Quantitative Assessment of mRNA Cap Analogues as Inhibitors of in Vitro Translation†

Aili Cai,‡ Marzena Jankowska-Anyszka,§ Adrian Centers,|| Lidia Chlebicka,⊥ Janusz Stepinski,§ Ryszard Stolarski,⊥ Edward Darzynkiewicz,⊥ and Robert E. Rhoads*,‡

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, Louisiana 71130-3932, Department of Chemistry, University of Warsaw, 02-093 Warsaw, Poland, Department of Biophysics, University of Warsaw, 02-089 Warsaw, Poland, and Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536

Received December 22, 1998; Revised Manuscript Received May 5, 1999

ABSTRACT: Fifty-eight analogues of the 5′-terminal 7-methylguanosine-containing cap of eukaryotic messenger RNA were synthesized and tested for their ability to inhibit in vitro protein synthesis. A new algorithm was developed for extracting K_I, the dissociation constant for the cap analogue·eIF4E complex, from protein synthesis data. The results indicated that addition of a methyl group to the N2 of guanine produced more inhibitory compounds, but addition of a second methyl group to N2 decreased the level of inhibition dramatically. Aryl substitution at N7 improved the efficacy of guanine nucleoside monophosphate analogues. Substitution of the aromatic ring at the para position with methyl or NO_2 groups abolished this effect, but substitution with Cl or F enhanced it. By contrast, aryl substitution at N7 in nucleoside di- or triphosphate analogues produced only minor effects, both positive and negative. By far the strongest determinants of inhibitory activity for cap analogues were phosphate residues. The beneficial effect of more phosphate residues was related more to anionic charge than to the number of phosphate groups per se. The second nucleotide residue in analogues of the form m^7GpppN affected inhibitory activity in the order G > C > U > A, but there was no effect of 2′-*O*-modification. Opening the first ribose ring of m^7GpppG analogues dramatically decreased activity, but alterations at the 2′-position of this ribose had no effect. Non-nucleotide-based cap analogues containing benzimidazole derivatives were inhibitory, though less so than those containing 7-methylguanine.

Messenger RNA is recruited during the initiation of protein synthesis by binding to the 43S initiation complex to form the 48S initiation complex (*1*). Cis-acting elements in mRNA that stimulate this process include the 5′-terminal 7-methylguanosine-containing cap, the 3′-terminal poly(A) tract, and, in a small subset of viral and cellular mRNAs, an internal ribosome entry sequence (*2*). A number of factors determine the degree to which translation is dependent on each of these cis-acting elements. These include the mRNA in question, the cell type, and alterations of cells caused by heat shock (*3*), virus infection (*4*), and meiotic maturation (*5*). Cap-dependent initiation also requires trans-acting factors that include eIF3[1] and members of the eIF4 group (*1*). The latter group is composed of eIF4A, a 46 kDa ATP-dependent RNA helicase; eIF4B, a 70 kDa protein that anneals complementary RNA strands and also stimulates eIF4A activity; eIF4E, a 25 kDa cap-binding protein; and eIF4G, a linking protein that has specific binding sites for RNA, eIF3, eIF4A, eIF4E, and the poly(A)-binding protein (*6*−*10*).

eIF4E has been isolated from a wide variety of species, including human, rabbit, mouse, rat, *Xenopus laevis*, *Saccharomyces cerevisiae*, *Saccharomyces pombe*, wheat, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Aplysia californica* (*11*−*13*, and references therein). Intriguingly, multiple isoforms of eIF4E differing in primary structure have been found in a single species, e.g., human (*14*, *15*), wheat (*16*), and *C. elegans* (*12*). In all of these proteins, the amino acid sequences exhibit conservation of a central core domain, suggesting a similar tertiary structure of the protein. The tertiary structure of this core domain has been determined in the case of mouse (*17*) and yeast (*18*) eIF4E.

At least three regulatory mechanisms govern the availability of eIF4E for protein synthesis. Phosphorylation of mammalian eIF4E at Ser-209 in response to insulin and other mitogens increases the affinity for caps by 3−4-fold (*19*−*23*). Binding of PHAS-I (also called 4E-BP) sequesters eIF4E and prevents its interaction with eIF4G, but phosphorylation

† This work was supported by Grant GM20818 from the National Institute of General Medical Sciences, Grant MEN/NSF-98-337 from the U.S.-Poland Maria Sklodowska-Curie Joint Fund II, and Grant KBN6PO4A03409 from the Polish Committee for Scientific Research.

* To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, 1501 Kings Highway, Shreveport, LA 71130-3932. Telephone: (318) 675-5156. Fax: (318) 675-5180. E-mail: rrhoad@lsumc.edu.

‡ Louisiana State University Medical Center.

§ Department of Chemistry, University of Warsaw, 02-093 Warsaw.

|| University of Kentucky.

⊥ Department of Biophysics, University of Warsaw.

[1] Abbreviations: eIF, eukaryotic initiation factor; MP, monophosphate; DP, diphosphate; TP, triphosphate.

10.1021/bi9830213 CCC: $18.00 © 1999 American Chemical Society
Published on Web 06/11/1999

of PHAS-I in response to insulin and other mitogens releases eIF4E (*24*−*26*). Finally, the rate of eIF4E gene transcription is increased by a variety of mitogenic stimuli (*27*, *28*).

Analogues of the mRNA cap have been useful in studying eIF4E function and cap-dependent translation. Initial studies demonstrated that 7-methyl GMP and 7-methyl GTP prevent binding of mRNA to initiation complexes, prevent cross-linking of periodate-oxidized mRNA to eIF4E, and inhibit in vitro protein synthesis (reviewed in ref *29*). Cap analogue-sensitive cross-linking to periodate-oxidized mRNA and cap analogue inhibition of protein synthesis were the assays initially used for identification and purification of eIF4E (*30*, *31*). Immobilized cap analogues form the basis for affinity chromatography of eIF4E (*32*, *33*). The direct binding of cap analogues to eIF4E has been assessed by quenching of intrinsic Trp fluorescence in eIF4E (*34*−*36*) and enhancement of 7-methylguanosine fluorescence (*35*). Varying the structure of the cap analogue demonstrated that binding to eIF4E (or inhibition of protein synthesis) can still occur if (1) the 7-substituent is an aryl or alkyl group other than methyl, (2) the ribose rings are substituted at the 2′-position, and (3) the N2 of guanine is substituted with one but not more methyl groups (*37*−*41*). Cap analogues with more phosphate groups or that are part of an oligonucleotide bind eIF4E with greater affinity (*39*, *42*).

Messenger RNAs differ in their dependence on the cap structure for initiation. Cap analogues added to an in vitro protein synthesis system inhibit different mRNAs to varying degrees (reviewed in ref *29*). In vivo, stimulation of the signal transduction pathways leading from insulin receptor to phosphorylation of eIF4E and PHAS-I increases the rate of translation of c-Myc mRNA but not actin mRNA (*43*). Overexpression of eIF4E in cultured cells causes preferential translation of a small subset of mRNAs that are involved in cellular growth and cell cycle progression that have been termed "growth-regulated" (reviewed in refs *44* and *45*). These include the mRNAs encoding ornithine decarboxylase, ornithine aminotransferase, c-Myc, cyclin D1, ribonucleotide reductase, basic fibroblast growth factor, vascular endothelial growth factor, and several others. These growth-regulated mRNAs tend to have unusually long 5′-untranslated regions that contain high degrees of secondary structure, upstream open reading frames, or non-AUG initiation codons. By contrast, eIF4E is less important for translation of "housekeeping" mRNAs. Although the mechanism by which eIF4E preferentially increases the rate of translation of growth-regulated mRNAs is not well understood, it is likely to be related to the unwinding of mRNA secondary structure by complexes of eIF4 factors (*46*, *47*).

This selective stimulation of growth-regulated mRNA translation by eIF4E may explain the observation that excess intracellular levels of eIF4E are correlated with abnormal cell growth. Overexpression of eIF4E in cultured cells from transfected vectors causes accelerated growth, loss of contact inhibition, growth in soft agar, and formation of tumors in nude mice (*48*, *49*). Malignant breast carcinomas have greatly elevated levels of eIF4E (*50*−*52*), and eIF4E can serve as an independent prognostic indicator of clinical outcome (*53*, *54*). Similarly, recurrence of head-and-neck cancers is predicted by the level of eIF4E in tumor margins (*55*). Amplification of the eIF4E gene has been reported for breast carcinomas (*56*).

