METHOD

Synthesis and properties of mRNAs containing the novel “anti-reverse” cap analogs 7-methyl(3′-O-methyl)GpppG and 7-methyl(3′-deoxy)GpppG

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ABSTRACT

The ability to synthesize capped RNA transcripts in vitro using bacteriophage polymerases has been of considerable value in a variety of applications. However, Pasquinelli et al. [RNA (1995) 1:957–967] found that one-third to one-half of the caps are incorporated in the reverse orientation, that is, with the m 7 G moiety of m 7 GpppG linked by a 3′-5′ phosphodiester bond to the first nucleotide residue of the RNA chain. Such reverse caps are unlikely to be recognized by eIF4E, based on previous studies, and thus complicate any comparison of the translational efficiencies of in vitro-synthesized mRNAs. We therefore designed two novel cap analogs, P1-3′-deoxy-7-methyguanosine-5′P3′-guanosine-5′triphosphate and P1-3′-O,7dimethyguanosine-5′P3′-guanosine-5′triphosphate, that are, theoretically, incapable of being incorporated in the reverse orientation. The key reactions of pyrophosphate bond formation were achieved in anhydrous dimethylformamide solutions employing the catalytic properties of zinc salts. Structures were proven by 1H NMR. Transcripts produced with SP6 polymerase using “anti-reverse” cap analogs (ARCAs) were of the predicted length and indistinguishable in size and homogeneity from those produced with m 7 GpppG or GpppG. Analysis of the transcripts with RNase T2 and tobacco acid pyrophosphatase indicated that reverse caps were formed with m 7 GpppG but not with ARCAs. Both of the ARCAs inhibited cell-free translation with a K I similar to that of m 7 GpppG. Finally, the translational efficiency of ARCA-capped transcripts in a rabbit reticulocyte lysate was 2.3- to 2.6-fold higher than that of m 7 GpppG-capped transcripts. This suggests the presence of reverse caps in conventional in vitro-synthesized mRNAs reduces their translational efficiency.

Keywords: bacteriophage polymerases; cap analog inhibition; cap-dependent translation; in vitro transcription; ribonuclease T2; tobacco acid pyrophosphatase; translational efficiency

INTRODUCTION

Because all cellular, nonorganellar mRNAs are capped (Shatkin, 1987), as well as most of the U-type snRNAs (Mattaj, 1986), the ability to synthesize capped RNA transcripts in vitro is a useful research tool. The most frequently employed technique is to transcribe a DNA template with either a bacterial (Contreras et al., 1982) or bacteriophage (Konarska et al., 1984; Yisraeli & Melton, 1989) RNA polymerase in the presence of all four ribonucleoside triphosphates and a cap dinucleotide such as m 7 G(5′)PPP(5′)G. The polymerase initiates transcription with a nucleophilic attack by the 3′-OH of the Guo moiety in m 7 GpppG on the α-phosphate of the next templated nucleoside triphosphate, resulting in the initial product m 7 GpppGpN. Suppression of the alternative, GTP-initiated product GpppGpN is achieved by setting the ratio of m 7 GpppG to GTP to between 5 and 10 in the transcription reaction mixture.

Somewhat unexpectedly, Pasquinelli et al. (1995) found that bacteriophage polymerases also utilize the 3′-OH of the 7-methylguanosine moiety of m 7 GpppG to initiate transcription, resulting in one-third to one-half of the products containing a “reverse cap,” that is,
Gpppm$^7$GpN. These authors demonstrated that reverse-capped pre-U1 RNA transcripts, when injected into Xenopus laevis nuclei, are exported more slowly than natural transcripts. Similarly, cytoplasmic reverse-capped U1 RNAs fail to be imported into the nucleus. The presence of a cap on mRNA has a strong stimulatory effect on translation (reviewed by Shatkin, 1985; Rhoads, 1985). The ability of reverse-capped mRNAs to be translated was not tested by Pasquinelli et al. (1995), but based on what is known about recognition of the cap structure by elf4E (Marcotrigiano et al., 1997; Matsuo et al., 1997; Cai et al., 1999), one would not expect reverse-capped mRNAs to be translated any more efficiently than uncapped RNAs.

