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Synthesis and application of a new 2′,3′-isopropylidene guanosine substituted cap analog

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ABSTRACT

The synthesis and biological evaluation of a new cap analog, which is modified at the C2′ and C3′ positions of N^7-methylguanosine is reported. The new cap analog, P^1-2′,3′-isopropylidene, 7-methylguanosine-5′P^3-guanosine-5′triphosphate was assayed with respect to its effects on efficiency of incorporation into RNAs during in vitro transcription, and intracellular stability and translational activity of its 5′-capped mRNAs, upon transfection into HeLa cells. The intracellular stability of 5′-capped and uncapped full length test mRNAs was measured by using a real-time RT-PCR assay. The RNA with the 5′-modified cap was found to be ~1.7 times more stable than the RNA with the 5′-standard cap and ~2.5 times more stable than the uncapped control RNA. The translational efficiency was monitored by measuring the luciferase activity of a variety of in vitro synthesized and capped RNAs coding for a luciferase fusion protein after transfection into HeLa cells. The RNA capped with the 2′,3′-isopropylidene substituted analog, ($m^{7,2',3'\text{-isopropylidene}}$G[5′]ppp[5′]G), was translated the most efficiently, with ~ 2.9-fold more activity than the standard cap (m^7G[5′]ppp[5′]G). The observed increase in the level of protein synthesis is likely resulted as a consequence of exclusively forward capped transcripts and increased cellular stability of the 5′-modified capped mRNA (Poly A).

The 5′-terminus of eukaryotic mRNA molecules synthesized by RNA polymerase II contains a unique 5′,5′-dinucleoside triphosphate structure, where the terminal nucleoside is N^7-methylguanosine.[1] This modification, known as a 5′-cap, plays a pivotal role in the function of mRNA in a variety of cellular processes including translation, splicing, intracellular transport, and turnover.[2–6] The cap on pre-mRNA interacts with the nuclear cap-binding complex, which remains bound and plays an active role during RNA processing and export.[7] The presence of the 5′-cap structure is known to increase both the accuracy and efficiency of pre-mRNA splicing.[8,9] The best studied role of the cap is in translation, where it is specifically recognized by the translational initiation factor eIF4E.[8] Synthetic capped mRNAs are useful tools to study and understand number of biological processes. To create capped mRNAs, DNA templates are transcribed with either a bacterial or bacteriophage RNA polymerase in the presence of all four ribonucleoside triphosphates and a synthetic cap 'analog', the dinucleotide m^7G[5′]ppp[5′]G.[8,10–12] By having the first transcribed base be G, the polymerase initiates transcription with a nucleophilic attack by the 3′-OH of either GTP or the guanosine moiety of m^7G[5′]ppp[5′]G on the α-phosphate of the next encoded nucleoside triphosphate, resulting in the initial product pppGpN or m^7G[5′]ppp[5′]GpN (Fig. 1).[13] Due to the fact that the cap analog can only be incorporated as the initial base, the latter product can be favored by an excess of the cap analog over GTP. However, since both nucleosides in the analog have a free 3′-OH group, this results in an in vitro product in which 30–50% of the RNA transcripts are capped in the reverse orientation, with the methylated guanosine internal. As only the mRNAs possessing correctly incorporated caps are properly recognized during cellular processes such as translation initiation and intracellular transport, the high fraction of reverse-capped transcripts can produce misleading data.[13]

The recent literature has described substituted caps such as $m_2^{7,3'\text{-}O}$G[5′]ppp[5′]G and m^7,3′dG[5′]ppp[5′]G. These are called anti-reverse cap analogs (ARCAs), because the 3′-OH group of the ribose connected to the m^7G cannot serve as the initiating nucleophile due to the absence of a free 3′-OH group.[14,15] In addition to 3′-OH modifications, 2′-OH modifications on m^7G moiety can also incorporate solely in the forward orientation, even though the 2′-OH group does not participate in the phosphodiester linkage.[16] In continuation of our efforts[17,18] to test the chemically modified cap analog and gain a further insight into its structure–function relationship with biological systems, we have synthesized new cap analog, in which both 2′ and 3′-OH groups from m^7Guo were substituted with the bridging 2′,3′-isopropylidene group. Due to this substitution, the new cap analog $m^{7,2',3'\text{-isopropylidene}}$G[5′]ppp[5′]G is incorporated into RNA