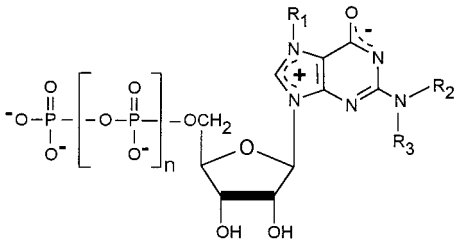
These findings suggest the possibility that inhibiting the action of eIF4E pharmacologically might preferentially interfere with the growth and division of rapidly growing cells, e.g., those that are malignantly transformed. Indeed, the expression of antisense RNA directed against eIF4E mRNA slows growth and produces a more normal phenotype in *ras*-transformed fibroblasts (*57*), and expression of PHAS-I and -II, which sequester eIF4E, reverses the oncogenic phenotype of *src*-transformed cells (*58*). Similarly, rapamycin, which inhibits the branch of the phosphatidylinolitol 3-kinase pathway leading to PHAS-I phosphorylation (*43*, *59*, *60*), does not affect basal protein synthesis but completely blocks insulin-stimulated synthesis of the growth-regulated protein c-Myc as well as DNA synthesis and cell cycle progression (*43*).

One possibility for interfering with the action of eIF4E would be to develop cap analogues with high affinity and specificity for eIF4E. However, dissociation constants measured by direct binding between cap analogues and purified eIF4E often do not correlate well with efficacy as inhibitors of cap-dependent protein synthesis (see the Discussion). Therefore, we chose in this study to test cap analogues as inhibitors of in vitro translation. To compare various cap analogues quantitatively, we developed a new algorithm for extracting dissociation constants from in vitro translation data. Using this algorithm, we re-evaluated a number of cap analogues previously tested and also examined a series of new cap analogues.

MATERIALS AND METHODS

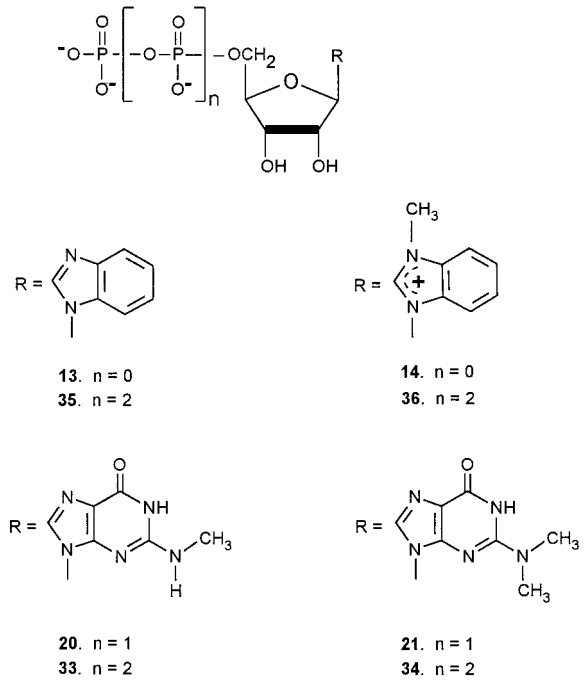
Cap Analogues. Cap analogues (Figures 1−5) were synthesized by the procedures referenced in Table 1. The concentration of each cap analogue was calculated from absorbance measurements as follows. For compounds **1**−**12**, **15**−**19**, and **22**−**32**, the absorbance was read at two pH values and two wavelengths, since 7-methylguanine exists as a mixture of cationic and zwitterionic forms at neutral pH (*61*). The concentration was calculated from each absorbance reading using the following molar extinction coefficients: pH 6.0, 258 nm, 11.4×10^3; pH 6.0, 282 nm, 7.34×10^3; pH 9.0, 258 nm, 6.05×10^3; and pH 9.0, 282 nm, 8.78×10^3. The average concentration was calculated from the four readings. For other cap analogues, a single absorbance reading was taken at pH 7.0, and the following λ_{max} and extinction coefficients were used: **13**, 245 nm, 7.4×10^3; **14**, 270 nm, 7.0×10^3; **20** and **33**, 253 nm, 11.8×10^3; **21** and **34**, 260 nm, 13.7×10^3; **35**, 245 nm, 7.0×10^3; **36**, 270 nm, 6.9×10^3; **37**, 255 nm, 23.0×10^3; **38** and **48**, 259 nm, 16.0×10^3; **39**, 255 nm, 21.4×10^3; **40**, **50**, and **51**, 255 nm, 22.6×10^3; **41** and **55**, 251 nm, 25.5×10^3; **42**, 255 nm, 19.6×10^3; **43**, 255 nm, 19.3×10^3; **44**, 259 nm, 21.3×10^3; **45**, 262 nm, 21.1×10^3; **46**, 262 nm, 16.7×10^3; **47**, 260 nm, 18.5×10^3; **49**, 256 nm, 23.5×10^3; **52**, 251 nm, 19.8×10^3; **53**, **54**, and **58**, 255 nm, 21.0×10^3; **56**, 258 nm, 17.0×10^3; and **57**, 256 nm, 23.0×10^3.

In Vitro Translation. Micrococcal nuclease-treated reticulocyte lysate was prepared from anemic rabbits as described previously (*62*). Several preparations of reticulocyte lysate were used in the course of this study. Rabbit globin mRNA was prepared from polyribosomes of reticulocyte lysate and used at a concentration of 5 μg/mL (*63*). Uncapped rabbit



1. $R_1 = CH_3$, $R_2 = R_3 = H$, n = 0
2. $R_1 = C_6H_5CH_2CH_2$, $R_2 = R_3 = H$, n = 0
3. $R_1 = C_6H_5CH_2$, $R_2 = R_3 = H$, n = 0
4. $R_1 = p\text{-}CH_3C_6H_4CH_2$, $R_2 = R_3 = H$, n = 0
5. $R_1 = p\text{-}NO_2C_6H_4CH_2$, $R_2 = R_3 = H$, n = 0
6. $R_1 = p\text{-}ClC_6H_4CH_2$, $R_2 = R_3 = H$, n = 0
7. $R_1 = R_2 = CH_3$, $R_3 = H$, n = 0
8. $R_1 = CH_3$, $R_2 = CH_3CH_2$, $R_3 = H$, n = 0
9. $R_1 = CH_3CH_2$, $R_2 = CH_3$, $R_3 = H$, n = 0
10. $R_1 = C_6H_5CH_2CH_2$, $R_2 = CH_3$, $R_3 = H$, n = 0
11. $R_1 = p\text{-}ClC_6H_4CH_2$, $R_2 = CH_3$, $R_3 = H$, n = 0
15. $R_1 = CH_3$, $R_2 = R_3 = H$, n = 1
16. $R_1 = p\text{-}ClC_6H_4CH_2$, $R_2 = R_3 = H$, n = 1
17. $R_1 = p\text{-}ClC_6H_4CH_2$, $R_2 = CH_3$, $R_3 = H$, n = 1
18. $R_1 = R_2 = R_3 = CH_3$, n =1
19. $R_1 = p\text{-}ClC_6H_4CH_2$, $R_2 = R_3 = CH_3$, n = 1
22. $R_1 = CH_3$, $R_2 = R_3 = H$, n = 2
23. $R_1 = C_6H_5CH_2$, $R_2 = R_3 = H$, n = 2
24. $R_1 = p\text{-}ClC_6H_4CH_2$, $R_2 = R_3 = H$, n = 2
25. $R_1 = p\text{-}FC_6H_4CH_2$, $R_2 = R_3 = H$, n = 2
26. $R_1 = R_2 = CH_3$, $R_3 = H$, n = 2
27. $R_1 = C_6H_5CH_2$, $R_2 = CH_3$, $R_3 = H$, n = 2
28. $R_1 = p\text{-}ClC_6H_4CH_2$, $R_2 = CH_3$, $R_3 = H$, n = 2
29. $R_1 = p\text{-}FC_6H_4CH_2$, $R_2 = CH_3$, $R_3 = H$, n = 2
30. $R_1 = R_2 = R_3 = CH_3$, n = 2
31. $R_1 = p\text{-}ClC_6H_4CH_2$, $R_2 = R_3 = CH_3$, n = 2
32. $R_1 = p\text{-}FC_6H_4CH_2$, $R_2 = R_3 = CH_3$, n = 2

FIGURE 1: 7-Substituted guanosine 5′-mono-, di-, and triphosphate cap analogues.



13. n = 0
35. n = 2
14. n = 0
36. n = 2
20. n = 1
33. n = 2
21. n = 1
34. n = 2

FIGURE 2: 7-Unsubstituted guanosine and benzimidazole riboside 5′-mono-, di-, and triphosphate cap analogues.