The presence of a significant proportion of reverse-capped RNAs in the products of in vitro transcription should decrease the overall translational activity of the RNA preparation. In addition, heterogeneous populations of RNA molecules would compromise the determination of translational efficiency and other functional properties of mRNA. For these reasons, we designed two cap analogues that, theoretically, cannot be incorporated in the reverse orientation. We found that these “anti-reverse” cap analogs (ARCAs) are, in fact, incorporated exclusively in the normal orientation. Furthermore, they behave like the natural caps in their interactions with the translational machinery, resulting in mRNAs that are translationally more active.

RESULTS AND DISCUSSION

Transcription by bacteriophage RNA polymerases in the presence of m$^7$GpppG is initiated with a nucleophilic attack by the 3’-OH of either the m$^7$Guo or Guo moiety on the electrophilic α-phosphate of the first templated nucleoside triphosphate. The basic strategy of our approach was to eliminate one of these two 3’-OH groups. In the case of P$^1$-3’-deoxy-7-methylguanosine-5’-P$^3$-guanosine-5’ triphosphate (Fig. 1, compound 9, henceforth abbreviated m$^7$3’-GpppG), the substitution was −H for −OH. In the case of P$^1$-3’-O,7-dimethylguanosine-5’-P$^3$-guanosine-5’ triphosphate (Fig. 1, compound 10, henceforth abbreviated m$^7$O,7-GpppG), the substitution was −OCH$_3$ for −OH.

Synthesis of ARCAs

Chemical synthesis of the ARCAs was performed by a methodology that differed in some respects from methodologies reported previously (Stepinski et al., 1995; Jankowska et al., 1996). To avoid preparation of imidazole derivatives from 7-methylated substrates, the activation of which is often difficult, we developed a new coupling strategy involving guanosine 5’-phosphorimidazolide and the modified 7-methylated nucleoside diphosphate (Fig. 1). Moreover, carrying out the coupling reaction in the presence of ZnCl$_2$ (Kadokura et al., 1997) instead of Mn$^{2+}$, and using anhydrous dimethylformamide (DMF) instead of water as a solvent, allowed us to obtain high yields (see Materials and Methods). In a similar reaction, the synthesis of Ch$_2$pppG from GDP and the imidazole of methyl phospho in DMF, Kadokura et al. (1997) obtained a yield of 39% without ZnCl$_2$ compared with 98% with ZnCl$_2$. Furthermore, carrying out the reactions in aqueous Mn$^{2+}$ resulted in yields of 42% and 37%, respectively, for the coupling of Im(m$^7$GDP) plus GMP (Sawai et al., 1999) and Im(m$^7$GMP) plus GDP (Sawai et al., 1991), whereas we obtained yields of 78% and 88%, respectively, for the same reactions using DMF and ZnCl$_2$.

$^{13}$C NMR and UV spectra for intermediates were in good agreement with the predicted structures (data not shown). The $^1$H NMR assignments of the protons in both ARCAs confirmed their chemical structures (Table 1). Two sets of sugar $^1$H signals in each spectrum point to dinucleotides. The presence of the methyl signals at 4.068 ppm (compound 10) and 4.027 (compound 9) together with the disappearance of the H(8) resonances, due to exchange for solvent deuterium (Darzynkiewicz et al., 1990), testify to the presence of 7-methylguanine. In the case of compound 10, the additional methyl group was observed at 3.483 ppm, accompanied by a characteristic upfield shift of the H3’ signal. Lack of the 3’-hydroxyl in compound 9 gives the characteristic “deoxy” pattern of H3’/H3” at 2.086–2.148 ppm with further scalar couplings to H4’ and H2’.

Information regarding conformational parameters is gathered in Table 2. This shows populations of the N form in the N ⇔ S dynamic equilibrium of the sugar ring, populations of the +sc (gauche-gauche) conformer about C4’-C5’, and populations of the ap (gauche-‘gauche’) conformer of the phosphate group. The 7-substituted Guo moieties show the characteristic “deoxy” pattern of H3’/H3” at 2.086–2.148 ppm with further scalar couplings to H4’ and H2’.