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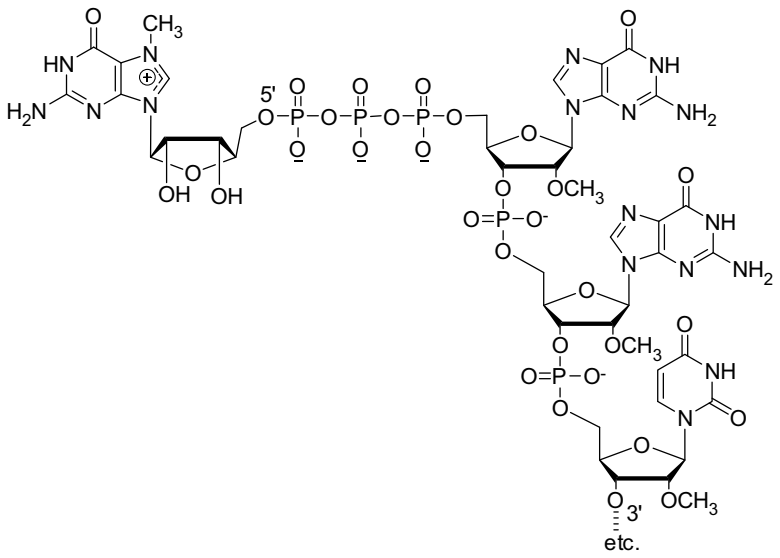
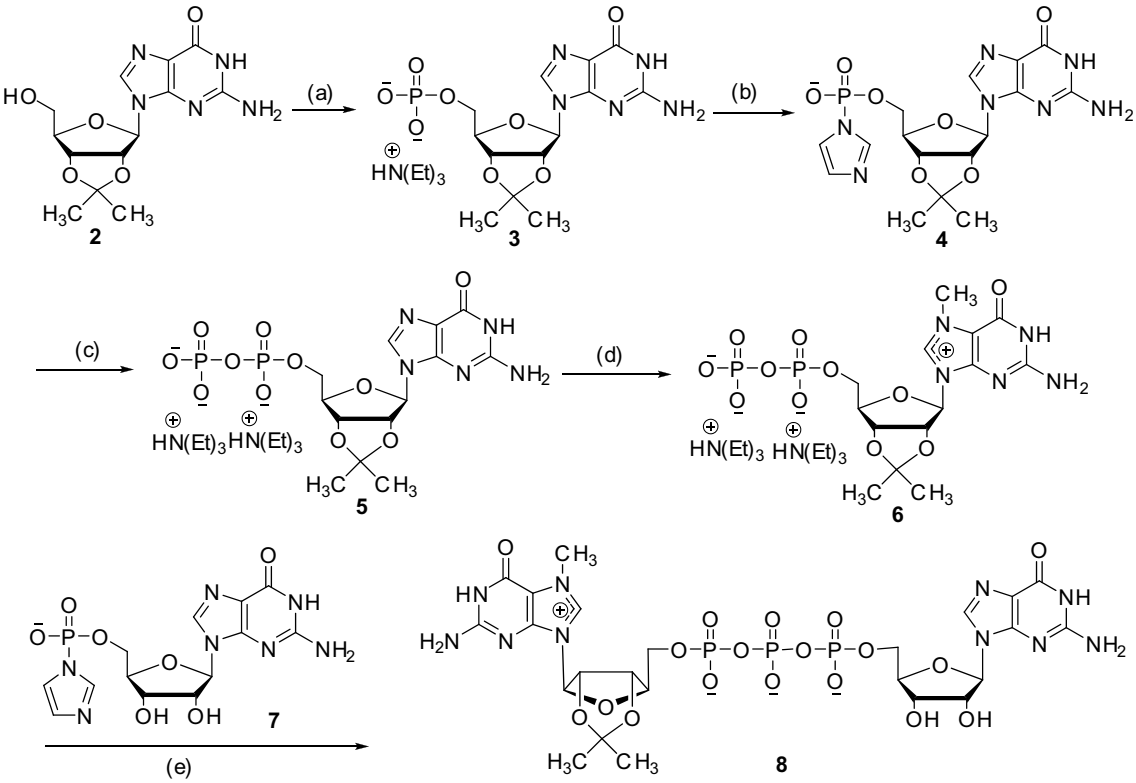


