1954) Biochem. J. 56, 31P.

Inhibition of Eukaryotic Translation by Nucleoside 5′-Monophosphate Analogues of mRNA 5′-Cap: Changes in N7 Substituent Affect Analogue Activity†

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Received October 7, 1988; Revised Manuscript Received January 23, 1989

ABSTRACT: Nucleotide cap analogues of 7-methylguanosine 5′-monophosphate (m^7GMP) were synthesized in which the 7-methyl moiety was replaced with 7-ethyl (e^7), 7-propyl (p^7), 7-isopropyl (ip^7), 7-butyl (b^7), 7-isobutyl (ib^7), 7-cyclopentyl (cp^7), 7-(carboxymethyl) (cm^7), 7-benzyl (bn^7), 7-(2-phenylethyl) [7-(2-PhEt)], and 7-(1-phenylethyl) [7-(1-PhEt)]. These derivatives were assayed as competitive inhibitors of capped mRNA translation in reticulocyte lysate. We observed that N7 alkyl and alicyclic substituents larger than ethyl significantly decreased the inhibitory activity of these cap analogues presumably by decreasing their affinity for cap binding proteins, which participate in the initiation of translation. This result defined a maximum size for this class of N7 substituents in the nucleotide binding domain of cap binding proteins. Like m^7GMP, the N7-substituted GMP derivatives synthesized in this study were found to be predominantly in the anti conformation as determined by proton NMR analyses. However, bn^7GMP and 7-(2-PhEt)GMP, which have aromatic N7 substituents, were more effective than m^7GMP as competitive inhibitors of translation. The increased affinity of bn^7GMP for cap binding proteins was further examined by synthesis of β-globin mRNA containing 5′-bn^7G, 5′-m^7G, or 5′-e^7G cap structures. These modified mRNAs were tested as translation templates. Messenger RNA capped with bn^7G was observed to increase the translation activity of the template 1.8-fold relative to that of its m^7G-capped mRNA counterpart. By contrast, e^7G-capped mRNA was 25% less active than m^7G-capped mRNA. UV photo-cross-linking of m^7G-capped mRNA to cap binding proteins was also inhibited to a greater extent by bn^7GMP than by m^7GMP or e^7GMP. Thus, from these data the inhibitory effect of bn^7GMP was due to its increased affinity for cap binding proteins and not by inhibition at another step of initiation.

One role of the 5′-cap structure [m^7G(5′)ppp(5′)N][1] found in all eukaryotic mRNAs is to facilitate ribosome binding during the initiation phase of translation (Shatkin, 1985; Banerjee, 1980). In eukaryotic translation, several key interactions between the 5′-cap of mRNAs and cap binding proteins are required for optimum binding of 5′-caps to these ligand binding proteins [Adams et al., 1978; Shatkin, 1985; Darzynkiewicz et al., 1981, 1985, 1987; for review of initiation, see Pain (1985)]. Individually, these proteins have been identified as eIF-4A (Grifo et al., 1982; Edery et al., 1983), eIF-4B, (Grifo et al., 1982), eIF-4E or CBP I (Sonenberg et

† E.D. was supported in part by the Polish Academy of Sciences (CPBR 3.13) and the Ministry of Education of Poland (CPBP 01.06). N.S. was supported by a grant from the Medical Research Council of Canada. S.M.T. was supported by grants from the Life and Health Insurance Medical Research Fund, the Wright Foundation, the Margaret Early Research Trust, the American Cancer Society (MV-257), and the NIH (GM38512). N.S. is the recipient of a Medical Research Council Scientist award from the Medical Research Council of Canada. C.G. is a recipient of a predoctoral fellowship from the Cancer Research Society (Montreal).

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[1] Abbreviations: m^7G, 7-methylguanosine; m^7GMP, 7-methylguanosine 5′-monophosphate; e^7GMP, 7-ethylguanosine 5′-monophosphate; p^7GMP, 7-propylguanosine 5′-monophosphate; a^7GMP, 7-allylguanosine 5′-monophosphate; ip^7GMP, 7-isopropylguanosine 5′-monophosphate; b^7GMP, 7-butylguanosine 5′-monophosphate; ib^7GMP, 7-isobutylguanosine 5′-monophosphate; cp^7GMP, 7-cyclopentylguanosine 5′-monophosphate; cm^7GMP, 7-(carboxymethyl)guanosine 5′-monophosphate; bn^7GMP, 7-benzylguanosine 5′-monophosphate; 7-(2-PhEt)-GMP, 7-(2-phenylethyl)guanosine 5′-monophosphate; 7-(1-PhEt)GMP, 7-(1-phenylethyl)guanosine 5′-monophosphate; CBP, cap binding protein; eIF, eukaryotic initiation factor; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; TEAB, triethylammonium bicarbonate; TLC, thin-layer chromatography.

0006-2960/89/0428-4771$01.50/0

al., 1978; Tahara et al., 1981), and eIF-4F or CBP II (Tahara et al., 1981; Grifo et al., 1982, 1983; Edery et al., 1983). Of these, only eIF-4F and eIF-4E have been shown to possess true cap binding activity; eIF-4A probably does not bind the cap directly but instead binds at a site very near to the cap structure, as does eIF-4B (Pelletier & Sonenberg, 1985). The chemical features of the cap which are recognized include (1) the N7 substituent of the capping guanine nucleotide (Adams et al., 1978; Furuichi et al., 1979), (2) the purine C2 amino and C6 keto substituents (Adams et al., 1978), and (3) the α-phosphate of the capping nucleotide (Darzynkiewicz et al., 1981, 1987).

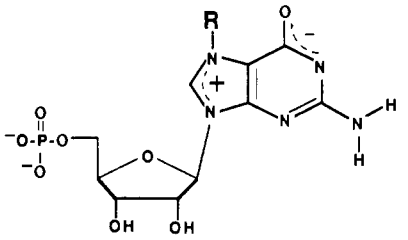
The interaction of the 5′-cap of mRNA with eukaryotic cellular components is also important for other biological processes. Other nontranslation roles for the cap that have been described include stabilization against 5′-exonucleolytic activities (Furuichi et al., 1977; Green et al., 1983), transcription initiation in influenza (Ulmanen et al., 1983), RNA splicing (Konarska et al., 1984; Edery & Sonenberg, 1985), 3′-processing of histone mRNA (Georgiev et al., 1984), and termination of transcription of influenza virus mRNAs (Beaton & Krug, 1986).

Previously it was suggested that the basis for function of the cap structure might lie with the delocalized positive charge, resulting from N7 alkylation, in the imidazole portion of the capping guanine nucleotide. As such, the size of the N7 substituent was not believed to affect function or activity of the cap structure primarily because 7-benzyl-GDP was as effective an inhibitor of translation as m^{7}GDP (Adams et al., 1978). Our group has systematically examined the structural features of nucleotide cap analogues which are required for competitive inhibition of mRNA 5′-cap dependent processes in translation. For the studies reported here, a new series of N7-substituted cap analogues was synthesized and analyzed in translation assays. Contrary to previous reports, we observed that there was a size limit to the N7 substituent and that bn^{7}G probably binds to CBPs in a mechanism unlike other N7 alkyl derivatives.