β-globin mRNA was synthesized by in vitro transcription of plasmid pOGβ34 (*64*) using SP6 polymerase following the manufacturer's instructions (*65*) and used in translation reactions at a concentration of 40 μg/mL. [^{3}H]Leu (60 Ci/mmol) was purchased from DuPont-New England Nuclear. The concentrations of all components in translation reactions were as described previously (*63*) except that $MgCl_2$ was at 1.0 mM and potassium acetate was adjusted to 150−170 mM to give maximal translation for each preparation of lysate.

Assay of Cap Analogues. The biological activity of each cap analogue was tested at six or seven concentrations by inhibition of translation at 30 °C for 1 h. For groups I and compounds **35** and **36** (see Table 1), the range of concentrations was 16−1000 μM. For groups IVa and IVb, the range was 16−500 μM. For groups II, III, and IVc, the range was 6−200 μM. In each assay, a standard cap analogue was included at each of the same concentrations (see below).

Calculation of K_I Values. The competition between a cap analogue and an authentic mRNA cap for binding to the active site of eIF4E is formally similar to the competition between a competitive inhibitor and a substrate for binding to the active site of an enzyme. However, protein synthesis is the result of a large number of sequential catalytic reactions and does not depend solely on eIF4E activity. Furthermore, as the initiation phase of protein synthesis is progressively inhibited, the rate of protein synthesis is increasingly limited by initiation rather than by elongation or termination, so dependence on eIF4E increases. A kinetic model was previously developed that takes into account the rate constants of both initiation and elongation (*66*). This was subsequently expanded to include terms for the cap analogue·eIF4E complex, K_I (*63*). In this formulation, the number of new polypeptide chains initiated per minute, Q_i, is given by the expression

$$Q_i = k_1 m_0 (1 - nL)[(R_0 - m_0 nN)/(1 + I/K_I)] \quad (1)$$

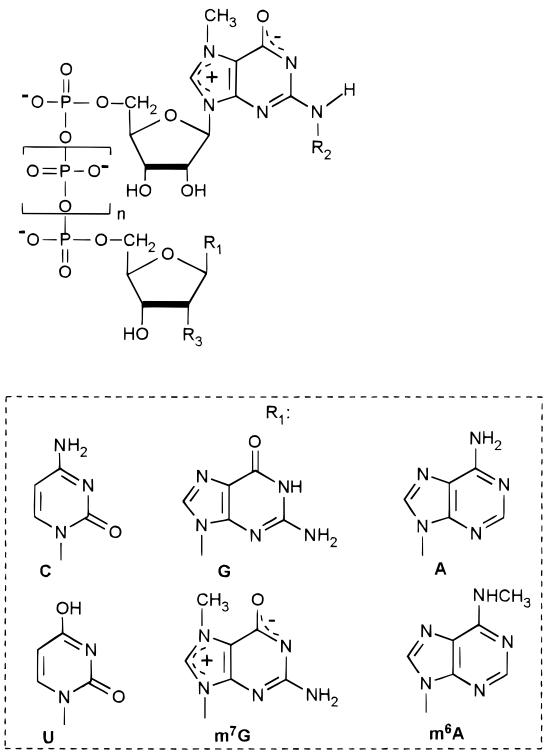
where k_1 is the rate constant for initiation, m_0 is the total concentration of mRNA, n is the probability that a codon is covered by a ribosome, L is the number of codons covered by one ribosome, R_0 is the concentration of ribosomes, and N is the number of codons in the mRNA. The value of n is obtained by setting Q_i equal to the flux of ribosomes across a particular codon, Q_e, which is the situation that occurs at steady state (*63*).

A modification of the kinetic model previously developed (*63*) was made in the study presented here to take into account the fact that a certain fraction of translation is independent of the cap. This has been shown in several ways, e.g., the fact that uncapped mRNAs are translated at all, albeit at a lower rate (*67*, *68*), and that cap analogues fail to inhibit translation completely (*67*).

Thus,

$$Q_t = Q_{ci} + Q_i \quad (2)$$

where Q_t is the total rate of translation, Q_{ci} is the cap-independent component, and Q_i is the cap-dependent component, as defined in eq 1. The relative contributions of Q_{ci} and Q_i to Q_t vary as a function of many factors, including



37. R_1 = **G**, R_2 = H, R_3 = OH, n = 0
38. R_1 = **m^{7}G**, R_2 = H, R_3 = OH, n = 0
40. R_1 = **G**, R_2 = H, R_3 = OH, n = 1
42. R_1 = **G**, R_2 = H, R_3 = OCH_3, n = 1
43. R_1 = **G**, R_2 = R_3 = H, n = 1
44. R_1 = **A**, R_2 = H, R_3 = OH, n = 1
45. R_1 = **m^{6}A**, R_2 = H, R_3 = OH, n = 1
46. R_1 = **C**, R_2 = H, R_3 = OH, n = 1
47. R_1 = **U**, R_2 = H, R_3 = OH, n = 1
48. R_1 = **m^{7}G**, R_2 = H, R_3 = OH, n = 1
49. R_1 = **G**, R_2 = CH_3, R_3 = OH, n = 1
54. R_1 = **G**, R_2 = H, R_3 = OH, n = 2
56. R_1 = **m^{7}G**, R_2 = H, R_3 = OH, n = 2
57. R_1 = **G**, R_2 = CH_3, R_3 = OH, n = 2

FIGURE 3: Dinucleotide cap analogues with 7-methylguanine.

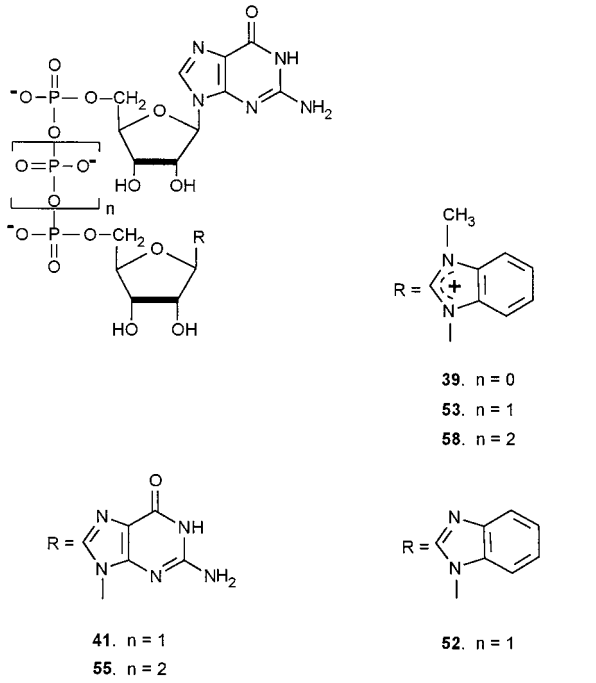
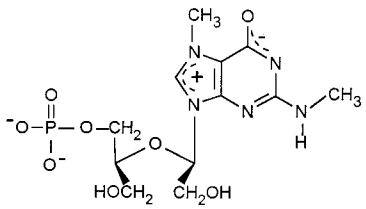
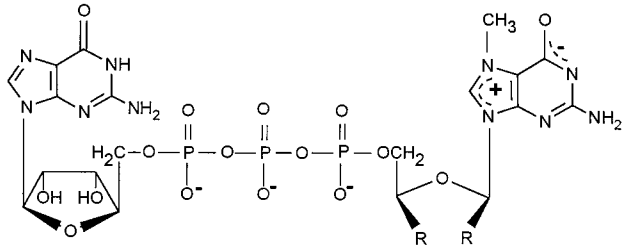


FIGURE 4: Dinucleotide cap analogues with benzimidazole and unsubstituted guanine.



12.



50. R = CH_2OH
51. R = H

FIGURE 5: Ribose ring-opened cap analogues.

the type of mRNA being translated, the pH, the ionic strength, the mRNA concentration, and the particular batch of reticulocyte lysate that is used (reviewed in ref *29*). Q_{ci} was therefore treated as an unknown to be determined in the curve-fitting algorithm (see below).

The program KaleidaGraph (version 3.06; Synergy Software, Reading, PA) was used to fit curves in which the extent of inhibition of in vitro translation was measured as a function of cap analogue concentration. The fit of eq 2 to the experimental data was obtained by iterative least-squares minimization. The independent variable was the cap analogue concentration, I (in micromolar). The dependent variable was radioactivity incorporated into globin, Q_t (in counts per minute). Three constants were optimized by the curve-fitting algorithm: Q_{ci}, K_I, and Q_i^0, the latter being the cap-dependent rate of translation with no inhibitor present.