### Table 1

$^1$H NMR chemical shifts in parts per million (±0.001) versus internal sodium 3-trimethylsilyl-2,2,3,3-$^2$H$_2$)-propionate.

<table>
<thead>
<tr>
<th></th>
<th>m$^7$3’-GpppG (9)</th>
<th>m$^7$O,7-GpppG (10)</th>
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<tr>
<td>m$^3$G</td>
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<tr>
<td>H8</td>
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<td>H1’</td>
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<td>H2’</td>
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<tr>
<td>H5”</td>
<td>4.196</td>
<td>4.26$^b$</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>4.027</td>
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$^a$Deuterated.

$^b$Signal overlapping.

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also more pronounced in the 7-substituted guanosines (Darzynkiewicz et al., 1990; Wieczorek et al., 1997). The conformation of the Guo moiety of AR-CAs is similar to that of Guo in normal caps, 64% of the S form (36% N) and 63% of the +sc form (Las- sota et al., 1984). Thus, m$_7$Guo and m$_7$3’dG show conformational features characteristic of m$_7$Guo rather than Guo.

Synthesis of ARCA-capped RNA transcripts

The ARCA-capped RNA transcripts were next tested in an in vitro transcription system. A template DNA was generated by digesting the plasmid pSP-luc+ with EcoRI. The theoretical size of an RNA transcript from this template was 1,706 nt. This was near the size of the products observed in reactions carried out in the presence of [α-32P]ATP and either GpppG, m$_7$GpppG, m$_7$3’dGpppG, or m$_2$7’-GpppG (Fig. 2). In six separate experiments, the yield of product in the presence of AR-CAs was not statistically different from the yield in the presence of m$_7$GpppG.

Analysis of cap orientation in ARCA-capped RNA transcripts

From the structure of the AR-CAs, it should not be possible for them to be incorporated in the reverse orientation. We verified this experimentally by digestion with RNase T2 and tobacco acid pyrophosphatase (TAP). To obtain a higher proportion of radioactivity in the cap versus internal positions, a shorter DNA template was produced by cleaving pSP-luc+ with Ncol instead of EcoRI. This was expected to yield an RNA product of 43 nt (plus a cap). The size of this product was confirmed by polyacrylamide gel electrophoresis in Tris/borate/EDTA/urea (data not shown). RNase T2 digests RNA with no base specificity. Thus, it will generate mostly 3’-NMPs from this
mRNAs containing anti-reverse caps

RNA. Those nucleotide residues located 5' to an A residue will acquire a 32P-labeled 3'-phosphate by nearest neighbor transfer. The pyrophosphate bonds in the cap, however, are not susceptible to RNase T2. Because the first nucleotide residue after the cap in the synthetic RNA is A, the α-phosphate of [α-32P]ATP will be transferred to the cap upon RNase T2 digestion. Thus, for RNAs initiated in the normal orientation with m7GpppG, the product will be m7GpppGp* (where p* represents 32P). RNase T2-digestion products expected from RNAs initiated with GTP or with each of the four cap analogs in either normal or reverse orientations are shown in Table 3.

The RNase T2-digestion products of normal and reverse m7GpppG-capped RNAs (m7GpppGp* and Gppppm7Gp*, respectively) have identical masses and charges; they would therefore be expected to elute from an anion exchange column at nearly the same time. However, digestion of normal and reverse-capped mRNAs with TAP yields two alternate labeled products, pGp* and pm7Gp*, that differ in both charge and mass, because the m7 group confers a positive charge on G (Hendler et al., 1970). The nucleotides pm73'dGp* and pm7-3'O3Gp* have the same charge as pm7Gp*. Thus, whereas RNase T2 digestion alone would not be expected to distinguish between normal and reverse orientations, the combination of RNase T2 and TAP would do so (see Table 3).