Experimental Procedures

Materials. Guanosine 5′-monophosphate and Sephadex A-25 were from Sigma. Alkyl and aryl halides were obtained from Aldrich, Sigma, and POCH (Poland). [^{35}S]Methionine (sp. act. 1347 Ci/mmol) was obtained from Amersham; m^{7}G(5′)ppp(5′)G and T7 RNA polymerase were from New England Biolabs. Ribonucleoside triphosphates and nucleotide chromatography markers were from Pharmacia P-L Biochemicals. [α-^{32}P]GTP (sp. act. 3000 Ci/mmol) was from New England Nuclear. All other chemicals were of reagent grade or higher quality.

Synthesis of Cap Analogues. The 7-substituted GMP cap analogues were synthesized from GMP (Na^+ salt, 2.8 mmol) and the corresponding alkyl halide (10 mmol) by the general method described earlier (Darzynkiewicz et al., 1985, 1987). Reactants were incubated at room temperature for 24 h in 10–12 mL of Me_2SO; specific conditions and product yields are listed below. Upon completion of the reaction, water was added, and free halide was removed by several extractions with diethyl ether. The aqueous phase was applied to a 3.5 × 70 cm column of DEAE-Sephadex (A-25, HCO_3^- form). The column was washed with water and eluted with a 0–0.6 M linear gradient of TEAB, pH 7.5 (4 L). The N7-substituted GMP products eluted from the column as sharp peaks between 0.35 and 0.45 M TEAB. Product peaks were pooled, desalted, and converted to the corresponding Na^+ salts by ion exchange as before (Darzynkiewicz et al., 1985).



R =	
-CH$_3$	
-CH$_2$CH$_3$	(1)
-(CH$_2$)$_2$CH$_3$	(2)
-CH$_2$CH=CH$_2$	(3)
-CH(CH$_3$)$_2$	(4)
-(CH$_2$)$_3$CH$_3$	(5)
-CH$_2$CH(CH$_3$)$_2$	(6)
-C$_5$H$_9$	(7)
-CH$_2$COOH	(8)
-CH$_2$C$_6$H$_5$	(9)
-CH$_2$CH$_2$C$_6$H$_5$	(10)
-CH(CH$_3$)C$_6$H$_5$	(11)

FIGURE 1: Structures of N7-substituted cap analogues. For names of compounds, see Experimental Procedures.

(A) 7-Ethylguanosine 5′-monophosphate (e^{7}GMP, **1**). Ethyl iodide was reacted with GMP. The product was worked up as above to afford 618 mg of **1** (50.7% yield).

(B) 7-Propylguanosine 5′-monophosphate (p^{7}GMP, **2**). Propyl iodide was reacted with GMP and worked up to give 430 mg of **2** (35% yield).

(C) 7-Allylguanosine 5′-monophosphate (a^{7}GMP, **3**). Allyl bromide was reached with GMP as above to give 771 mg of **3** (61.6% yield).

(D) 7-Isopropylguanosine 5′-monophosphate (ip^{7}GMP, **4**). Isopropyl bromide was reacted with GMP for 3 days as above to give 49 mg of **4** (4% yield).

(E) 7-Butylguanosine 5′-monophosphate (b^{7}GMP, **5**). Butyl iodide was reacted with GMP as above to give 282 mg of **5** (22% yield).

(F) 7-Isobutylguanosine 5′-monophosphate (ib^{7}GMP, **6**). Isobutyl bromide (40 mmol) was reacted with GMP as above for 3 days to afford 96 mg of **6** (7.2% yield).

(G) 7-Cyclopentylguanosine 5′-monophosphate (cp^{7}GMP, **7**). Cyclopentyl bromide (40 mmol) was reacted with GMP for 3 days as described to give 13 mg of **7** (1% yield).

(H) 7-(Carboxymethyl)guanosine 5′-monophosphate (cm^{7}GMP, **8**). Bromoacetic acid (40 mmol) was reacted with GMP as above to give 431 mg of **8** (31.7% yield).

(I) 7-Benzylguanosine 5′-monophosphate (bn^{7}GMP, **9**). Benzyl chloride (40 mmol) was reacted with GMP for 2 days as above to afford 360 mg of **9** (25.8% yield).

(J) 7-(2-Phenylethyl)guanosine 5′-monophosphate [7-(2-PhEt)GMP, **10**]. 2-Phenylethyl bromide was reacted with GMP for 3 days as above to give 91 mg of **10** (6.3% yield).

(K) 7-(1-Phenylethyl)guanosine 5′-monophosphate [7-(1-PhEt)GMP, **11**]. 1-Phenylethyl bromide was reacted with GMP as above to afford 145 mg of **11** (10% yield).

The structures of the above-described cap analogues are shown in Figure 1.

Synthesis of Extended Cap Dinucleotides. A full description of the synthesis and chemical characterization of bn^{7}G(5′)ppp(5′)G and e^{7}G(5′)ppp(5′)G will be published elsewhere. Briefly, these dinucleotides were synthesized by the general method described by Nakagawa et al. (1980).

Spectroscopy. Proton NMR spectra were obtained with a Bruker AM-360 spectrometer. Samples were dissolved in D_2O (100% D; Merck Sharp & Dohme), and NMR spectra were taken at 300 K at the indicated pH. Chemical shifts were

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

compd	no.	system A	system B
m^{7}GMP		0.64	0.10
e^{7}GMP	**1**	0.61	0.16
p^{7}GMP	**2**	0.55	0.22
a^{7}GMP	**3**	0.61	0.20
ip^{7}GMP	**4**	0.58	0.21
b^{7}GMP	**5**	0.49	0.33
ib^{7}GMP	**6**	0.52	0.32
cp^{7}GMP	**7**	0.41	0.26
cm^{7}GMP	**8**	0.66	0.07
bn^{7}GMP	**9**	0.37	0.19
7-(2-PhEt)GMP	**10**	0.24	0.28
7-(1-PhEt)GMP	**11**	0.44	0.36

[a] Chromatography was performed on cellulose F_{254} thin-layer plates (Merck) in the indicated solvent systems. System A is saturated $(NH_4)_2SO_4$/2-propanol/0.1 M potassium phosphate (pH 7.4) (79:2:19 v/v/v). System B is 1% $(NH_4)_2SO_4$/2-propanol (1:2 v/v).

measured relative to internal 3-(trimethylsilyl)propanesulfonic acid (TSP), sodium salt. Conformational analyses of the GMP derivatives were performed as described earlier (Darzynkiewicz et al., 1981, 1985, 1987).

UV spectra were obtained with a Zeiss (Jena, GDR) VSU-2P spectrophotometer (Darzynkiewicz et al., 1985, 1987).