Normalization of K_I Values between Experiments. As noted above, the degree to which in vitro translation is dependent on the cap is influenced by many factors, including the batch of lysate that is used. To allow comparison between experiments, we included a standard cap analogue in every assay of an unknown cap analogue. However, since K_I values for the various cap analogues ranged from 2 to 1000 μM, it was not possible to use a single standard cap analogue for all compounds. Instead, a standard cap analogue was used that had a K_I most similar to that expected for the unknown cap analogue (based on structural similarities). Thus, 7-benzyl GMP was used as a standard for 7-(*p*-chlorobenzyl) GMP, 7-methyl GpppG was used as a standard for 7-methyl GpppA, etc. The standard cap analogues used for each unknown cap analogue are listed in Table 1.

A primary standard of 7-methyl GTP (**22**) was chosen, against which all other standard cap analogues were com-

Table 1: Comparison of Cap Analogues as Inhibitors of in Vitro Protein Synthesis

number	compound	ref for synthesis[a]	ref for previous assay[a]	no. of different syntheses[b]	no. of different assays[b]	standard[c]	apparent K_I	SEM
group I: nucleoside monophosphates								
1	7-methyl GMP	*39*	*38*, *40*, *69*, 72, *73*, *78*	1	12	**22**	382	65
2	7-(2-phenylethyl) GMP	*69*	*69*	1	2	**3**	233	11
3	7-benzyl GMP	*69*	*69*	1	3	**1**	113	8
4	7-(*p*-methylbenzyl) GMP	*78*		1	2	**1**	NI[d]	
5	7-(*p*-nitrobenzyl) GMP	*78*		1	2	**1**	NI	
6	7-(*p*-chlorobenzyl) GMP	*78*		2	3	**1**, **3**	51.2	15.5
7	2,7-dimethyl GMP	*41*	*38*, *41*	1	1	**1**	151	22
8	2-ethyl-7-methyl GMP	*78*		1	2	**1**	136	3.0
9	2-methyl-7-ethyl GMP	*78*		1	1	**1**	338	116
10	2-methyl-7-(2-phenylethyl) GMP	*78*		1	1	**1**	173	26
11	2-methyl-7-(*p*-chlorobenzyl) GMP	*78*		2	2	**1**	13.9	2.1
12	2,7-dimethyl-*seco* GMP	*79*		1	1	**1**	NI	
13	benzimidazole riboside MP	*80*		2	3	**1**	NI	
14	3-methylbenzimidazole riboside MP	*80*		2	2	**1**	NI	
group II: nucleoside diphosphates								
15	7-methyl GDP	*72*, *73*		1	2	**22**	7.50	0.13
16	7-(*p*-chlorobenzyl) GDP	*78*		1	2	**15**	6.76	1.59
17	2-methyl-7-(*p*-chlorobenzyl) GDP	*78*		1	1	**15**	4.20	1.06
18	2,2,7-trimethyl GDP	*78*		1	1	**22**	132	33
19	2,2-dimethyl-7-(*p*-chlorobenzyl) GDP	*78*		1	1	**15**	109	32
20	2-methyl GDP	*78*		1	1	**22**	114	14
21	2,2-dimethyl GDP	*78*		1	1	**15**	271	92
group III: nucleoside triphosphates								
22	7-methyl GTP	*39*	*72*, *73*	1	10	none	4.39	0.89
23	7-benzyl GTP	*78*		2	3	**22**	8.42	0.53
24	7-(*p*-chlorobenzyl) GTP	*78*		2	2	**22**	7.04	1.27
25	7-(*p*-fluorobenzyl) GTP	*78*		1	2	**22**, **23**	7.35	0.85
26	2,7-dimethyl GTP	*78*		2	3	**22**	4.31	0.46
27	2-methyl-7-benzyl GTP	*78*		1	1	**22**	3.40	0.22
28	2-methyl-7-(*p*-chlorobenzyl) GTP	*78*		2	2	**22**	2.94	0.24
29	2-methyl-7-(*p*-fluorobenzyl) GTP	*78*		1	2	**22**, **23**	3.39	0.07
30	2,2,7-trimethyl GTP	*78*		1	1	**22**	75.8	18.3
31	2,2-dimethyl-7-(*p*-chlorobenzyl) GTP	*78*		1	1	**22**	38.6	10.8
32	2,2-dimethyl-7-(*p*-fluorobenzyl) GTP	*78*		1	1	**22**	83.6	11.3
33	2-methyl GTP	*78*		1	1	**22**	75.7	11.1
34	2,2-dimethyl GTP	*78*		1	1	**22**	206	23
35	benzimidazole riboside TP	*80*		1	1	**1**	284	100
36	3-methylbenzimidazole riboside TP	*80*		1	1	**1**	411	155
group IV: dinucleoside polyphosphates								
(a) diphosphates								
37	7-methyl GppG	*81*		1	1	**40**	128	9
38	7-methyl Gpp(7-methyl G)	*81*		1	1	**40**	371	52
39	3-methylbenzimidazole riboside ppG	*80*		2	2	**40**	39.4	13.8
(b) triphosphates								
40	7-methyl GpppG	*41*	*70*	2	13	**22**	17.1	1.0
41	GpppG	*81*	*72*	1	2	**40**, **54**	246	66
42	7-methyl Gppp(2′-*O*-methyl G)	*82*	*72*, *83*	1	2	**40**	20.8	2.2
43	7-methyl Gppp(2′-deoxy G)	*82*		1	1	**40**	27.8	1.9
44	7-methyl GpppA	*73*, *82*	*38*, *73*	1	1	**40**	31.5	1.9
45	7-methyl Gppp(6-methyl A)	82		1	1	**40**	33.7	2.4
46	7-methyl GpppC	*82*		1	1	**40**	22.7	1.8
47	7-methyl GpppU	*82*		1	1	**40**	24.2	1.9
48	7-methyl Gppp(7-methyl G)	*81*		1	1	**40**	31.0	4.2
49	2,7-dimethyl GpppG	*41*		1	1	**40**	14.7	2.3
50	7-methyl-*seco* GpppG	*79*		1	2	**40**	371	30.6
51	7-methyl-acyclo GpppG	*79*		1	1	**40**	73.3	15.0
52	benzimidazole riboside pppG	*80*		2	2	**40**	622	74
53	3-methylbenzimidazole riboside pppG	*80*		2	2	**40**	747	333
(c) tetraphosphates								
54	7-methyl GppppG	*81*	*70*	2	2	**40**	3.62	0.46
55	GppppG	*81*		1	1	**54**	181	28
56	7-methyl Gpppp(7-methyl G)	*81*	*70*	1	1	**54**	2.67	0.52
57	2,7-dimethyl GppppG	*81*		1	1	**54**	2.15	0.20
58	3-methylbenzimidazole riboside ppppG	*80*		2	2	**40**	268	9

[a] Literature citations are given for the synthesis of each compound and for any previously published assay of its ability to inhibit in vitro translation. [b] The number of different syntheses for each compound is given as well as the number of different times that compound was assayed for inhibition of in vitro translation in this work. [c] The cap analogue that was used as an internal standard in determining the inhibition of in vitro translation. [d] No inhibition (apparent $K_i > 1000$ μM).

pared. An average K_I for 7-methyl GTP of 4.39 ± 0.89 μM was obtained from 10 separate experiments using several different batches of reticulocyte lysate. Each of the six secondary cap analogue standards, 7-methyl GMP, 7-benzyl GMP, 7-methyl GDP, 7-benzyl GTP, 7-methyl GpppG, and 7-methyl GppppG, was assayed in the same experiment as the primary standard, 7-methyl GTP, and its K_I was determined. When an unknown cap analogue was assayed, the K_I values for both the unknown and the standard were determined. The K_I of the unknown was then normalized on the basis of the K_I determined for the standard. This procedure is illustrated below. Since the final values reported for unknown cap analogues were derived by normalization of the primary data, we refer to them as apparent K_I values.

RESULTS

Estimation of Apparent K_I Values. A total of 58 different cap analogues were synthesized for this study (Figures 1−5). In the case of 14 of these, a second synthesis was performed to confirm the apparent K_I values obtained with the first (see Table 1). Each cap analogue was assayed for its ability to inhibit synthesis of rabbit globin from exogenous globin mRNA in a rabbit reticulocyte translation system. A series of six concentrations of each cap analogue was tested in the same experiment with the same range of concentrations of the appropriate standard cap analogue (given in Table 1). The standard cap analogue was used to normalize variations between experiments. In the case of 28 of the 58 compounds, two or more independent assays were performed to confirm results of apparent K_I determinations.