RNAs were synthesized from the short DNA template in the presence of [α-32P]ATP, the other three nonradioactive NTPs, and either no cap analog, GpppG, m7GpppG, m73'dGpppG, or m7-3'O3GpppG. The products were digested with RNase T2 and resolved by anion exchange HPLC (Fig. 3). Uncapped RNA yielded primarily 3'-NMPs (Fig. 3A, 20–30 min) with a small amount of material that may have been the partially degraded product ppGp* (Fig. 3A, 76 min). The expected product pppGp* was not observed. Based on its high negative charge, it may not have eluted from the column. Its presence, however, is likely, as RNase T2 plus TAP digestion yielded pGp* (Fig. 3B, 56 min) where none existed previously (Fig. 3A).

In the case of GpppG-capped RNAs, RNase T2 alone yielded a structure eluting at 89 min (Fig. 3C) that is likely to be GpppGp* (the presence of a second phosphate ester reduces the charge relative to pppGp*). The minor peak at 77 min may be the partially degraded product pGp*. Consistent with these assignments, both compounds disappeared upon TAP digestion simultaneously with the appearance of a new peak corresponding to pGp* at 56 min (Fig. 3D). No pm7Gp* (42 min) was formed, as expected.

The major highly charged RNase T2-resistant product from m7GpppG-capped RNA eluted at 73 min (Fig. 3E) and is likely to be m7GpppGp*. This compound elutes earlier than the peak at 89 min in Figure 3C, tentatively assigned the structure GpppGp*,
FIGURE 3. Analysis of in vitro-synthesized RNAs by enzymatic digestion and anion exchange HPLC. mRNAs were generated by transcription of Nco I-digested pSP-luc with [$\alpha$-32P]ATP and either no cap dinucleotide (A,B), GpppG (C,D), m7GpppG (E,F), m7,5'dGpppG (G,H), or m2,5'GpppG (I,J). Aliquots of 5 to 13 ng of RNA were digested with RNase T2 (left panels) or RNase T2 plus tobacco acid pyrophosphatase (TAP; right panels) as described in Materials and Methods. Nucleotides and caps were separated on a Partisil 10SAX/25 column developed with a gradient of potassium phosphate, pH 3.5, as described in Materials and Methods. Fractions of 1 mL were collected and the Cerenkov radiation determined with a Beckman LS 6500 scintillation counter. The elution times of the following standard compounds, detected by UV absorption, are shown: 3'-CMP, 3'-UMP, 3'-AMP, 3'-GMP, 5'-GDP, 5'-GTP, 3',5'-GDP (pGp), 3',5'-m7GMP (pm7Gp), and GpppG.
because of the additional positive charge. The minor peak at 77 min may be the reverse cap Gpppm"7"Gp*, suggesting that the proximity of the 3'-P to the positive charge of the m7G ring may influence the charge on P. These assignments are strengthened by the fact that TAP digestion converted these products to two labeled compounds eluting earlier, pGp* (56 min) and pm7Gp* (42 min) (Fig. 3F). The ratios of pGp* and pm7Gp* suggest that they were derived from the 73- and 77-min peaks of Figure 3E, respectively.

With the ARCA m73'dGpppG, an RNase T2-resistant product was observed at 78 min that is likely to be m73'dGpppG* (Fig. 3G). It elutes at nearly the same time as the compound thought to be m7GpppG* (78 min versus 77 min for Fig. 3G and E, respectively). Interestingly, whereas there were two peaks in this region for RNA synthesized with m7GpppG (Fig. 3E), there was only one for RNA synthesized with the ARCA (Fig. 3G), consistent with the inability of the ARCA to be incorporated in the reverse orientation. When digested with TAP, this peak at 78 min disappeared and a new one appeared at the elution time of pGp* (Fig. 3H, 56 min). The fact that no pm7Gp* appeared at 42 min with the ARCA (Fig. 3H), whereas it did with m7GpppG (Fig. 3F), is further proof that the ARCA can be incorporated in only one orientation.

The products observed upon digestion of RNA synthesized with the second ARCA, m27,03"GpppG (Fig. 3I,J), eluted the same as those obtained with m73'dGpppG-capped RNA.