In Vitro Translation. Rabbit reticulocytes were prepared from phenylhydrazine-treated animals as previously described (Tahara et al., 1981). Native globin mRNA was purified from salt-washed rabbit reticulocyte ribosomes with 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. Synthetic globin mRNAs were synthesized with T7 RNA polymerase and a β-globin cDNA template prepared from pT7rG4 (Darzynkiewicz et al., 1988). The 5′-noncoding region of the β-globin transcript is m^{7}GpppGGGAGACCCAAGCUUGGACUGUGUUUA-CUUGCAAUCCCCCAAAACAGACAGAAUG. The translation start codon is underlined.

Translation assays were performed in micrococcal nuclease treated rabbit reticulocyte lysate, prepared essentially as described (Pelham & Jackson, 1976). Reaction conditions used for in vitro translation were as previously described (Darzynkiewicz et al., 1987). Globin mRNA was used at a concentration of 4 μg/mL in a 25-μL reaction volume.

UV Photo-Cross-Linking of mRNA to Protein. Photocross-linking of radiolabeled mRNA to proteins was performed as described by Pelletier and Sonenberg (1985) with yeast rp51AΔ2 mRNA (Ruskin et al., 1986). Uncapped ^{3}H-labeled mRNA was synthesized by transcription of rp51AΔ2 cDNA with SP6 RNA polymerase. Messenger RNA was capped with vaccinia guanylyltransferase (BRL) in the presence of [α-^{32}P]GTP. ^{32}P-Cap-labeled mRNA [(0.5–1) × 10^5 cpm; ~5 × 10^6 cpm/μg] was incubated with ~100 μg of ribosomal high-salt wash from rabbit reticulocytes. Incubation was for 10 min at 30 °C followed by UV irradiation. Samples were treated with 20 μg of RNase A for 30 min at 37 °C and analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography.

Results

Characterization of Cap Analogues. N7-Substituted GMP cap analogues were analyzed for purity by thin-layer chromatography (TLC). All cap analogues migrated as single fluorescent spots in two solvent systems. This intrinsic fluorescence is characteristic of N7 substitution (Table I).

The UV absorption maxima and minima were determined for each of the cap analogues listed in Table I. No remarkable differences in minima or maxima were observed. All of the analogues had a λ_{max} between 256 and 259 nm and a λ_{min} between 230 and 235 nm, at pH 2. At pH 7 the analogues exhibited a dual λ_{max} centered at 257–258 and 280–282 nm. Dual λ_{min} centered at 236–241 and 271–273 nm were also observed at this pH. Spectra were not obtained at pH 12 due to sample decomposition. The only notable difference in UV absorption among the cap analogues was observed for those with aromatic substituents, which tended to have slightly higher λ_{min} and λ_{max} values.

Confirmation of Cap Analogue Identity by NMR Spectroscopy. All compounds exhibited proton spectra consistent with N7-substituted GMP (Table II). The ^{1}H signal for H8 was notably absent, indicating increased proton exchange with solvent. This exchange is characteristic of N7 substitution (Darzynkiewicz et al., 1981). Furthermore, the identity of all N7 substituents was confirmed by homonuclear decoupling, analysis of coupling constant patterns, and chemical shift values as well as signal integration. In the case of 7-(1-PhEt)GMP (**11**), two overlapping multiplets were observed for each signal position. These two compounds, present in equal amounts, were provisionally identified as two stereoisomers of this cap analogue.

Conformation of N7-Substituted Nucleotide Cap Analogues. We showed earlier for C8-substituted m^{7}GMP derivatives that the syn–anti conformation of the glycosidic bond is affected on the basis of the nature of the C8 group (Darzynkiewicz et al., 1987). The effect of N7 substitution, with alkyl groups larger than methyl, on syn–anti conformation had not been previously determined. For this reason it was necessary to establish the nucleotide conformation of the N7-

Table II: ^{1}H NMR Chemical Shifts of Cap Analogues (in ppm vs Internal TSP)

			chemical shifts (in ppm)						
compd	no.	pH	1′	2′	3′	4′	5′	5″	other signals
GMP[a]		8.0	5.92	4.79	4.49	4.31	4.00	3.97	8.20 (8-H)
m^{7}GMP[a]		7.0	5.99	4.68	4.37	4.27	3.94	3.83	4.07 (N–CH_3)
e^{7}GMP	**1**	6.4	6.07	4.67	4.48	4.38	4.16	4.04	1.54 (CH_3); 4.49 (CH_2–N)
p^{7}GMP	**2**	6.7	6.07	4.68	4.47	4.37	4.12	4.02	0.93 (CH_3); 1.93 (CH_2); 4.42, 4.43 (CH_2–N)
a^{7}GMP	**3**	6.5	6.07	4.69	4.48	4.38	4.16	4.05	6.10 (H-1 allyl); 5.31 (H-2t allyl); 5.40 (H-2c, allyl)
ip^{7}GMP	**4**	6.7	6.06	4.72	4.48	4.37	4.12	4.04	1.63 (CH_3); 5.08 (CH–N)
b^{7}GMP	**5**	6.8	6.06	4.68	4.47	4.37	4.12	4.02	0.91 (CH_3); 1.35 (CH_2); 1.89 (CH_2); 4.46 (CH–N)
ib^{7}GMP	**6**	7.4	6.07	4.69	4.46	4.37	4.08	3.99	0.95 (CH_3); 0.91 (CH_3); 2.25 (CH); 4.27, 4.31 (CH_2–N)
cp^{7}GMP	**7**	7.5	6.04	4.74	4.48	4.36	4.07	4.01	5.13 (1-CH, cyclopentyl); 1.74, 1.90, 2.09, 2.27 (4 × CH_2, cyclopentyl)
cm^{7}GMP	**8**	6.5	6.10	4.70	4.49	4.38	4.17	4.05	5.04 (CH_2)
bn^{7}GMP	**9**	7.2	6.07	4.70	4.50	4.38	4.14	4.03	5.67 (CH_2); 7.37–7.41 (benzene ring)
7-(2-PhEt)GMP	**10**	7.1	5.94	4.39	4.18	4.28	4.05	3.93	4.70, 4.74 (CH_2–N); 3.23 (CH_2 phenyl); 7.1–7.4 (phenyl)
7-(1-PhEt)GMP	**11**	6.6	6.07	4.78	4.52	4.39	4.16	4.07	2.01, 2.02 (2 CH_3); 6.15, 6.17 (CH–N); 7.4 (phenyl)
			6.06	4.78	4.51	4.39	4.15	4.06	

[a] Values from Kim and Sarma (1978).