Typical results for three cap analogues (two unknowns and one standard) are shown in Figure 6A. The curves represent least-squares fits of eq 2 to the data points. In this particular experiment, cap-independent translation (Q_{ci}) was found to represent 11.2% of total translation (Q_t). The K_I values obtained in this experiment were 2.83 ± 0.22 μM for one of the unknowns, 2,7-dimethyl GTP (**26**), 43.0 ± 10.4 μM for the other of the unknowns, 2,2,7-trimethyl GTP (**30**), and 2.49 ± 0.25 μM for the standard, 7-methyl GTP (**22**). In 10 experiments conducted over several years in which several different batches of reticulocyte lysate and exogenous globin mRNA were utilized, the average K_I for 7-methyl GTP was 4.39 μM. The K_I values of the unknowns were therefore normalized by multiplying by a factor of 1.76 (4.39/2.49) to yield 4.99 ± 0.39 μM for **26** and 75.8 ± 18.3 μM for **30**. Two other independent assays of **26** produced uncorrected K_I values of 4.91 and 8.83 μM, which were normalized by the same procedure to 3.43 and 4.50 μM, respectively. The average of all three normalized values for **26** (4.31 ± 0.46 μM) appears in Table 1. The average without normalization would have been 5.52 ± 1.76 μM.

With some of the weaker cap analogues, significant inhibition was not observed except at high concentrations, raising the question of whether the compound could be competing with GTP in the numerous GTP-requiring steps of initiation, elongation, and termination (*1*). To test this, we assayed some of them (**1**, **22**, **33**, **35**, and **36**) with uncapped globin mRNA (Figure 6B). In none of these cases was inhibition of uncapped mRNA translation observed, ruling out a general inhibition of GTP-dependent reactions.

Effect of a Single Alkyl Substituent at N2. The exocyclic amino group N2 of the 7-methylguanine moiety is known

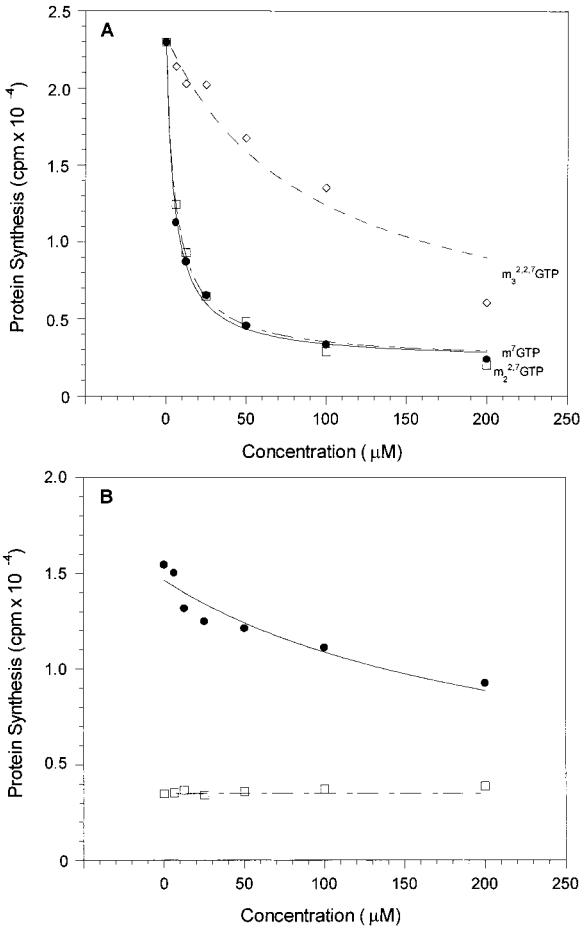


FIGURE 6: (A) Extraction of apparent K_I values from translational inhibition data. A nuclease-treated rabbit reticulocyte lysate translation system was programmed with 5 μg/mL rabbit globin mRNA, and the rate of protein synthesis was measured in the presence of the indicated concentrations of **22** (solid line with ●), **26** (dashed line with □), and **30** (dashed line with ◇). The data were fit to eq 2 by least-squares minimization, from which an apparent K_I was determined for each cap analogue. (B) Comparison of cap-dependent and cap-independent translation. The extent of inhibition of translation of capped rabbit globin mRNA (●) and uncapped β-globin mRNA (□) was measured at the indicated concentrations of **33**.

to be important for binding, since cap analogues containing 7-methylinosine or 7-methylxanthosine fail to inhibit mRNA binding to ribosomes (*37*). Previous studies have shown that a single methyl substituent at N2 is tolerated (*37*, *38*, *41*). Quantitative analysis shows that addition of a single methyl group to N2 increases the effectiveness of cap analogues as translational inhibitors (Table 1). This generalization holds at the level of 7-methyl-substituted nucleoside MP (**7** vs **1**), 7-(2-phenylethyl)-substituted nucleoside MP (**10** vs **2**), 7-benzyl-substituted nucleoside TP (**27** vs **23**), 7-(*p*-chlorobenzyl)-substituted nucleoside TP (**28** vs **24**), 7-(*p*-fluorobenzyl)-substituted nucleoside TP (**29** vs **25**), and dinucleoside tetraphosphate (**57** vs **54**). An improvement is also seen with an ethyl group at N2 (**8** vs **1**). Interestingly, addition of the N2 methyl group converts even GDP (**20**) and GTP (**33**) into weak inhibitors of cap-dependent (but not cap-independent) translation.

Effects of Two Methyl Groups at N2. Previous studies indicated that, while one methyl group on N2 can be tolerated, the presence of two methyl groups greatly diminishes the effectiveness of the cap (*38*, *41*). The results of

our analysis confirm this observation. Thus, the apparent K_I values of dimethylated N2 cap analogues were consistently higher than those of the corresponding N2-unsubstituted analogues (Table 1). This was true at the level of 7-methylated nucleoside DP (**18** vs **15**), 7-(*p*-chlorobenzyl)-substituted nucleoside DP (**19** vs **16**), 7-methylated nucleoside TP (**30** vs **22**), 7-(*p*-chlorobenzyl)-substituted nucleoside TP (**31** vs **24**), and 7-(*p*-fluorobenzyl)-substituted nucleoside TP (**32** vs **25**). The antagonistic effect of the second methyl group is observed even with 7-unsubstituted cap analogues (**21** vs **20** and **34** vs **33**). Interestingly, approximately 70% of the mRNAs in *C. elegans* contain the 2,2,7-trimethyl G cap, but there are different isoforms of eIF4E that are capable of recognizing it (*12*).

Aryl Substituents at N7. Previously, it was shown that 7-benzyl GMP was more effective as an inhibitor of translation than 7-methyl GMP (*69*). This result was confirmed in this study (Table 1, **3** vs **1**). We explored various modifications of the aromatic ring to see if such cap analogues could be made even more effective. Substituting at the para position with methyl or NO_2 groups made the cap analogue a poorer inhibitor (**4** and **5** vs **3**). Substituting at the para position with Cl, however, improved the cap analogue 2-fold as an inhibitor (**6** vs **3**). Combining the positive effect of a 7-(*p*-chlorobenzyl) substituent with the positive effect of an N2-methyl substituent produced the most effective inhibitor in the nucleoside MP series (**11**), 27-fold more effective than the parent compound (**1**). Another aromatic substituent at N7 also improved efficacy as an inhibitor slightly, but not as much as benzyl (**2** vs **1**). This effect was observed for N2-unsubstituted analogues but not for N2-methyl analogues (**10** vs **7**).

At the level of nucleoside TP, by contrast, the substitution of benzyl for methyl at N7 was not beneficial (**23** vs **22**). This contrasts with a previous report that 7-methyl GDP and 7-benzyl GDP exhibited similar activities in preventing reovirus mRNA binding to ribosomes (*37*). A marginal improvement was caused by *p*-halo substitutions (**25** and **24** vs **23**), but even these were less effective than the 7-methyl analogue (**22**). In the DP series, the 7-(*p*-chlorobenzyl) substituent is only slightly more inhibitory than the 7-methyl group (**16** vs **15**). For the N2-methyl nucleoside TP series, the effect of substituting aryl for methyl at N7 produced a small improvement (**27**−**29** vs **26**). However, the improvement (between 1.3 and 1.7-fold) was considerably smaller than that seen with the nucleoside MP series (between 3- and 7-fold). As noted above, the presence of two methyl groups on N2 greatly diminishes the inhibitory effect of cap analogues. In such analogues, a 7-(*p*-chlorobenzyl) substituent nonetheless improved the inhibitory properties in the TP series (**31** vs **30**). However, there was no significant effect of 7-(*p*-chlorobenzyl) substitution in the DP series (**19** vs **18**) or 7-(*p*-fluorobenzyl) substitution in the TP series (**32** vs **30**).