Figure 1. The chemical structure of the 5′ terminus of a 'capped' mRNA with standard mCAP **1**.

transcripts exclusively in the correct orientation. In this communication, we report the first example of the biological effect of 2′ and 3′-disubstituted analog, that is, m $^{7,2',3'\text{-isopropylidene}}$G[5′]ppp[5′]G on capping efficiency, in vitro transcription, RNA intracellular stability, and translational activity by comparing with standard mCAP, that is, m^7G[5′]ppp[5′]G **1** as a control.

The reaction pathway leading to the formation of desired m$^{7,2',3'\text{-isopropylidene}}$G[5′]ppp[5′]G **8** for biological testing is depicted in Scheme 1. The commercially available 2′,3′-isopropylidene guanosine **2** was converted into the corresponding 2′,3′-isopropylidene GMP **3** using $POCl_3$ and trialkyl phosphate, and reaction was quenched within 30 min.[19] It is important to note that any further increase of the reaction time led to a ring opening of 2′,3′-isopropylidene to give the corresponding GMP as the side product. The monophosphorylated product **3** was converted into the corresponding imidazolide salt **4** with 39% yield using imidazolide, triphenylphosphine, and aldirthiol. Next, the resulting imidazolide salt **4** was phosphorylated using $(Et_3NH)_3PO_4$ in the presence of zinc chloride as the catalyst that afforded the corresponding 2′,3′-isopropylidene GDP **5** with 45% yield.[20] The methylation



Scheme 1. Reagents and conditions: (a) $POCl_3$, $(OMe)_3P$, 0 °C, 30 min; (b) imidazole, aldrithiol, PPh_3, DMF, rt, 15 h, 39%; (c) $(Et_3NH)_3PO_4$, DMF, rt, 4 h, 45%; (d) dimethyl sulfate, water, pH 4.0, rt, 3 h, 75%; (e) $ZnCl_2$, DMF, rt, 4 h, 53%.

reaction of **5** using dimethyl sulfate as the methylating agent is highly regioselective, affording $m^{7,2',3'\text{-isopropylidene}}GDP$ **6** as the sole product with 75% yield.[21] No other isomer was detected based on the proton and phosphorous NMR spectral data. Finally, the coupling reaction of $m^{7,2',3'\text{-isopropylidene}}GDP$ **6** with ImGMP **7** in the presence of zinc chloride as the catalyst afforded $m^{7,2',3'\text{-isopropylidene}}G[5']ppp[5']G$ **8** with 53% yield.[22] The structure of **8** was confirmed by ^1H and ^{31}P NMR and mass spectral data.[23]