Table III: Vicinal Proton Coupling Constants (Hz)

compd	no.	pH	$J(1',2')$	$J(2',3')$	$J(3',4')$	$J(4',5')$	$J(4',5'')$	$J(5'P)$	$J(5''P)$	$J(4'P)$
GMP[a]		8.0	6.1	5.2	3.4	4.3	2.9	4.7	4.7	1.1
m^7GMP[a]		7.0	3.6	4.9	5.1	2.3	2.0	4.5	4.5	1.7
e^7GMP	**1**	6.4	4.0	4.9	5.1	2.6	2.5	4.2	4.9	2.1
p^7GMP	**2**	6.7	4.2	5.1	4.8	2.6	2.6	4.3	5.0	2.1
a^7GMP	**3**	6.5	3.9	4.9	4.9	2.6	2.5	4.3	5.0	2.0
ip^7GMP	**4**	6.7	4.6	5.0	4.9	2.7	2.7	4.1	5.3	2.2
b^7GMP	**5**	6.8	4.2	4.7	4.9	2.7	2.7	4.3	5.2	2.1
ib^7GMP	**6**	7.4	4.6	5.2	4.8	2.7	2.8	4.3	5.1	2.1
cp^7GMP	**7**	7.5	4.9	4.8	4.3	3.1	3.2	4.4	5.4	1.3
cm^7GMP	**8**	6.5	3.8	4.8	5.4	2.5	2.7	4.3	5.0	*d*
bn^7GMP	**9**	7.2	4.1	4.7	4.9	2.7	2.6	4.2	5.0	2.1
7-(2-PhEt)GMP	**10**	7.1	3.8	4.8	5.4	2.9	3.1	4.3	5.2	*d*
7-(1-PhEt)GMP[b]	**11**	6.6	4.8	5.0	4.5	*c*	*c*	4.4	5.7	*c*
					4.4	*c*	*c*	4.5	5.3	

[a] Values from Kim and Sarma (1978). [b] Values given for two stereoisomers. [c] Values not available due to overlap. [d] Values not available.

substituted series of cap analogues.

The conformation of each cap analogue was determined from the ^{1}H NMR parameters given in Tables II and III. With the exception of 7-(2-PhEt)GMP (**10**), all derivatives had nearly the same chemical shifts for ribose protons (Table II). This information indicated that different N7 substituents did not significantly alter nucleotide conformation about the glycosidic bond. All N7-substituted cap analogues, described in this study, therefore exhibited a preference for the anti conformation, similar to m^7GMP, m^7GDP, m^7GTP, and the extended cap structure (Hickey et al., 1977; Kim & Sarma, 1978; Darzynkiewicz et al., 1981).

For 7-(2-PhEt)GMP (**10**; see Table II), significant (in comparison to other compounds) upfield shifts were observed for H2′ and H3′ ($\Delta\delta \approx 0.3$ ppm). Smaller chemical shifts ($\Delta\delta \leq 0.1$ ppm) were observed for the other ribose protons of **10**. Such changes in chemical shift were unlike those expected for a change in the syn–anti equilibrium (Davies, 1978) and therefore were more likely a result of an influence of the benzene ring current by the N7 substituent. Upfield shifts in proton signals could originate from a nucleotide conformation in which protons H2′ and H3′ are above the plane of the substituent aromatic ring (Johnson & Bovey, 1958).

All derivatives exhibited similar values for coupling constants (Table III). Therefore, it was concluded that the furanose ring conformations among the cap analogues were similar, with nearly equal populations of N and S conformers (Table IV). This distribution was essentially identical with that observed for m^7GMP. As follows from Table III, for the cap analogues, the exocyclic 4′-CH_2OP groups had a preference for the g^+ rotamer (67–79%, Table IV). All derivatives also had a similar preference for the g^{+} conformation of the phosphate group. Thus it was concluded that the presence of an N7 substituent, much less its chemical nature, was important for the observed nucleotide conformations. Nonetheless, some small differences were observed between derivatives (see Table IV). For example, cp^7GMP (**7**) had lower populations of both g^+ and N states compared to other derivatives. However, such small differences, not exceeding 10% relative to m^7GMP, cannot be wholly responsible for the observed differences in biological activity as shown below.

Effect of Cap Analogues on in Vitro Translation. The nucleotide cap analogues described above were assayed for inhibition of globin synthesis in an in vitro translation assay. If the N7 substituent prevented the cap analogue from competing with the 5′-capped ends of mRNA, then it could be inferred that the nucleotide binding domain of the cap binding protein was unable to accommodate such a modification. Cap analogues were defined to be noninhibitory if the concentration required for 50% inhibition was greater than 0.5 mM. Since eIF-4E and the M_r 26 000 component of eIF-4F appear to be the only true cap binding proteins, the observed effects are thought to be primarily due to competitive inhibition of these factors.

Table IV: Conformational Parameters[a]

compd	no.	% N state	% g^+	% g'^+
GMP[b]		36	58	81
m^7GMP[b]		54	87	83
e^7GMP	**1**	54	79	83
p^7GMP	**2**	51	78	82
a^7GMP	**3**	52	79	82
ip^7GMP	**4**	52	76	81
b^7GMP	**5**	52	76	81
ib^7GMP	**6**	51	75	81
cp^7GMP	**7**	45	67	79
cm^7GMP	**8**	57	78	82
bn^7GMP	**9**	52	77	82
7-(2-PhEt)GMP	**10**	57	70	81
7-(1-PhEt)GMP	**11**	47	*c*	77
		46	*c*	79

[a] Accuracy = ±5%. [b] From Darzynkiewicz et al. (1981). [c] Values not available due to overlapping spectra.

When added to translation assays, the straight-chain derivatives (**2**, **5**), with the exception of e^7GMP (**1**) and a^7GMP (**3**), were not inhibitory (Figure 2A). The branched derivatives (**4**, **6**) and the only alicyclic derivative tested (**7**) were also not inhibitory. The lack of inhibitory activity of the various cap analogues generally correlated with the size of the N7 substituent. N7 groups larger than ethyl significantly diminished the activity of the cap analogue as an inhibitor. 7-Allyl-GMP (**3**; 50% inhibition at 0.39 mM) was more inhibitory than p^7GMP (**2**; 50% inhibition at >0.5 mM) but generally less than e^7GMP (**1**; 50% inhibition at 0.18 mM). The presence of the double bond apparently increased the affinity of **3** relative to **2** for CBPs. As shown in Figure 2B, of the cap analogues containing aromatic substituents, only bn^7GMP (**9**; 50% inhibition at 0.05 mM) and 7-(2-PhEt)GMP (**10**; 50% inhibition at 0.055 mM) had significant inhibitory activity. Surprisingly, the latter two analogues were more active as inhibitors than m^7GMP. The lower inhibitory activity of **11** (50% inhibition at 0.21 mM) was consistent with the lack of inhibition also seen with **4**, **6**, and **7**, where either a branched N7 substituent or one containing a secondary carbon bonded to N7 prevented competitive inhibition.