In summary, 7-aryl substitutions have a strong positive influence on the weaker nucleoside MP inhibitors (as long as the aromatic ring does not have methyl or NO_2 groups), but the effect of 7-aryl substituents on the stronger nucleoside DP and TP inhibitors is marginal and varies from compound to compound. Interestingly, the 2-methyl-7-(*p*-chlorobenzyl) analogue is the best inhibitor in either the nucleoside MP (**11**), DP (**17**), or TP (**28**) series.

Phosphate Groups. By far the largest determinants of inhibitory activity for cap analogues are the phosphate residues. Adding the β phosphate in the 7-methylguanosine series improves inhibitory activity 51-fold (Table 1, **15** vs **1**), and adding the γ phosphate improves it another 1.7-fold (**22** vs **15**). Derivatives containing substituents that increase inhibitory activity compared to that of the 7-methyl analogue are further improved by addition of β and γ phosphates. However, more inhibitory analogues benefited progressively less from phosphate addition. In the DP series, the improvement was 8-fold for 7-(*p*-chlorobenzyl) (**16** vs **6**) but only 3-fold for 2-methyl-7-(*p*-chlorobenzyl) (**17** vs **11**). In the TP series, the improvement for adding both β and γ phosphates was 35-fold for 2,7-dimethyl (**26** vs **7**), 13-fold for 7-benzyl (**23** vs **3**), 7-fold for 7-(*p*-chlorobenzyl) (**24** vs **6**), and 5-fold for 2-methyl-7-(*p*-chlorobenzyl) (**28** vs **11**), with the phosphates again making less of a contribution for the more inhibitory cap analogues.

The beneficial effect of more phosphate residues seems to be more related to anionic charge than to the number of phosphate groups per se. Thus, **22**, with a charge of −3.5 at neutral pH, is more inhibitory than **40**, with a charge of −2.5, even though they both contain three phosphates. (At neutral pH, the 7-methylguanine moiety is half-protonated.) The nucleoside TP is also more inhibitory than the corresponding dinucleoside TP for the 2,7-dimethyl analogues (**49** vs **26**). More anionic charge converts the ribose ring-opened analogue from a noninhibitor (**12**) to an inhibitor (**50**). This is also true for analogues that do not even contain a 7-methylguanine moiety; members of the benzimidazole riboside series become more inhibitory as the anionic charge is increased. Thus, compounds **35**, **52**, and **13** have charges of −3.5, −3, and −1, respectively, and decrease in the same order as inhibitors. Similarly, the 3-methylbenzimidazole ribosides **58**, **35**, **53**, and **14** have charges of −3, −2.5, −2, and −1.5, respectively, and decrease in the same order as inhibitors. One member of the 3-methylbenzimidazole series does not fit this pattern for unknown reasons, the nucleoside diphosphate **39**, which is the best inhibitor of the series.

A further improvement in inhibitory activity is seen by addition of a fourth phosphate. Thus, the tetraphosphates **54**−**58** are more inhibitory than their TP homologues **40**, **41**, **48**, **49**, and **53** by 5-, 1.4-, 12-, 7-, and 2.8-fold, respectively. A previous study reported that **54** and **56** were 25−50-fold more potent as inhibitors than their TP counterparts (*70*), but we do not see such a large effect. Another previous study indicated that there was little effect of adding the γ phosphate (*39*). However, our results indicate that TPs are more effective than DPs for 7-methyl (**22** vs **15**), 2-methyl-7-(*p*-chlorobenzyl) (**28** vs **17**), 2,2,7-trimethyl (**30** vs **18**), 2,2-dimethyl-7-(*p*-chlorobenzyl) (**31** vs **19**), 2-methyl (**33** vs **20**), and 2,2-dimethyl (**34** vs **21**) substituents. The best inhibitors in the entire group of 58 analogues is **57**, which combines two favorable modifications, high anionic charge and N2 methylation.

Second Nucleotide Moiety. Although a recent report notes that 2′-*O*-methylation of the first nucleotide residue after the TP stimulates c-*mos* mRNA recruitment to the ribosome (*71*), we find little difference between **40** and **42**. This agrees with previous studies with *Artemia salina* and wheat germ extracts which indicated that nucleoside diphosphates and their corresponding 2′-*O*-methyl analogues had similar inhibitory

activity (*72*, *73*) and that 2′-*O*-methyl, 3′-*O*-methyl, and 2′-deoxy-7-methyl GMP analogues have similar inhibitory activity (*39*). However, **43** is slightly less inhibitory. Similarly, 6-methylation of A causes no change in inhibition (**45** vs **44**). The two pyrimidine-containing caps **46** and **47** have similar activity, but none of the "natural" cap analogues is as effective as 7-methyl GpppG (**42** and **44**−**47** vs **40**).

As noted above, and somewhat surprisingly, the dinucleoside TPs are considerably less potent inhibitors than the corresponding nucleoside TPs. Thus, **40**, **42**, and **44**−**47** are between 4- and 8-fold less potent than **22**, and **49** is 3-fold less potent than **26**. This contrasts with previous reports (*72*, *73*) which indicated that the nucleoside TPs and dinucleoside TPs have similar activities.

Ribose Ring. Earlier studies showed that ribose ring-opened derivatives of 7-methyl GMP are less active translational inhibitors than 7-methyl GMP itself (*39*, *40*). Consistent with this, we find a dramatic reduction in inhibitory activity upon opening the ribose ring, whether considered at the nucleoside MP (**12** vs **7**) or dinucleoside TP (**50** vs **40**) level. However, the acyclo derivative **51** is more inhibitory than the seco derivative **50**. The acyclo moiety can easily adopt a conformation close to that of the normal ribose ring, but this is impossible for the seco derivative because of steric effects (*74*). This raises questions of specificity in experiments in which periodate-oxidized mRNAs are utilized for cross-linking studies (*75*).

Benzimidazole Derivatives. The possibility of developing derivatives based on ring systems other than the purine ring is attractive because of the large number of additional compounds potentially made available. We examined one such alternative ring system, the benzimidazoles. Like 7-methylguanine, 3-methylbenzimidazole is positively charged, but its charge does not vary with pH in the neutral range as does that of 7-methylguanine. Also, like 7-methylguanine, it may form stacking interactions with Trp, since it does so with guanine (*76*). However, unlike 7-methylguanine, it is unable to form hydrogen bonds. Such a compound would therefore be useful in trying to estimate the individual contributions of stacking and hydrogen bond formation to the binding with eIF4E.

Some of the benzimidazole derivatives were active as inhibitors of translation (Table 1, **35**, **36**, **52**, **53**, and **58**). At all levels of phosphate, however, benzimidazole analogues were considerably less active than their 7-methylguanine-based counterparts (**13** vs **1**, **35** vs **22**, **52** vs **40**, and **58** vs **54**). Addition of a methyl group to the 3-position of benzimidazole, which is analogous to the 7-position of guanine (see Figure 2), might have been expected to improve inhibitory activity. However, the benzimidazoles and 3-methylbenzimidazoles had similar activity at all levels of phosphate groups (**13** vs **14**, **35** vs **36**, and **53** vs **52**).

DISCUSSION

The interactions of cap analogues with eIF4E may be assessed either by physical chemical methods, e.g., fluorescence quenching, or by functional assays, e.g., inhibition of protein synthesis (see the introductory section). Each method has advantages and disadvantages. An advantage of the fluorescence quenching method is that it is carried out with purified eIF4E and does not reflect the influence of any other proteins. The correlation of binding constants with the individual interactions between groups in the active site of eIF4E and specific structural features of the cap can best be made with data obtained by this method. An advantage of the second method, inhibition of translation, is that it measures the effect of the cap analogue in a more physiological setting, in which eIF4E is present at very low concentrations but other proteins that might interact nonspecifically with the cap analogue are present at very high concentrations (e.g., 50 mg/mL in reticulocyte lysate). For the purpose of screening compounds of potential therapeutic value, the translational assay is more appropriate. Such compounds must have high selectivity, stability, and affinity for eIF4E in the presence of the entire complement of cellular proteins.

Previously published data on comparison of cap analogues as inhibitors of protein synthesis is fragmentary at best. Generally, only compounds assayed in the same study can be compared, and most previous studies have not provided quantitative data at all. In vitro translational systems and different types of mRNAs differ widely in their response to cap analogues. In one study, for instance, the *A. salina* and wheat germ systems translating the same mRNA differed nearly 4-fold in sensitivity to cap analogues, and even within the same translational system, different mRNAs varied up to 3-fold in sensitivity to cap analogue (*72*). Consequently, a survey of the literature cannot produce quantitative comparisons among the cap analogues assayed to date. By using the same translational system under strict conditions of pH, mRNA type, mRNA concentration, salt concentration, etc., we have obtained a data set that is reasonably self-consistent. By including standard cap analogues in each assay and normalizing the data, we have further improved the internal agreement of this data set. Using this assay, we have redetermined the apparent translational K_I values of 11 cap analogues previously assayed and determined for the first time the apparent K_I values of 47 new cap analogues.