### Inhibition of protein synthesis by ARCAAs

One criterion for assessing cap analog interaction with the translational machinery is inhibition of protein synthesis (Canaani et al., 1976; Hickey et al., 1976). The binding of cap analogs to eIF4E, measured in vitro with purified components, and the inhibition of protein synthesis, measured in a cell-free translation system, is well correlated for a variety of different cap analog structures (Carberry et al., 1990). We tested GpppG, m7GpppG, and the two ARCAAs for their ability to compete with natural globin mRNA for recognition by the translational machinery and thereby inhibit translation in a rabbit reticulocyte lysate (RRL) system (Fig. 4). GpppG was not an inhibitor, and in fact slightly stimulated protein synthesis at low concentrations. The two ARCAAs, on the other hand, were equally inhibitory as m7GpppG.

It is possible to compare cap analogs quantitatively as inhibitors by fitting a theoretical curve to the translation data (Cai et al., 1999). The curve is derived from an equation that treats cap analogs as competitive inhibi-

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Inhibition of translation by ARCAAs compared with m7GpppG and GpppG. Natural rabbit globin mRNA was translated at 5 μg/ml in a RRL system, and globin synthesis was detected by incorporation of [3H]Leu into protein as described in Materials and Methods. The following cap analogs were included during translation at the indicated concentrations: GpppG: circles; m7GpppG: squares; m73'dGpppG: triangles; m27,03"GpppG: diamonds.
tors of mRNA binding to eIF4E; the value of the dissociation constant for the cap analog-eIF4E complex, $K_d$, is varied until the best least-squares fit is obtained. The curves in Figure 4 for m$^7$GpppG, m$^7$3′dGpppG, and m$^7$2′,3′GpppG are, in fact, theoretical curves corresponding to $K_d$ values of 27.8 ± 12.6, 27.8 ± 7.1, and 14.3 ± 1.9 μM, respectively. Although it appears in this experiment that the $m_2^7$,3′ GpppG compound was more inhibitory, in a replicate of this experiment, the $K_d$ values for the ARCA did not differ statistically from those of m$^7$GpppG. This is also in agreement with a study showing that $m_2^7$,3′ GMP and m$^7$GMP were equally inhibitory for translation (Darzynkiewicz et al., 1985).

**Translation of ARCA-capped mRNAs**

Based on the results presented thus far, namely, that one-third to one-half of m$^7$GpppG is incorporated into RNA in reverse orientation (Pasquinielli et al., 1995; Fig. 2; Table 3), that ARCA are incorporated exclusively in the normal orientation (Fig. 3), and that ARCA are recognized to the same extent as m$^7$GpppG by the translational machinery (Fig. 4), one would predict that the homogeneous population of in vitro-synthesized ARCA-capped mRNAs would be more active translationally than m$^7$GpppG-capped mRNAs. We tested this with luciferase mRNAs that were either uncapped or capped with GpppG, m$^7$GpppG, or the two ARCA (Fig. 5). The fact that all the m$^7$G-containing mRNAs were translated more efficiently than the uncapped or GpppG-capped mRNAs indicates that the RRL system employed was highly dependent on the cap. In the experiment shown, the m$^7$,2′,3′ GpppG and m$^7$3′dGpppG-capped mRNAs were 2.3- and 2.6-fold more efficient than m$^7$GpppG-capped mRNA, respectively. In six experiments employing four separate batches of in vitro-synthesized mRNAs, the mRNAs produced with ARCA were reproducibly more active than with m$^7$GpppG.

Pasquinielli et al. (1995) found that reverse capping varied between 28 and 48%, depending on the pH of the in vitro-transcription reaction. In the experiment shown in Figure 3 and summarized in Table 3, reverse capping was approximately 33%. If ARCA and normal caps stimulate translation to the same degree, which seems likely based on the inhibition data (Fig. 4), one would expect the (homogeneous) preparation of ARCA-capped mRNA to be only 1.5-fold more active than the (heterogeneous) preparation of m$^7$GpppG-capped mRNAs. The fact that the RNA was even more active (2.3- to 2.6-fold) suggests that reverse-capped mRNAs may actually be inhibitory rather than merely neutral in their effect on translation.