The lack of inhibition observed with cm^7GMP (**8**; Figure 2A) confirmed the earlier result by Adams et al. (1978). However, we differ with their interpretation that the nucleotide conformation of this analogue precluded its binding to cap binding proteins. As we observed, **8** was found to be primarily

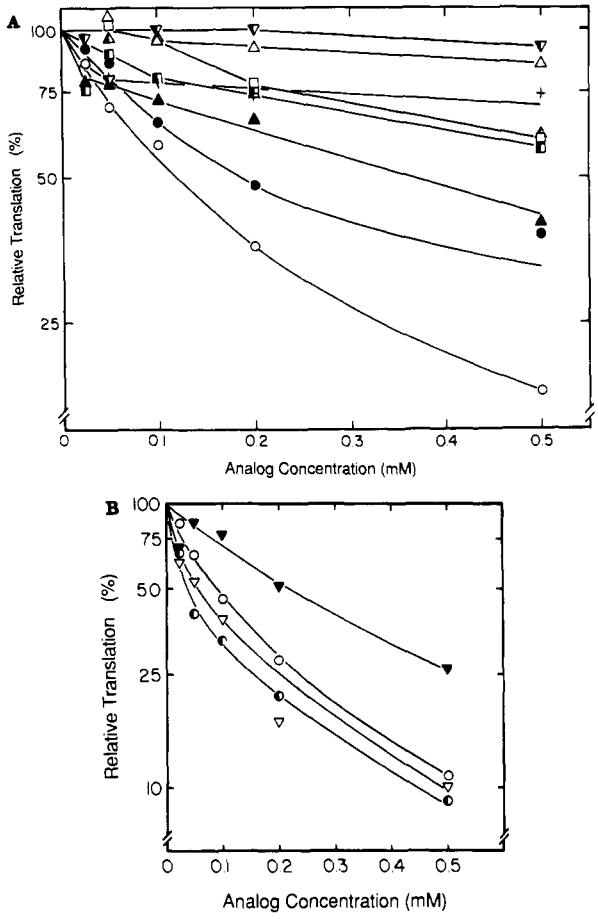


FIGURE 2: Effect of cap analogues on globin translation. Translation of rabbit β-globin mRNA in reticulocyte lysate was assayed in the presence of cap analogues at the indicated concentrations. Data were normalized relative to [^{35}S]Met incorporation in the absence of analogues. (A) Effect of 7-alkyl-substituted cap analogues: m^{7}GMP (○), e^{7}GMP (●), p^{7}GMP (□), a^{7}GMP (▲), ip^{7}GMP (△), b^{7}GMP (□), ib^{7}GMP (▲), cp^{7}GMP (▽), and cm^{7}GMP (+). (B) Effect of 7-aryl-substituted cap analogues: m^{7}GMP (○), bn^{7}GMP (◐), 7-(2-PhEt)GMP (▽), and 7-(1-PhEt)GMP (▼).

anti, and the furanose conformation was similar to that for m^{7}GMP (Tables III and IV). On the basis of the inactivity of the analogues with substituents larger than ethyl, we would instead propose that **8** did not bind efficiently to CBPs due to a combination of unfavorable electrostatic and/or steric effects between the nucleotide binding domain of CBPs and the negatively charged N7 carboxymethyl moiety. However, further studies must be performed before a definitive statement can be made regarding the nature of this interaction with the cap binding proteins.

Intramolecular Effect of bn^{7}G- and e^{7}G-Capped mRNA. On the basis of the inhibition patterns of cap analogues in translation, it appeared that bn^{7}GMP (**9**; 50% inhibition at 0.05 mM) was a more potent inhibitor than m^{7}GMP (50% inhibition at 0.09 mM); by contrast, e^{7}GMP (**1**; 50% inhibition at 0.19 mM) was less active than m^{7}GMP as an inhibitor. These data implied that differences in activity between nucleotide derivatives **1** and **9** could be explained by their relative affinity for cap binding proteins. However, the inhibitory activity of **9** appeared to be anomalous because the translation results obtained with the alkyl series (Figure 2A) indicated that N7 substituents larger than ethyl precluded interaction of the cap analogue with cap binding proteins. One possible explanation for this apparent anomaly is that **9** and **10** do not inhibit the same partial reactions of initiation as do m^{7}GMP and **1**. From the current model for initiation of translation, eIF-4F binds to the mRNA cap prior to or simultaneously with binding of mRNA to the 43S initiation complex (Pain, 1986). It is possible that **9** and **10** inhibited a partial reaction of initiation in addition to, or other than, the interaction of eIF-4F with mRNA. In an effort to address this issue we analyzed the intramolecular or cis effect of modified mRNA cap structures on mRNA activity.

Dinucleotides of the form bn^{7}G(5′)ppp(5′)G and e^{7}G(5′)ppp(5′)G were synthesized and incorporated into artificial globin mRNAs by transcriptional priming. We and others have shown that m^{7}G(5′)ppp(5′)G and related dinucleotides of this form can be efficiently incorporated into RNA transcripts by use of phage SP6 or T7 RNA polymerase such that the 5′ ends of the synthetic mRNAs are quantitatively capped (Konarska et al., 1984; Edery et al., 1985; Darzynkewicz et al., 1988). A rabbit β-globin cDNA, linearized with *Ava*I (pT7rG4; Darzynkiewicz et al., 1988), was used as template for transcription by T7 RNA polymerase. The capped dinucleotides bn^{7}GpppG and e^{7}GpppG were added to separate transcription mixtures to obtain synthetic mRNAs with the corresponding 5′-cap structures. In order to verify that the 5′-ends were correctly modified to the extent expected, the differently capped transcripts were internally labeled, by use of [α-^{32}P]GTP in the transcription mixtures, and digested to completion with RNase T2. The total digests were then examined by two-dimensional TLC and autoradiography for nearest-neighbor transfer of the radioactive phosphate into the 5′-cap dinucleotide (Konarska et al., 1984). As shown in Figure 3 (panels B and C), both capped dinucleotides were incorporated into the 5′-ends of T7 transcripts as indicated by the absence of pppG^{32}p, which would be produced from an unmodified 5′-end. (Compare panels B and C to panel A for analysis of m^{7}G-capped transcript and to panel D for uncapped mRNA.) Instead, for each digest a new spot appeared which corresponded to the position of the 3′-phosphorylated 5′-terminal-capped dinucleotide (NpppG^{32}p). From the absence of pppG^{32}p or its degradation products in the autoradiograms, we estimated that the 5′-ends of the synthetic mRNAs were capped to the extent of 95% or better. Thus, both capped dinucleotides were efficiently incorporated into T7 RNA polymerase transcripts.

The bn^{7}G- and e^{7}G-capped β-globin mRNAs were assayed in micrococcal nuclease treated reticulocyte lysate and compared for translation activity relative to that of m^{7}G-capped mRNA. As shown in Figure 4, mRNA capped with bn^{7}GpppG was 1.8-fold more active than control m^{7}G-capped transcripts on the basis of initial rates of radiolabel incorporation. By contrast, e^{7}G-capped mRNA was 25% less active than its m^{7}G-capped counterpart. These results were consistent with the observed relative activity of the corresponding nucleoside monophosphate cap analogues as inhibitors of translation and their differences in affinity for components of the translation machinery.