Analogs of the mRNA cap are widely employed to study processes involved in mRNA metabolism and are useful in biotechnology and medicinal applications. The compound **1**, $m^7G[5']ppp[5']G$ is a standard cap analog and the limitation of mCAP analog is that it is incorporated bidirectionally. The 3′-OH of either the G or m^7G can serve as the initiating nucleophile for transcriptional elongation, leading to the synthesis of two isomeric RNAs with their cap dinucleotide in either forward or reverse orientation in approximately equal proportions, depending upon the ionic conditions of the transcription reaction. The reverse form of capped mRNAs, that is, $G[5']pppm^7G[pN]^n$, will not be recognized during the translation process, while only forward orientated sequences, that is, $m^7G[5']pppG[pN]^n$, will be translated.[13] To address the reverse capping problem, we have synthesized a cap analog in which the C2′ and C3′ positions of N^7-methylguanosine are both blocked with a single 2′,3′-isopropylidene moiety. Because of this substitution of the 2′ and 3′-OH groups from m^7Guo moiety, there is only one 3′-OH group available for the RNA polymerase to initiate transcription from, resulting in all capped products being in a forward orientation, $m^{7,2',3'\text{-isopropylidene}}G[5']ppp[5']GpN$. Biochemical utility of this new cap analog, P^1-2′,3′-isopropylidene,7-methylguanosine-5′P^3-guanosine-5′triphosphate analog, ($m^{7,2',3'\text{-isopropylidene}}G[5']ppp[5']G$) **8** was assayed with respect to its effect on capping efficiency, efficiency of incorporation into RNAs during in vitro transcription, and intracellular stability and translational activity of these RNAs upon transfection into HeLa cells.

In order to determine the capping efficiency of the $m^{7,2',3'\text{-isopropylidene}}G[5']ppp[5']G$ **8**, it was next included in an in vitro transcription system using a modified protocol with the MAXIscript® Kit off the pTri β actin (Ambion, Inc.), followed by a gel shift assay.[24] Appropriate controls with no-cap or standard cap $m^7G[5']ppp[5']G$ **1** were run in separate reactions. Under these modified reaction conditions, only ATP and GTP were used out of the four NTPs, while CTP and UTP were omitted from the transcription reaction. Due to this omission, transcription was terminated at the first coded pyrimidine at position +7, producing a transcript of only six nucleotides in length. For the transcription reactions with cap analogs, the GTP concentration was diminished 20% from the no-cap control reaction, while the cap analog concentration compensated for this 80% loss, so that the net concentration of 'GTP equivalents' was maintained. All reactions were performed in the presence of (α-^{32}P) ATP to internally label the transcript. The resulting 6-mer transcription products were analyzed by 20% denaturing polyacrylamide/8 M urea gel. The outcome of the gel shift assay, shown in Figure 2, indicates that the capped RNAs, which migrate slower than uncapped RNAs, are produced in slightly different amounts with compound **8**. These reactions were performed in triplicate and the capping efficiency was determined by quantitating the intensities of capped versus uncapped RNA by normalizing with the background intensity. From these gel shift assays, it was clear that standard cap $m^7G[5']ppp[5']G$ **1** has a capping efficiency of 62%, while the new modified cap, $m^{7,2',3'\text{-isopropylidene}}G[5']ppp[5']G$ **8** has a 49% capping efficiency. The capping efficiency experiment clearly indicates that the modified cap **8** was substrate for T7 RNA polymerase. The slightly lower capping efficiency of compound **8** is likely due to the inhibition of the reverse incorporation, that is, out of the two potential complexes the new cap analog can form with the transcription machinery (either nucleotide can presumably fit into the enzymatic cleft), only one is productive.

To study the ability of the modified cap analog to function as a translation in $m^{7,2',3'\text{-isopropylidene}}G[5']ppp[5']G$ **8** was used in a transcription reaction.[25] The template used, Blp 1-linearized AmbLuc Poly(A), contains a 60 base Poly(A) tail and a T7 promoter site for use in in vitro transcription. The in vitro transcription was performed with standard cap **1**, modified cap **8**, and a control reaction containing no-cap. After DNase digestion of the template, the transcripts containing 5′-caps or no-caps were purified by using the MEGAclear™ Kit (Ambion, Inc.). The transcript produced with T7 RNA polymerase using modified cap **8** was of the predicted length (1.85 kb) and indistinguishable in size and homogeneity from those produced with either standard cap **1**, or control reaction. Analysis of each transcribed mRNA was performed on an Agilent 2100 Bioanalyzer, which revealed that mRNAs were not degraded and were of similar size (data not shown). The yield of T7 RNA polymerase transcription reaction with standard cap **1**, and modified cap **8**, along with control reactions is shown in Figure 3. The modified cap **8** analog produced optimum yield as compared to standard mCAP **1**.