A newly improved fluorescence quenching method (*36*) yields affinity constants for cap analogues that are in the same relative order but that are lower than those reported here (*77*). The reasons for the discrepancies between fluorescence and translational K_I values are not known but could include some or all of the following. Cap analogue may bind nonspecifically to other proteins in the reticulocyte lysate translational system, thereby lowering their effective concentrations. This would not necessarily be the same for all cap analogues. For instance, if nucleoside MPs bind nonspecifically to proteins to a greater extent than do nucleoside TPs, it would explain the greater disparity that is observed between K_I values determined by fluorescence versus translation for the former class of compounds. Another possibility is that not all of the eIF4E is in the pathway for translation. The eIF4E-binding protein PHAS-I prevents eIF4E interaction with eIF4G but does not prevent eIF4E interaction with the cap. (eIF4E·PHAS-I complexes are isolated on 7-methyl GTP-Sepharose affinity columns.) Thus, some of the cap analogue may bind to nonfunctional eIF4E. Finally, our knowledge of the actual mechanism of protein synthesis initiation is incomplete, and the relatively simple kinetic model used for curve fitting may be inappropriate. It is not known, for example, if eIF4E initially binds the cap as part of the eIF4F complex, as part of an even larger

complex containing eIF3, or as part of the 43S initiation complex. Nor is it known whether eIF4E remains bound to the cap or dissociates as the 43S initiation complex begins to scan for the first AUG.

The functional groups of cap analogues that make them more effective inhibitors of protein synthesis can be rationalized to some extent in terms of the tertiary structure of the eIF4E·cap analogue complex (*17*, *18*). Introduction of two methyl groups on the N2-amino group of 7-methylguanine disrupts a hydrogen bond with Glu-103, but one alkyl substitution at N2 leaves the hydrogen bond intact. This part of the cap is close to the protein surface, so the additional methyl substituent is easily accommodated in the complex. This methyl group closes the hydrophobic pocket of eIF4E more tightly and thus may enhance its hydrophobicity. The substantial increase in the inhibitory properties upon addition of the β phosphate is due to three additional hydrogen bonds with Arg-112, Arg-157, and Lys-162. The stabilizing interactions for the γ phosphate have not been identified, since the structural studies were performed with eIF4E·7-methyl GDP complexes. However, another Lys residue occurs in this region, Lys-159, and there is also the possibility of forming a hydrogen bond with Asp-161.

The effects of para substituents on the 7-benzyl group are harder to rationalize. In terms of electron withdrawing properties, the order is $NO_2 > F > Cl > H > CH_3$. In terms of size, the order is $NO_2 > CH_3 \approx Cl > F > H$. The order of inhibition of in vitro protein synthesis for the MP series is $Cl > H > CH_3 \approx NO_2$. For all three of the TP series [2-NH_2, 2-$NH(CH_3)$, or 2-$N(CH_3)_2$], the order is $Cl > H \approx F$. Neither of these series correlated, either positively or negatively, with the electron withdrawing series or the steric bulk series. One possibility is that electron-withdrawing groups are favorable until they become too large. Another possibility is that substitutions that enhance π electron density of the benzyl ring favor cap binding. From the location of the 7-methyl group in the known tertiary structures, it is likely that the 7-benzyl group forms a π stacking interaction with Trp-166. Strongly electron-withdrawing groups such as NO_2 and F should weaken these π stacking interactions. However, Cl at a para position forms resonance structures that actually increased π electron density.

REFERENCES

1. Merrick, W. C., and Hershey, J. W. B. (1996) in *Translational Control* (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., Eds.) pp 31−69, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Sachs, A. B., Sarnow, P., and Hentze, M. W. (1997) *Cell 89*, 831−838.
3. Rhoads, R. E., and Lamphear, B. (1995) in *Cap-Independent Translation* (Sarnow, P., Ed.) pp 131−153, Springer-Verlag, Berlin.
4. Ehrenfeld, E. (1996) in *Translational Control* (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., Eds.) pp 549−573, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
5. Richter, J. D. (1991) *BioEssays 13*, 179−183.
6. Lamphear, B. J., Kirchweger, R., Skern, T., and Rhoads, R. E. (1995) *J. Biol. Chem. 270*, 21975−21983.
7. Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995) *Mol. Cell. Biol. 15*, 4990−4997.
8. Tarun, S. Z., and Sachs, A. B. (1996) *EMBO J. 15*, 7168−7177.
9. Le, H., Tanguay, R. L., Balasta, M. L., Wei, C. C., Browning, K., Metz, A. M., Goss, D. J., and Gallie, D. R. (1997) *J. Biol. Chem. 272*, 16247−16255.
10. Keiper, B. D., and Rhoads, R. E. (1999) *Dev. Biol. 206*, 1−14.
11. Rhoads, R. E., Joshi-Barve, S., and Rinker-Schaeffer, C. (1993) *Prog. Nucleic Acid Res. Mol. Biol. 46*, 183−219.
12. Jankowska-Anyszka, M., Lamphear, B. J., Aamodt, E. J., Harrington, T., Darzynkiewicz, E., Stolarski, R., and Rhoads, R. E. (1998) *J. Biol. Chem. 273*, 10538−10542.
13. Dyer, J. R., Pepio, A. M., Yanow, S. K., and Sossin, W. S. (1998) *J. Biol. Chem. 273*, 29469−29474.
14. Rychlik, W., Domier, L. L., Gardner, P. R., Hellmann, G. M., and Rhoads, R. E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 945−949.
15. Rom, E., Kim, H. C., Gingras, A.-C., Marcotrigiano, J., Favre, D., Olsen, H., Burley, S. K., and Sonenberg, N. (1998) *J. Biol. Chem. 273*, 13104−13109.
16. Browning, K. S., Lax, S. R., and Ravel, J. M. (1987) *J. Biol. Chem. 262*, 11228−11232.
17. Marcotrigiano, J., Gingras, A.-C., Sonenberg, N., and Burley, S. K. (1997) *Cell 89*, 951−961.
18. Matsuo, H., Li, H., McGuire, A. M., Fletcher, C. M., Gingras, A.-C., Sonenberg, N., and Wagner, G. (1997) *Nat. Struct. Biol. 4*, 717−724.
19. Rychlik, W., Gardner, P. R., Vanaman, T. C., and Rhoads, R. E. (1986) *J. Biol. Chem. 261*, 71−75.
20. Joshi, B., Cai, A.-L., Keiper, B. D., Minich, W. B., Mendez, R., Beach, C. M., Stepinski, J., Stolarski, R., Darzynkiewicz, E., and Rhoads, R. E. (1995) *J. Biol. Chem. 270*, 14597−14603.
21. Morley, S. J., and Traugh, J. A. (1990) *J. Biol. Chem. 265*, 10611−10616.
22. Minich, W. B., Balasta, M. L., Goss, D. J., and Rhoads, R. E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 7668−7672.
23. Wang, X., Flynn, A., Waskiewicz, A. J., Webb, B. L. J., Vries, R. G., Baines, I. A., Cooper, J. A., and Proud, C. G. (1998) *J. Biol. Chem. 273*, 9373−9377.
24. Lin, T., Kong, X., Haystead, T. A. J., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J. C. (1994) *Science 266*, 653−656.
25. Pause, A., Belsham, G. J., Gingras, A., Donze, O., Lin, T., Lawrence, J. C., and Sonenberg, N. (1994) *Nature 371*, 762−767.
26. Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995) *EMBO J. 14*, 5701−5709.
27. Rosenwald, I. B., Rhoads, D. B., Callanan, L. D., Isselbacher, K. J., and Schmidt, E. V. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6175−6178.
28. Jones, R. M., Branda, J., Johnston, K. A., Polymenis, M., Gadd, M., Rustgi, A., Callanan, L., and Schmidt, E. V. (1996) *Mol. Cell. Biol. 16*, 4754−4764.
29. Rhoads, R. E. (1985) *Prog. Mol. Subcell. Biol. 9*, 104−155.
30. Sonenberg, N., Morgan, M. A., Merrick, W. C., and Shatkin, A. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4843−4847.
31. Hellmann, G. M., Chu, L.-Y., and Rhoads, R. E. (1982) *J. Biol. Chem. 257*, 4056−4062.
32. Sonenberg, N., Rupprecht, K. M., Hecht, S. M., and Shatkin, A. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4345−4349.
33. Webb, N. R., Chari, R. V. J., DePillis, G., Kozarich, J. W., and Rhoads, R. E. (1984) *Biochemistry 23*, 177−181.
34. McCubbin, W. D., Edery, I., Altmann, M., Sonenberg, N., and Kay, C. M. (1988) *J. Biol. Chem. 263*, 17663−17671.
35. Carberry, S. E., Rhoads, R. E., and Goss, D. J. (1989) *Biochemistry 28*, 8078−8083.
36. Wieczorek, Z., Darzynkiewicz, E., and Lonnberg, H. (1998) *J. Photochem. Photobiol., B 43*, 158−163.
37. Adams, B. L., Morgan, M., Muthukrishnan, S., Hecht, S. M., and Shatkin, A. J. (1978) *J. Biol. Chem. 253*, 2589−2595.
38. Miura, K. I., Kodama, Y., Shimotohna, K., Fukui, T., Ikehara, M., Nakagawa, I., and Hata, T. (1979) *Biochim. Biophys. Acta 564*, 264−274.
39. Darzynkiewicz, E., Ekiel, I., Tahara, S. M., Seliger, L. S., and Shatkin, A. (1985) *Biochemistry 24*, 1701−1707.