One possibility for the difference in activity of bn^{7}GMP vs m^{7}GMP was that the former analogue affected individual cap binding proteins differently. Since several initiation factors have been shown to interact with a portion of the mRNA at or near the cap (Grifo et al., 1982; Pelletier & Sonenberg, 1985), it is possible that the affected protein(s) for the observed inhibition by bn^{7}GMP is (are) not the same as that for m^{7}GMP. In order to help answer this question, we examined the ability of m^{7}GMP, e^{7}GMP, and bn^{7}GMP to inhibit UV cross-linking of radiolabeled mRNA to cap binding proteins.

^{32}P-Cap-labeled rp51AΔ2 mRNA was synthesized from an SP6 transcription template and incubated with rabbit reticu-

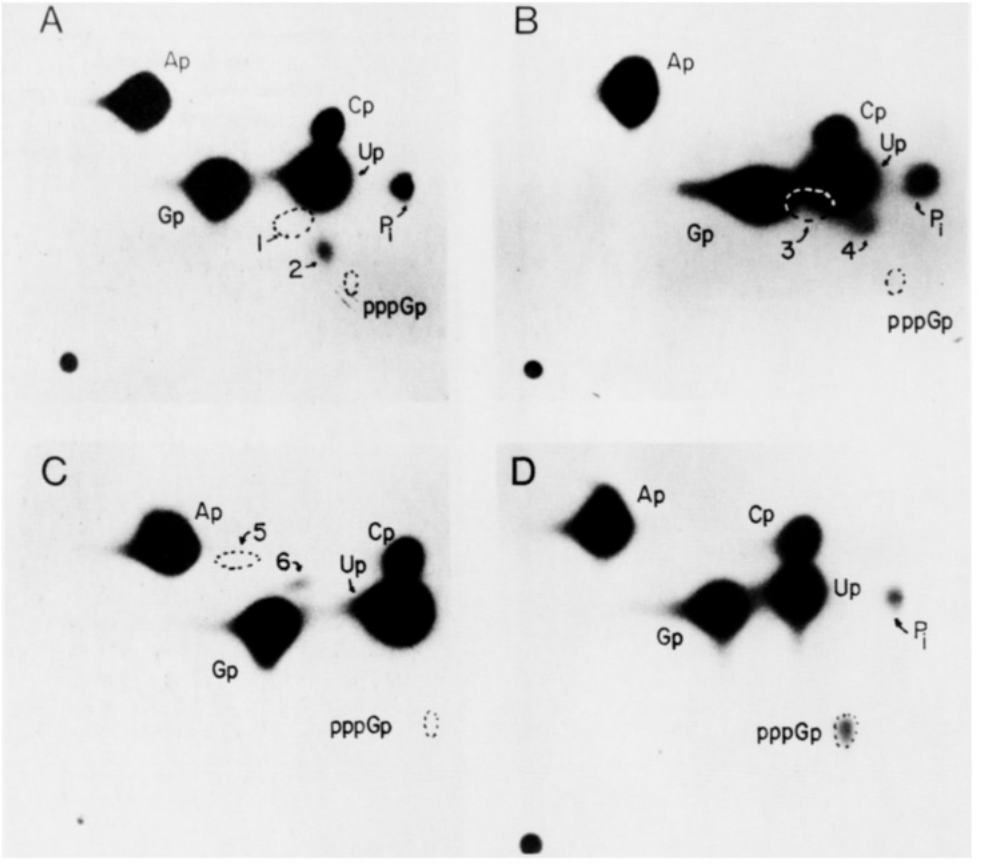


FIGURE 3: 5′-End analysis of differently capped globin transcripts. [α-^{32}P]GTP was incorporated into β-globin mRNA transcripts from a β-globin cDNA template with T7 RNA polymerase. Transcripts were transcriptionally capped with m^{7}G, e^{7}G, and bn^{7}G by addition of the corresponding NpppG dinucleotide in the transcription mixture; uncapped mRNA was synthesized by omitting the cap dinucleotide. The samples (50000 cpm) were digested with T2 RNase for 15 min at 37 °C, and the resulting products were analyzed by two-dimensional chromatography (Konarska et al., 1984). The autoradiograms are shown: (A) m^{7}G-capped globin mRNA; (B) e^{7}G-capped globin mRNA; (C) bn^{7}G-capped globin mRNA; (D) uncapped mRNA. Positions of 3′-NMPs, pppGp, and NpppG were determined by cochromatography with known nucleotide standards: (1) m^{7}GpppG; (2) m^{7}GpppGp; (3) e^{7}GpppG; (4) e^{7}GpppGp; (5) bn^{7}GpppG; (6) bn^{7}GpppGp.

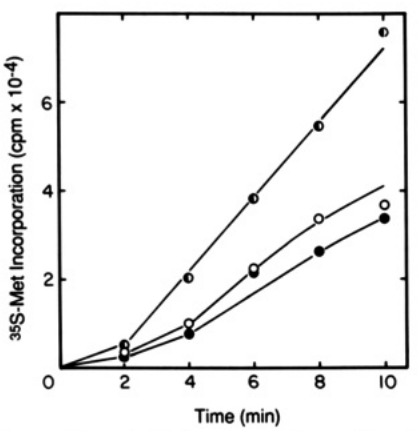


FIGURE 4: Comparison of relative translation activity of bn^{7}G- and e^{7}G-capped β-globin mRNAs. β-Globin mRNA was transcribed from a β-globin cDNA template with T7 RNA polymerase. Transcripts were capped with m^{7}G (○), e^{7}G (●), and bn^{7}G (◐) and translated in reticulocyte lysate. Five-microliter aliquots were taken at the time points indicated and hot TCA precipitated. The amount of incorporated radiolabel is plotted vs time.

locyte salt wash, followed by UV irradiation to cross-link RNA–protein complexes (Pelletier & Sonenberg, 1985). After RNase treatment the complexed proteins were analyzed by SDS gel electrophoresis and autoradiography. As shown in Figure 5, cross-linking to polypeptides corresponding to eIF-4B (M_r 80000) and eIF-4E (M_r 26000) was inhibited by all three cap analogues, but to varying total extents. The degree of RNA cross-linking to either polypeptide changed in parallel with the relative activity of each analogue. From this analysis bn^{7}GMP antagonized UV cross-linking to the same cap binding proteins as did m^{7}GMP but to a greater extent. By contrast e^{7}GMP was a slightly less effective inhibitor in the UV cross-linking assay relative to m^{7}GMP for eIF-4B and not significantly different from m^{7}GMP for eIF-4E. The observed activity of bn^{7}GMP (9) as a relatively potent inhibitor of translation in comparison to m^{7}GMP was corroborated both by the translation and by UV cross-linking assays. We obtained similar results by using human β-globin SP6 transcripts in UV cross-linking studies (data not shown).