We next measured the effect of $m^{7,2',3'\text{-isopropylidene}}G[5']ppp[5']G$ **8** on the mRNA stability in cultured cells, and compared to mRNAs

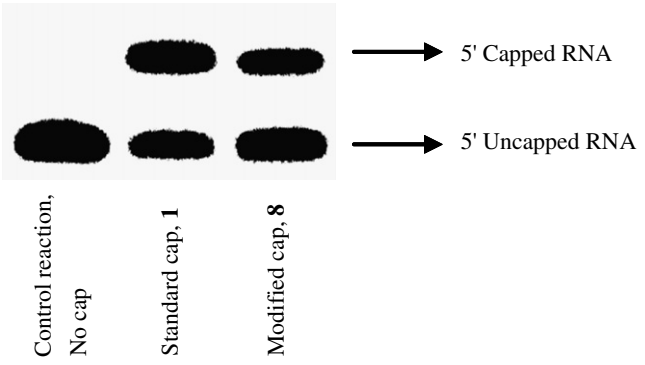


Figure 2. dPAGE gel (20%) showing capping efficiency of standard cap **1** and modified cap **8**. The control reaction was normal in vitro transcription reaction, in which no-cap analog was added. Radiation in the gel bands of interest were quantified by a phosphorimager (GE Healthcare).[24]

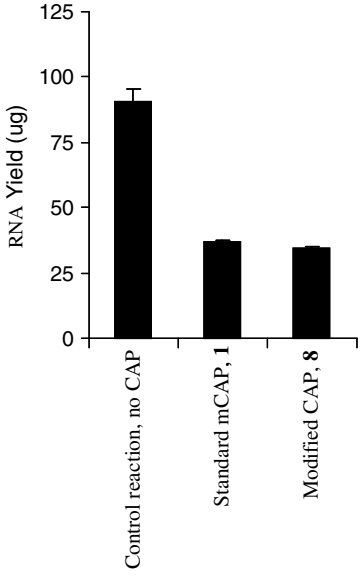


Figure 3. Yield of T7 RNA polymerase transcription reaction with standard **1** and modified CAP **8** analog.[25]

bearing standard cap **1** and no-cap. The experimental procedure to determine the cellular stability assay for 5′-capped luciferase mRNA was carried out as reported previously with little modification.[26,27] In vitro transcribed 5′-capped mRNAs from standard mCAP **1**, mixed population of 5′-capped and uncapped mRNA (Poly A) from modified cap **8**, and uncapped control reactions were electroporated into HeLa cells. The resulting 1.85 kb transcripts contain an open reading frame for luciferase and a 60 base Poly(A) tail at their 3′ end, both known to be essential for function and consequential stability in vivo. Following mRNA addition and electroporation, HeLa cells were allowed to recover in growth media, then harvested at intervals up to 10 h. The amount of intact cellular luciferase mRNA was measured by real-time PCR using TaqMan assays as described in User Bulletin No. 2 for the ABI Prism 7700 Sequence Detection System. Luciferase mRNA (5′-capped and uncapped populations) with modified cap **8** was found to be ~1.7 times more stable than mRNA with standard cap **1** and ~2.5 times more stable compared to uncapped control mRNA as shown in Figure 4.

The AmbLuc Poly(A) vector was transcribed with T7 RNA polymerase, creating an RNA encoding the firefly luciferase protein with a polyA tail. Three transcripts were made: the control was a standard in vitro transcription reaction having a final concentration of 7.5 mM of GTP, while the capped transcript reactions had final concentrations of 1.5 mM of GTP and 6.0 mM of either m^7G[5′]ppp[5′]G **1** or $m^{7,2',3'\text{-isopropylidene}}$G[5′]ppp[5′]G **8**, in an otherwise standard reaction mixture. The RNA product generated from this transcription reaction was next transfected by using HeLa cells.[28] Protein production post-transfection was measured as luciferase activity by using the Luciferase Assay System from Promega (E1483). Cells were harvested and lysed at 5, 10, 15, 20, 25, 30, and 40 h of post-transfection and luciferase activity was measured according to kit protocols. Luciferase activity data were normalized to the control reaction, that is, the no-cap, transcript transfection results. This comparison is illustrated in Figure 5. Both capped luciferase RNAs, luciferase expression exceeded that from the uncapped transcript at all time points, with peak amounts at 15–20 h after transfection. However, the gains in peak amounts of luciferase enzyme from modified cap analog **8** almost tripled those made by standard mCAP **1**. This could reflect both the lack of nonfunctional (reverse-capped) capped transcripts and the longer half-life of the cap-**8** transcripts.