These results indicate that ARCA are very similar to normal cap analogs. The modifications at the 3′-O-position of m$^7$GGuo do not appear to affect conformation (Table 2) or interaction with the translational machinery (Figs. 4 and 5). This is in agreement with the X-ray-derived structure of an NH$_2$-terminally truncated fragment of mouse eIF4E in complex with m$^7$GDP, which shows that the 3′-OH of the m$^7$Guo moiety projects away from the protein and is accessible to solvent (Marcotrigiano et al., 1997). Consequently, the ARCA have the advantage of being incorporated exclusively in the normal orientation but no apparent disadvantages. To our knowledge, the degree to which m$^7$G is incorporated in place of G in internal positions of a synthetic RNA chain by bacteriophage polymerases has not been rigorously determined. Regardless of the level of this misincorporation, the ARCA would be incapable of donating m$^7$G internally as well as at the 5′ end. A different type of ARCA, for example, m$^7$,2′,3′ GpppG or m$^7$,2′,3′dGpppG, would be useful for in vitro synthesis of U-type snRNAs with 100% normal cap orientation.

**MATERIALS AND METHODS**

**Column chromatography**

Both final products (9 and 10) and intermediate nucleotides (3–8) were isolated from reaction mixtures by column chromatography on DEAE-Sephadex (A-25, HCO$_3^-$ form) using a linear gradient of triethylammonium bicarbonate (TEAB), pH 7.5, in water. Fractions were collected, and products peaks (monitored at 260 nm) were pooled and evaporated to dryness, with ethanol added repeatedly to remove the TEAB buffer. Thus, the products were obtained as TEA salts.
mRNAs containing anti-reverse caps

The purity of intermediates and products was monitored at 260 nm by analytical HPLC using a Spectra-Physics SP8800 apparatus on a 25-cm LC-18-T reverse phase column (Supelco). The mobile phase was a linear gradient of methanol from 0 to 25% in 0.1 M KH₂PO₄, pH 6, over 15 min with flow rate of 1.3 mL/min.

Mono- and dinucleotides obtained by enzymatic digestion of in vitro-synthesized RNAs were analyzed by HPLC using a Waters 625 instrument with a 996 PDA detector on a 4.5 × 250 mm Partisil 10SAX/25 column (Whatman). The program for elution of nucleotides consisted of water for the first 5 min, a linear gradient of 0 to 87.5 mM KH₂PO₄, pH 3.5, over 35 min, a linear gradient of 87.5 to 500 mM of KH₂PO₄ over 30 min, and isocratic elution at 500 mM KH₂PO₄ for 21 min, all at a flow rate of 1 mL/min.

Spectroscopy

¹H NMR and ¹³C NMR spectra were recorded on a Varian UNITYplus 500 MHz instrument in dimethylsulfoxide-d₆ (for nucleoside intermediates) and ²H₂O (for mono- and dinucleotides). The absorption spectra were obtained on a Cary 3E spectrophotometer.

¹H NMR spectra of 9 and 10 were run at 25 °C at 1.4 mg/0.7 mL and 0.4 mg/0.7 mL in ²H₂O, respectively. Conformations of the sugar moieties were derived from the vicinal ¹H-¹H coupling constants (Haasnoot et al., 1980). Conformations of the phosphate groups were determined from the ¹H-³¹P coupling constants (Lankhorst et al., 1984).

Synthesis of mono- and dinucleotides

3’-Deoxyguanosine 5’-monophosphate (3)

3’-Deoxyguanosine (1, commercial product from Sigma, 50 mg, 0.19 mmol) was stirred overnight with trimethylphosphite (2 mL) and phosphorus oxychloride (53 µL, 0.57 mmol) at 6 °C. Adding 20 mL of water and neutralizing with 1 M NaHCO₃ quenched the reaction. DEAE-Sephadex chromatography using a linear gradient of 0–0.9 M TEAB afforded 3 (yield: 45 mg, 43%).