Discussion

Previous nucleotide cap analogue studies showed that substitution of the N7 methyl by ethyl, carboxymethyl, or benzyl does not alter inhibitory activity of the analogue in translation assays. These data led to the proposal that the N7 substituent is necessary to ensure that a delocalized positive charge resides in the imidazole portion of the purine ring. This positive charge is thought to be required for electrostatic interaction with the negatively charged 5′-phosphate group(s) to maintain the "rigid" anti conformation of the capping nucleotide (Kim & Sarma, 1978; Darzynkiewicz et al., 1981, 1987). By contrast, N7 alkyl and alicyclic derivatives of GMP synthesized for this study differed in activity apparently due to simple steric exclusion of the larger cap analogues from the nucleotide binding domain of CBPs. Although a direct examination of the affinity of purified eIF-4F for cap analogues was not made, we are confident that our conclusions can be extended to the nature

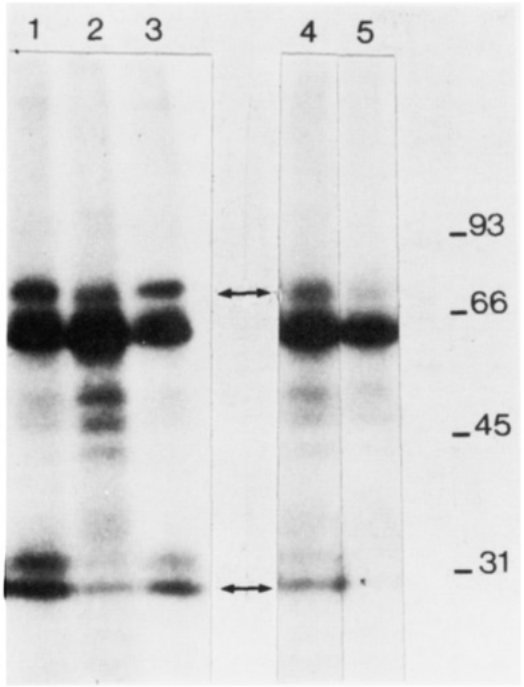


FIGURE 5: Inhibition of UV cross-linking of mRNA to cap binding proteins. The extent of ^{32}P-labeled mRNA UV cross-linked to crude reticulocyte initiation factors was assayed in the presence of cap analogues at a concentration of 1 mM, as listed below. Values for extent of cross-linking relative to control (lane 1) for eIF-4B and eIF-4E, respectively, are in parentheses. Reaction mixtures were resolved on an SDS–polyacrylamide gel. The autoradiogram is shown. Arrows denote positions of eIF-4B (M_r 80000) and eIF-4E (M_r 26000). (Lane 1) Control (100%, 100%); (lane 2) m^{7}GMP (29%, 25%); (lane 3) GMP (82%, 47%); (lane 4) e^{7}GMP (32%, 23%); (lane 5) bn^{7}GMP (9%, 2%).

of the protein–ligand interaction since eIF-4E and eIF-4F are the only true m^{7}G binding activities in translation. From our studies, alkyl groups larger than ethyl were not well recognized by CBPs. 7-Propyl-GMP (**2**) was not inhibitory, in comparison to m^{7}GMP and e^{7}GMP. Also a secondary carbon bonded to N7 precluded binding and competition as observed for ip^{7}GMP (**4**). The inhibitory effect of a^{7}GMP (**3**) was not clear. Although larger than **1**, **3** was more inhibitory than **2** (Figure 2A). It is not apparent at this time if the olefinic nature of this substituent resulted in a different substituent conformation that allowed it to enter the N7 substituent binding domain or whether it interacted with the cap binding protein through a π–π interaction similar to that proposed for cap analogues **9** and **10** (see below). As we also demonstrated, an increase in the size of the N7 moiety did not alter the syn–anti conformation of the analogues relative to m^{7}GMP. Darzynkiewicz et al. (1987) showed that a cap analogue with syn conformation [i.e., 7,8-dimethylguanosine 5′-monophosphate, $m_2^{7,8}$GMP; also Adams et al. (1978)] was inhibitory in translation assays, although less so than m^{7}GMP. These data do suggest a preference for the anti conformation in ligand binding. Therefore, this type of conformational change was not responsible for the loss of binding of the larger N7-substituted cap analogues to the cognate CBPs. By contrast, benzyl and its higher order analogue 2-phenylethyl, when present at the N7 position, did not diminish the activity of the cap analogue as might be expected from simple size considerations. A similar inhibitor effect of 7-benzyl substitution of GDP was observed earlier (Adams et al., 1978). On the basis of our recent results, the earlier model proposed by Adams et al. (1978) must be modified by inclusion of a size and shape limitation of the N7 moiety. We now propose that the carbon bonded to N7 must be a primary carbon and that the entire N7 substituent must be smaller than ethyl in size for facile recognition by cap binding proteins. It should be stated that bn^{7}GMP (**9**) and 7-(2-PhEt)GMP do not follow the behavior of the alkyl, allyl, and alicyclic substituents which were tested and thus must be considered separately in terms of their interactions with CBPs.

The incorporation of bn^{7}G and e^{7}G as the 5′-cap of synthetic globin mRNAs afforded a direct test of the relative affinity of these cap structures for cap binding proteins. By this approach we demonstrated that the inferred higher affinity for cap binding proteins shown by bn^{7}GMP was corroborated by an observed increase in translation efficiency of bn^{7}G-capped globin mRNA relative to its m^{7}G-capped counterpart. The lower degree of competition by e^{7}GMP was consistent with a lowered affinity of this cis cap structure for cap binding proteins, although this was not apparent from the UV cross-linking experiment.

Our results indicated that the N7 ligand binding site of the cap binding protein is of limited size and may have an unusual structure since a branched or secondary carbon atom attached to the N7 position of the guanine cap was apparently too bulky for efficient interaction with the cap binding protein. Yet this domain did appear to be capable of accepting a phenyl moiety as in analogues **9** and **10**. Perhaps this domain is a deep pocket with a narrow opening. Along these lines, model studies show a potential interaction of the cap with Trp residue(s) of eIF-4E/eIF-4F (Ishida et al., 1988; Ueda et al., 1988). These workers suggest that aromatic ring stacking interactions between the guanine base and indolyl residue(s) of the mRNA cap and protein might occur during ligand binding. Hence, binding of **9** and **10** to CBPs could be further stabilized by stacking interactions involving the N7 substituents even though these are much larger than ethyl. eIF-4E is known to have a high tryptophan content (8 out of 247 amino acid residues; Rychlik et al., 1987; Altmann et al., 1988) compared to an average value of 1.4 Trp/100 amino acid residues in proteins of eukaryotes (Doolittle, 1986). Thus, it is possible that some of these tryptophanyl residues may be involved in ligand binding. This is supported by recent mutagenesis studies performed with yeast eIF-4E which demonstrated that substitution of two specific Trp residues resulted in a loss of initiation factor activity (Altmann et al., 1988).