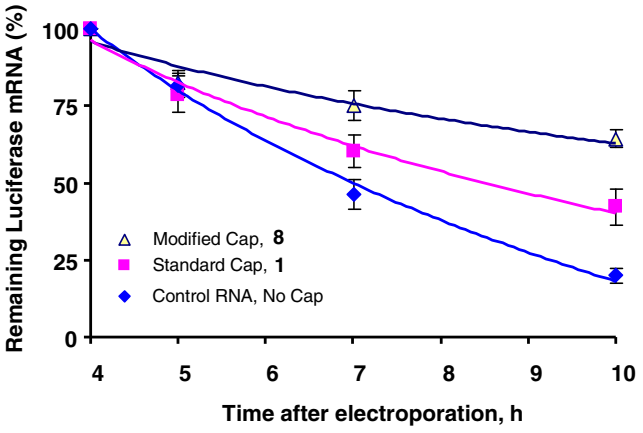


Figure 4. The amount of intact cellular luciferase mRNA was measured by real-time PCR using TaqMan assays with mixed population of 5′-capped and uncapped mRNA (Poly A) from modified cap **8**, standard cap **1**, and uncapped mRNAs as measured by real-time PCR. In vitro transcribed, 5′-capped mRNAs were electroporated into HeLa cells. Following discharge, cells were incubated at 37 °C and harvested at various times (4–10 h). Cells were lysed, total RNA isolated with TaqMan® Gene Expression Cells-to-CT™, and levels of fLuc mRNA were determined by real-time PCR.[27]

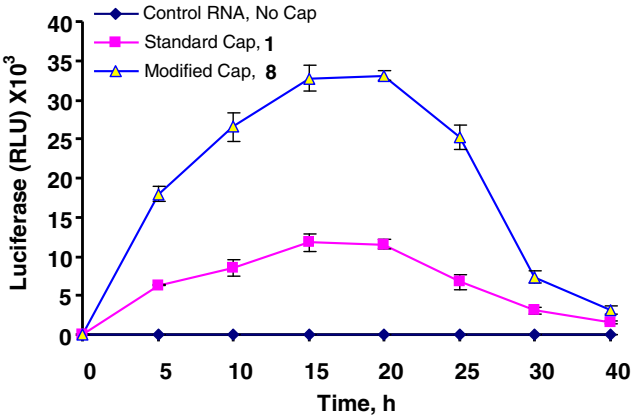


Figure 5. Translation efficiency of 5′-capped mRNA Poly(A) from m^7G[5′]ppp[5′]G **1** and $m^{7,2',3'\text{-isopropylidine}}$G[5′]ppp[5′]G **8.** The standard mCAP **1**, and modified cap **8,** 5′-capped in vitro transcribed Poly(A) tailed luciferase RNA (400 ng) were transfected into HeLa cells. The fold induction of luciferase protein data was normalized to the control reaction, that is, no-cap, mRNA Poly(A) transfection results.[28]