3’-O-Methyguanosine 5’-monophosphate (4)

This compound was obtained by a procedure analogous to that of 3, but starting from 59 mg of 3’-O-methyguanosine (2), which was prepared as previously described (Kusmierek & Shugar, 1978) (yield: 80 mg, 69%).

3’-Deoxyguanosine 5’-diphosphate (5)

Compound 3 (55 mg, TEA salt, 0.1 mmol), imidazole (34 mg, 0.5 mmol), and 2,2’-dithiodipyridine (Aldrich, 44 mg, 0.2 mmol) were mixed in anhydrous DMF (1.2 mL) and TEA (14 µL). Triphenylphosphine (52 mg, 0.2 mmol) was added, and the mixture was stirred for 5 h at room temperature. The mixture was placed in a centrifuge tube, and sodium perchlorate (49 mg, anhydrous) dissolved in acetone (6 mL) was added. After cooling for 2 h in a refrigerator, the mixture was centrifuged and the supernatant was discarded. The precipitate was washed with a new portion of acetone, cooled, and centrifuged again. The process was repeated once more, and the precipitate was dried in a vacuum desiccator over P₂O₅.

The imidazolide thus obtained was dissolved in 1.2 mL of DMF, and 200 mg of tris(triethylammonium)phosphate was added. The latter was prepared from TEA and phosphoric acid followed by drying over P₂O₅ in a desiccator to obtain semicrystalline mass. Finally, 80 mg of ZnCl₂ were added, and the reaction mixture was stirred at room temperature for 6.5 h, poured into a beaker containing a solution of 250 mg EDTA in 15 mL water, and neutralized with 1 M NaHCO₃. Chromatographic isolation on DEAE-Sephadex using a linear gradient of 0–1 M TEAB gave 5 (yield: 41 mg, 66%).

3’-O-Methyguanosine 5’-diphosphate (6)

This compound was obtained by a procedure analogous to that of 5 except starting from 58 mg of 4 (yield: 32 mg, 49%).

3’-Deoxy-7-methyguanosine 5’-diphosphate (7)

Compound 5 (34 mg, 0.055 mmol) was mixed with 1 mL of dimethylsulfoxide, 1 mL of DMF, and 100 µL of methyl iodide at room temperature. After 3 h, the reaction mixture was treated with 30 mL of cold water and extracted three times with 10-mL portions of diethyl ether. Chromatography of the aqueous phase, after neutralization with NaHCO₃, on DEAE-Sephadex using a linear gradient of 0 to 0.8 M TEAB gave 7 (yield: 10 mg, 28%).

3’-O, 7-Dimethylguanosine 5’-diphosphate (8)

This compound was obtained by a procedure analogous to that of 7, except starting from 66 mg of 6 (yield: 64 mg, 95%).

P³′-3’-Deoxy-7-methyguanosine-5’ triphosphate (9)

P³′-guanosine-5’ triphosphate (10)

GMP (purchased from Sigma, converted to the TEA salt, 29 mg, 0.05 mmol), imidazole (17 mg, 0.25 mmol), and 2,2'-dithiodipyridine (22 mg, 0.1 mmol, from Aldrich) were mixed in anhydrous DMF (1.2 mL) and TEA (7 µL). Triphenylphosphine (26 mg, 0.1 mmol) was added, and the mixture was stirred for 5 h at room temperature. The mixture was placed in a centrifuge tube, and sodium perchlorate (25 mg, anhydrous) dissolved in acetone (6 mL) was added. The procedure for washing the precipitate with acetone and drying over P₂O₅ was the same as for 5. The imidazolide of GMP thus obtained was dissolved in DMF (1.2 mL) and 7 (10 mg, TEA salt, 0.015 mmol) was added. Next, ZnCl₂ (40 mg) was added, and the mixture was stirred at room temperature overnight, poured into a beaker containing a solution of 125 mg of EDTA in 15 mL of water, and neutralized with 1 M NaHCO₃. Chromatographic isolation as for 5 gave 9 (13 mg, 88% based on the amount of 7 used).

P³′-3’-O, 7-Dimethylguanosine-5’ triphosphate (10)

This compound was prepared from GMP and 8 (34 mg) by a procedure analogous to that for 9 (yield: 23 mg, 78%).