The translation results observed for bn^{7}GMP (**9**) are another example of a guanine nucleoside monophosphate cap analogue with greater activity than m^{7}GMP as a competitive inhibitor of capped mRNA translation. As we reported earlier, 2,7-dimethylguanosine 5′-monophosphate ($m_2^{2,7}$GMP) is also more inhibitory than m^{7}GMP. The former, when present as the cap structure on mRNA, also increases translation activity of the template above that observed for the m^{7}G-capped form of mRNA (Darzynkiewicz et al., 1988). Although $m_2^{2,7}$G cap structures are found on a small but significant proportion of togaviridae mRNAs (HsuChen & Dubin, 1976; van Duijn et al., 1986), there is no precedence for bn^{7}G cap structures, in vivo. Nonetheless, it still remains to be seen how the molecular interactions between m^{7}G, $m_2^{2,7}$G, and bn^{7}G caps with the cap binding protein compare.

ACKNOWLEDGMENTS

We thank Aaron Shatkin for helpful discussions and comments and Laura Steel for preparation of the manuscript.

Registry No. **1**, 111289-78-2; **2**, 111289-77-1; **3**, 120362-38-1; **4**, 111289-74-8; **5**, 111289-76-0; **6**, 111289-73-7; **7**, 120362-39-2; **8**, 120362-40-5; **9**, 111289-75-9; **10**, 120362-41-6; **11**, 120362-42-7; 5′-GMP, 85-32-5; bn^{7}G(5′)ppp(5′)G, 120362-43-8; e^{7}G(5′)ppp(5′)G,

120362-44-9; ethyl iodide, 75-03-6; propyl iodide, 107-08-4; allyl bromide, 106-95-6; isopropyl bromide, 75-26-3; butyl iodide, 542-69-8; isobutyl bromide, 78-77-3; cyclopentyl bromide, 137-43-9; bromoacetic acid, 79-08-3; benzyl chloride, 100-44-7; 2-phenylethyl bromide, 103-63-9; 1-phenylethyl bromide, 585-71-7.

References

Adams, B. L., Morgan, M., Muthukrishnan, S., Hecht, S. M., & Shatkin, A. J. (1978) *J. Biol. Chem. 253*, 2589–2595.

Altmann, M., Edery, I., Trachsel, H., & Sonenberg, N. (1988) *J. Biol. Chem. 263*, 17229–17232.

Banerjee, A. K. (1980) *Microbiol. Rev. 44*, 175–205.

Beaton, A. R., & Krug, R. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6282–6286.

Darzynkiewicz, E., Antosiewicz, J., Ekiel, I., Morgan, M. A., Tahara, S. M., & Shatkin, A. J. (1981) *J. Mol. Biol. 153*, 451–458.

Darzynkiewicz, E., Ekiel, I., Tahara, S. M., Seliger, L. S., & Shatkin, A. J. (1985) *Biochemistry 24*, 1701–1707.

Darzynkiewicz, E., Ekiel, I., Lassota, P., & Tahara, S. M. (1987) *Biochemistry 26*, 4372–4380.

Darzynkiewicz, E., Stepinski, J., Ekiel, I., Jin, Y., Haber, D., Sijuwade, T., & Tahara, S. M. (1988) *Nucleic Acids Res. 16*, 8953–8962.

Davies, D. B. (1978) *Prog. Nucl. Magn. Reson. Spectrosc. 12*, 135–225.

Doolittle, R. F. (1986) *Of URFs and ORFs*, University Science Books, Mill Valley, CA.

Edery, I., & Sonenberg, N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7590–7594.

Edery, I., Humbelin, M., Darveau, A., Lee, K. A. W., Milburn, S., Hershey, J. W. B., Trachsel, H., & Sonenberg, N. (1983) *J. Biol. Chem. 258*, 11398–11403.

Furuichi, Y., Morgan, M. A., & Shatkin, A. J. (1979) *J. Biol. Chem. 254*, 6732–6738.

Furuichi, Y., La Fiandra, A., & Shatkin, A. J. (1977) *Nature (London) 266*, 235–239.

Georgiev, O., Mous, J., & Birnstiel, M. S. (1984) *Nucleic Acids Res. 12*, 8539–8551.

Green, M. R., Maniatis, T., & Melton, D. A. (1983) *Cell 32*, 681–694.

Grifo, J. A., Tahara, S., Leis, J., Morgan, M. A., Shatkin, A. J., & Merrick, W. C. (1982) *J. Biol. Chem. 257*, 5246–5252.

Grifo, J. A., Tahara, S. M., Morgan, M. A., Shatkin, A. J., & Merrick, W. C. (1983) *J. Biol. Chem. 258*, 5804–5810.

Hickey, E. D., Weber, L. A., Baglioni, C., Kim, C. H., & Sarma, R. H. (1977) *J. Mol. Biol. 109*, 173–183.

HsuChen, C.-C., & Dubin, D. T. (1976) *Nature (London) 264*, 190–191.

Ishida, T., Doi, M., Ueda, H., Inoue, M., & Scheldrick, G. M. (1988) *J. Am. Chem. Soc. 110*, 2286–2294.

Johnson, C. E., Jr., & Bovey, F. A. (1958) *J. Chem. Phys. 29*, 1012–1014.

Kim, C. H., & Sarma, R. H. (1978) *J. Am. Chem. Soc. 100*, 1571–1590.

Konarska, M. M., Padgett, R. A., & Sharp, P. A. (1984) *Cell 38*, 731–736.

Krystosek, A., Cawthon, M. L., & Kabat, D. (1975) *J. Biol. Chem. 250*, 6077–6084.

Miura, K.-I., Kodama, Y., Shimotohna, K., Fukui, T., Ikehara, M., Nakagawa, I., & Hata, T. (1979) *Biochim. Biophys. Acta 564*, 264–274.

Nakagawa, I., Konya, S., Ohtani, S., & Hata, T. (1980) *Synthesis*, 556–557.

Pain, V. (1986) *Biochem. J. 235*, 625–637.

Pelham, H. P. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–256.

Pelletier, J., & Sonenberg, N. (1985) *Mol. Cell. Biol. 5*, 3222–3230.

Ruskin, B., Pikielny, C. W., Rosbash, M., & Green, M. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2022–2026.

Rychlik, W., Domier, L., Gardner, P. R., Hellman, G. M., & Rhoads, R. E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 945–949.

Shatkin, A. J. (1985) *Cell 40*, 223–224.

Sonenberg, N., Morgan, M. A., Merrick, W. C., & Shatkin, A. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4843–4847.

Tahara, S. M., Morgan, M. A., & Shatkin, A. J. (1981) *J. Biol. Chem. 256*, 7691–7694.

Ueda, H., Doi, M., Inoue, M., Ishida, T., Tanaka, T., & Uesugi, S. (1988) *Biochem. Biophys. Res. Commun. 154*, 199–204.

Ulmanen, I., Broni, B., & Krug, R. M. (1983) *J. Virol. 45*, 27–35.

van Duijn, L. P., Kasperaitis, M., Ameling, C., & Voorma, H. O. (1986) *Virus Res. 5*, 61–66.