In summary, the biological activity of our modified new cap, $m^{7,2',3'\text{-isopropylidene}}$G[5′]ppp[5′]G **8** was evaluated relative to the standard mCAP, m^7G[5′]ppp[5′]G **1**. It was tested with respect to its capping efficiency, intracellular stability, and impact on translation activity, the last two by using transfection of transcripts made in vitro into cultured HeLa cells. The C2′ and C3′ isopropylidene substituted at N^7-methylguanosine cap analog, when used in in vitro transcription protocols, allows the generation of 5′-capped RNA in yields consistent with those obtained using standard mCAP **1**. The intracellular stability of 5′-capped and uncapped RNAs was measured by looking for the amount of intact RNA after transfection by electroporation, as measured by real-time RT-PCR using a TaqMan assay. The RNA capped with modified cap **8** was found to be ~1.7 times more stable than mRNA with standard cap **1** and ~2.5 times more stable than the 5′-uncapped control RNA. The RNAs made with this new cap, $m^{7,2',3'\text{-isopropylidene}}$G[5′]ppp[5′]G **8**, were compatible with the mammalian translation apparatus, producing luciferase enzyme from capped transcripts transfected into HeLa cells. It is therefore useful for in vitro (cell culture or presumably purified systems) translation experiments. The 2′,3′-isopropylidene substituted cap analog, ($m^{7,2',3'\text{-isopropylidene}}$G[5′]ppp[5′]G) **8** actually proved to be an excellent substrate for cap-dependent translation, as it was translated the most efficiently, generating ~2.9-fold more luciferase from the same amount of RNA (as judged by activity) than the standard cap (m^7G[5′]ppp[5′]G) **1**. The high translation activity observed is likely resulted as a consequence of exclusively forward capped transcripts and increased cellular stability of the 5′-modified capped mRNA (Poly A). Current biotechnology efforts for both in vitro and in cyto protein production will benefit from these characteristics.