40. Darzynkiewicz, E., Ekiel, I., Lassota, P., and Tahara, S. M. (1987) *Biochemistry 26*, 4372−4380.
41. Darzynkiewicz, E., Stepinski, J., Ekiel, I., Jin, Y., Haber, D., Sijuwade, T., and Tahara, S. M. (1988) *Nucleic Acids Res. 16*, 8953−8962.
42. Goss, D. J., Carberry, S. E., Dever, T. E., Merrick, W. C., and Rhoads, R. E. (1990) *Biochim. Biophys. Acta 1050*, 163−166.
43. Mendez, R., Myers, M. G., White, M. F., and Rhoads, R. E. (1996) *Mol. Cell. Biol. 16*, 2857−2864.
44. Rhoads, R. E. (1991) *Curr. Opin. Cell Biol. 3*, 1019−1024.
45. De Benedetti, A., and Harris, A. L. (1999) *Int. J. Biochem. Cell Biol. 31*, 59−72.
46. Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abramson, R. D., Merrick, W. C., and Thach, R. E. (1985) *J. Biol. Chem. 260*, 7651−7658.
47. Darveau, A., Pelletier, J., and Sonenberg, N. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2315−2319.
48. Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. (1990) *Nature 345*, 544−547.
49. De Benedetti, A., and Rhoads, R. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8212−8216.
50. Kerekatte, V., Smiley, K., Hu, B., Smith, A., Gelder, F., and De Benedetti, A. (1995) *Int. J. Cancer 65*, 27−31.
51. Nathan, C. A., Carter, P., Liu, L., Li, B., Abreo, F., Tudor, A., Zimmer, S., and Benedetti, A. D. (1997) *Oncogene 15*, 1087−1095.
52. Scott, P. A. E., Smith, K., Poulsorn, R., De Benedetti, A., Bicknell, R., and Harris, A. L. (1998) *Br. J. Cancer 77*, 2120−2128.
53. Li, B. D., Liu, L., Dawson, M., and De Benedetti, A. (1997) *Cancer 79*, 2385−2390.
54. Li, B. D. L., McDonald, J., Nassar, R., and De Benedetti, A. (1998) *Ann. Surg. Soc. 227*, 756−762.
55. Nathan, C. A., Liu, L., Li, B. D. L., Abreo, F., and De Benedetti, A. (1997) *Oncogene 15*, 579−583.
56. Sorrells, D. L., Destin, R. B., Meschonat, C., Rhoads, R., De Benedetti, A., Gao, M., Williams, B. J., and Li, B. D. L. (1997) *Ann. Surg. Oncol. 5*, 232−237.
57. Rinker-Schaeffer, C. W., Graff, J. R., De Benedetti, A., Zimmer, S. G., and Rhoads, R. E. (1993) *Int. J. Cancer 55*, 841−847.
58. Rousseau, D., Gingras, A.-C., Pause, A., and Sonenberg, N. (1996) *Oncogene 13*, 2415−2420.
59. Beretta, L., Gingras, A. C., Svitkin, Y. V., Hall, M. N., and Sonenberg, N. (1996) *EMBO J. 15*, 658−664.
60. von Manteuffel, S. R., Gingras, A.-C., Ming, X.-F., Sonenberg, N., and Thomas, G. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4076−4080.
61. Rhoads, R. E., Hellmann, G. M., Remy, P., and Ebel, J. P. (1983) *Biochemistry 22*, 6084−6088.
62. Chu, L.-Y., and Rhoads, R. E. (1978) *Biochemistry 17*, 2450−2455.
63. Chu, L.-Y., and Rhoads, R. E. (1980) *Biochemistry 19*, 184−191.
64. Schwemmle, M., Schickinger, J., Bader, M., Sarre, T. F., and Hilse, K. (1991) *Eur. J. Biochem. 201*, 139−145.
65. Titus, D. E. (1991) in *Promega Protocols and Applications Guide*, pp 59−61, Promega Corp., Madison, WI.
66. Lodish, H. F. (1974) *Nature 251*, 385.
67. Chu, L. Y., Lockard, R. E., RajBhandary, U. L., and Rhoads, R. E. (1978) *J. Biol. Chem. 253*, 5228−5231.
68. Ohlmann, T., Rau, M., Morley, S. J., and Pain, V. M. (1995) *Nucleic Acids Res. 23*, 334−340.
69. Darzynkiewicz, E., Stepinski, J., Ekiel, I., Goyer, C., Sonenberg, N., Temeriusz, A., Jin, Y., Sijuwade, T., Haber, D., and Tahara, S. M. (1989) *Biochemistry 28*, 4771−4778.
70. Sasavage, N. L., Friderici, K., and Rottman, F. M. (1979) *Nucleic Acids Res. 6*, 3613−3624.
71. Kuge, H., Brownlee, G. G., Gershon, P. D., and Richter, J. D. (1998) *Nucleic Acids Res. 26*, 3208−3214.
72. Filipowicz, W., Furuichi, Y., Sierra, J. M., Muthukrishnan, S., Shatkin, A., and Ochoa, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1559−1563.
73. Hickey, E. D., Weber, L. A., Baglioni, C., Kim, C. H., and Sarma, R. H. (1977) *J. Mol. Biol. 109*, 173−183.
74. Stolarski, R., Lassota, P., Kazimierczuk, Z., and Shugar, D. (1988) *Z. Naturforsch. 43c*, 231−242.
75. Sonenberg, N. (1981) *Nucleic Acids Res. 9*, 1643−1656.
76. Wieczorek, Z., Zdanowski, K., Chlebicka, L., Stepinski, J., Jankowska, M., Kierdaszuk, B., Temeriusz, A., Darzynkiewicz, E., and Stolarski, R. (1997) *Biochim. Biophys. Acta 1354*, 145−152.
77. Wieczorek, Z., Niedzwiecka-Kornas, A., Chlebicka, L., Jankowska, M., Kiraga, K., Stepinski, J., Dadlez, M., Drabent, R., Darzynkiewicz, E., and Stolarski, R. (1999) *Z. Naturforsch. 54*, 278−284.
78. Jankowska, M., Stepinski, J., Stolarski, R., Temeriusz, A., and Darzynkiewicz, E. (1993) *Collect. Czech. Chem. Commun. 58*, 138−141.
79. Stepinski, J., Grabowska, L., Darzynkiewicz, E., Temeriusz, A., Stolarski, R., Tahara, S. M., Shugar, D., Jarvinen, P., and Lonnberg, H. (1990) *Collect. Czech. Chem. Commun. 55*, 117−120.
80. Chlebicka, L., Wieczorek, Z., Stolarski, R., Stepinski, J., Darzynkiewicz, E., and Shugar, D. (1995) *Nucleosides Nucleotides 14*, 771−775.
81. Stepinski, J., Bretner, M., Jankowska, M., Felczak, K., Stolarski, R., Wieczorek, Z., Cai, A.-L., Rhoads, R. E., Temeriusz, A., Haber, D., and Darzynkiewicz, E. (1995) *Nucleosides Nucleotides 14*, 717−721.
82. Jankowska, M., Stepinski, J., Stolarski, R., Wieczorek, Z., Temeriusz, A., Haber, D., and Darzynkiewicz, E. (1996) *Collect. Czech. Chem. Commun. 61*, S197−S202.
83. Suzuki, H. (1977) *FEBS Lett. 79*, 11−14.

BI9830213