The final products (9 and 10) were converted to their Na⁺ salts by ion exchange using a small column of Dowex 50Wx8 (Na⁺ form), evaporation of the eluates to a small volume,
precipitation with ethanol, and centrifugation to give amorphous white powders. Analyses of purity were essentially the same as described earlier (Darzynkiewicz et al., 1990). Parameters from the T H NMR spectra of 9 and 10 are shown in Tables 1 and 2.

7-Methylguanosine 3',5'-diphosphate.

Guanosine 3',5'-diphosphate was methylated to make the chromatographic standard pm7Gp (Fig. 3) by the same procedure as used for 7.

In vitro synthesis of RNA

RNAs that were either capped with various types of cap analogs or uncapped were synthesized by in vitro transcription in two lengths. The DNA template used for both lengths of RNA was pSP-luc+ (Promega), which contains an SP6 bacteriophage promoter. To generate the short RNAs (43 nt exclusive of the cap), the plasmid was digested with Ncol. To generate the long RNAs (1,706 nt, containing the entire luciferase coding region), the plasmid was digested with EcoRI. A typical in vitro-transcription reaction mixture contained, in 20 µL, 40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 2 µg BSA, 20 U of RNasin (Promega), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.1 mM GTP, 1 mM cap analog (GpppG, m7GpppG, m3'dGpppG, or m2',5'GpppG), 0.2–1.0 µg DNA, and 20 U of SP6 polymerase (Promega). Reactions to synthesize the short RNAs also contained 28 µCi of [α-32P]ATP (ICN), whereas those to synthesize the long RNAs contained 0.8 µCi of [α-32P]CTP (ICN). Reaction mixtures were incubated for 60 min at 37 °C, extracted with phenol and chloroform, the solution made 0.8 molar with sodium acetate, the nucleic acids precipitated with 3 vols of ethanol on dry ice for 5 min, and the solution centrifuged at 14,000 rpm for 30 min. The pellet was dissolved in water, the solution made 0.2 molar in sodium acetate, and the nucleic acid was again precipitated with 2.5 vols of ethanol at 4 °C for 30 min and centrifuged at 14,000 rpm for 30 min. The pellet was allowed to air dry and then dissolved in diethylpyrocarbonate-treated water.

Enzymatic digestion of RNAs

The short RNAs were digested with 67 U RNase T2 (Life Technologies) in 15 µL of 0.14 M sodium acetate, pH 4.6, at 37 °C for 60 min. In some cases, the RNAs were subjected to a two-step digestion instead. The first digestion was with 10 U TAP (Epigence Technologies) in 5 µL of 0.50 mM sodium acetate, pH 6.0, 1 mM EDTA, 0.1% β-mercaptoethanol, and 0.01% Triton X-100 at 37 °C for 60 min. The digestion was continued for 60 min at 37 °C with 67 U RNase T2 in a final volume of 16 µL of 0.12 M sodium acetate, pH 4.6. Samples were analyzed without further treatment by anion exchange HPLC as described above.

Cell-free translation

A micrococcal nuclease-treated RRL system was used for in vitro translation as described previously (Cai et al., 1999). In some cases, the added mRNA was natural rabbit globin mRNA and protein synthesis was measured by incorporation of [3H]Leu into trichloroacetic acid-precipitable form. In other cases, the added mRNAs were luciferase mRNAs (the long form), synthesized in vitro as described above, and protein synthesis was measured by assaying luciferase activity using beetle luciferin (Promega) as substrate and a Monolite 2010 luminometer to detect light emission.

The ability of cap analogs to inhibit cell-free translation in the RRL system programmed with globin mRNA was measured as described previously (Cai et al., 1999). Data were fit by least squares minimization to a theoretical rate equation. The concentrations of cap analog solutions were measured by UV absorption at pH 7.0 using the following parameters (λ, ελ): GpppG, 251 nm, 25.5×103; m7GpppG, m3'dGpppG, and m2',5'GpppG, 255 nm, 22.6×103.

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