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References and notes

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23. *Typical procedure to make* ***8***. To a stirred solution of m $^{7,2',3'\text{-isopropylidene}}$GDP **6** (0.14 g, 0.20 mmol) and ImGMP **7** (0.07 g, 0.17 mmol) in 10.0 mL dry DMF, zinc chloride (0.046 g, 0.34 mmol) was added under nitrogen atmosphere, and the reaction mixture was stirred at rt for 4 h. After 4 h, the reaction mixture was added to a solution of EDTA disodium (0.25 g, 0.68 mmol) in 100.0 mL of water at 0 °C. The resulting aqueous solution was adjusted to pH 5.5 and loaded on a DEAE Sephadex column. The desired product was eluted using a linear gradient of 0–1 M TEAB, and the fractions containing the product were pooled, evaporated, and concentrated to 10.0 mL TEA salt of isopropylidene cap analog. The resulting 10.0 mL was passed through a Strata-X-AW column and washed with 10.0 mL water followed by 10.0 mL MeOH. Then, the desired compound was eluted with 15.0 mL of $NH_4OH/MeOH/H_2O$ (2/25/73), and the collected solution was evaporated and dried to give a fine white powder **8** (Yield: 0.073 g, 53%). Data for **8**. ^{1}H NMR (D_2O, 400 MHz) δ 5.94 (d, J = 2.4 Hz, 1H), 5.82 (d, J = 6.0 Hz, 1H), 5.17 (m, 1H), 5.09 (d, J = 5.2 Hz, 1H), 4.76 (m, 1H), 4.66 (t, J = 5.2 Hz, 1H), 4.47 (t, J = 4.4 Hz, 1H), 4.36–4.14 (m, 5H), 4.05 (s, 3H), 1.63 (s, 3H), 1.42 (s, 3H); ^{31}P NMR (D_2O, 162 MHz) δ *−9.96* (d, J = 20.1 Hz), −11.18 (d, J = 18.8 Hz), −21.71 (t, J = 18.6 Hz); MS (m/z): 841 $[M-H]^-$.
24. *Gel shift assay*. The Ambion pTri β actin template was used in an in vitro transcription reaction omitting pyrimidine nucleotides, resulting in the termination of transcription after the first seven coded nucleotides, all purines. Syntheses of the capped and uncapped oligoribonucleotides performed by using the MAXIscript kit (Ambion, Inc.), following manufacturer's protocol. Typically, 20 μl of the transcription reactions contained the following final concentrations of components: linearized pTri β actin vector template, 25 ng/μl (0.5 μg total); ATP, 2 mM; GTP, 0.4 mM; standard mCAP **1** or modified cap **8**, 1.6 mM each in separate reaction; reaction buffer, 1×; T7 RNA polymerase, 50 U/μl; and (α-^{32}P) ATP, 800 (Ci/mmol). In the control reaction, no-cap analog was added. The transcription reactions were incubated at 37 °C for 2 h, after which the reaction mixtures (10 μl) were then applied to a 20% dPAGE gel. Radiation in the gel bands of interest was quantified by a phosphorimager (GE Healthcare).
25. *In vitro transcription of the luciferase mRNA*. T7 RNA polymerase transcription was performed by using the MEGAscript kit (Ambion, Inc.). All transcription reactions were performed in a 20 μl final volume at the following final concentrations of components: linearized AmbLuc Poly(A) DNA, (1.0 μg total); 1× reaction buffer; ATP, UTP, and CTP, 7.5 mM each; and 50 U/μl of T7 RNA polymerase. Additionally, GTP was present at 7.5 mM in the no-cap control; and in the reactions with cap analog included GTP was present at 1.5 mM, while the cap analog (standard mCAP **1** or modified cap **8**) was present at 6.0 mM. The transcription reactions were incubated at 37 °C for 2 h. In order to hydrolyze the remaining plasmid DNA, 1 μl of turbo DNase was added to the reaction mixture, and further incubated at 37 °C for 15 min. Purifications of the RNA from these transcription reactions were done by using the MEGAclear™ Kit (Ambion, Inc.) as per manufacturer's protocol.
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27. *Cellular stability assay for 5′-capped luciferase mRNA*. In vitro transcribed, 5′-capped RNAs from standard mCAP **1**, mixed population of 5′-capped and uncapped mRNA (Poly A) from modified cap **8**, and uncapped control luciferase mRNAs from the above transcriptions were transfected into HeLa cells by electroporation. Cells were centrifuged at 300*g* for 5 min and resuspended in siPORTER electroporation buffer. Cells (75,000) in a total volume of 75 μl were placed in 1 mm path length cuvettes, 1 μg of mRNA was added, and electroporation was performed with the Bio-Rad Genepulser at 400 V/250 mF. Following discharge, cells were incubated in cuvettes at 37 °C for 10 min, then transferred to plates with pre-warmed complete medium (10,000 cells per well of 96-well plate), and placed at 37 °C in a 5% CO_2 humidified atmosphere. Cells were harvested at various post-electroporation time points (4–10 h), washed twice with PBS, and levels of fLuc mRNA were determined directly from the cells using the TaqMan® Gene Expression Cells-to-CT™ Kit (Applied Biosystems). Luciferase mRNA remaining at each time point was converted to a percent of the RNA present at earliest time point (4 h). The results were plotted as remaining intact luciferase mRNA versus time post-electroporation.
28. *Luciferase assay*. HeLa cells (60,000/well in 24-well plates) were seeded at least 12 h before transfection in growth medium without antibiotics. A complex of 5′-capped RNA was prepared by mixing 600 ng (2 μl) of RNA, 2.5 μl of TFX-20 (Promega), and 300 μl of serum-free DMEM in polystyrene tubes and incubated for 15 min at room temperature. After the incubation, media from the pre-plated HeLa cells were removed and 200 μl of the complex was added to each well. The plates were incubated for 1 h at 37 °C, and then 1 mL of pre-warmed media with serum was added. The transfected plates were incubated at 37 °C. Cells were harvested and lysed at 5, 10, 15, 20, 25, 30, and 40 h post-transfection. The cells were harvested and lysed by removing the media and adding 100 μl of 1× passive lysis buffer (Promega). The plate was mixed carefully to disrupt the cells and 10 μl of cell lysates from each transfections was mixed with 100 μl of luciferase substrate (Promega) and measured immediately on a luminometer (POLARstar OPTIMA, BMG Labtech) in 96-well plates. The fold induction of luciferase protein data was normalized to the control reaction, that is, the no-cap, mRNA Poly(A) transfection results.