Differential Inhibition of mRNA Degradation Pathways by Novel Cap Analogs

Ewa Grudzien1, Marcin Kalek1, Jacek Jemiesty2, Edward Darzyynkiewicz1, and Robert E. Rhoads1

From the 1Department of Biophysics, Warsaw University, Warsaw, 02-089, Poland and the 2Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, Louisiana 71130-3932

mRNA degradation predominantly proceeds through two alternative routes: the 5′→3′ pathway, which requires deadenylation followed by decapping and 5′→3′ hydrolysis; and the 3′→5′ pathway, which involves deadenylation followed by 3′→5′ hydrolysis and finally decapping. The mechanisms and relative contributions of each pathway are not fully understood. We investigated the effects of different cap structure (GpppG, m7GpppG, or m7,3′-OpppG) and 3′ termini (ASp, A60, or G60) on both translation and mRNA degradation in mammalian cells. The results indicated that cap structures that bind eIF4E with higher affinity stabilize mRNA to degradation in vivo. mRNA stability depends on the ability of the 5′ terminus to bind eIF4E, not merely the presence of a blocking group at the 5′-end. Introducing a stem-loop in the 5′-UTR that dramatically reduces translation, but keeping the cap structure the same, does not alter the rate of mRNA degradation. To test the relative contributions of the 5′→3′ versus 3′→5′ pathways, we designed and synthesized two new cap analogs, in which a methylene group was substituted between the α- and β-phosphate moieties, m2,3′-OpppG and m2,7,3′-OpppG, that are predicted to be resistant to cleavage by Dcp1/Dcp2 and Dcp5, respectively. These cap analogs were recognized by eIF4E and conferred cap-dependent translation to mRNA both in vitro and in vivo. Oligonucleotides capped with m2,7,3′-OpppG were resistant to hydrolysis by recombinant human Dcp2 in vitro. mRNAs capped with m2,3′-OpppG, but not m2,7,3′-OpppG, were more stable in vivo, indicating that the 5′→3′ pathway makes a major contribution to overall degradation. Luciferase mRNA containing a 5′-terminal m2,3′-OpppG and 3′-terminal poly(G) had the greatest stability of all mRNAs tested.

The 5′ terminus of all eukaryotic cellular mRNAs is modified with a 5′-5′ m7GTP-containing cap (1). Caps fulfill a variety of functions in the synthesis, translation, and degradation of mRNA. The presence of the 5′ cap structure increases both the accuracy and efficiency of pre-mRNA splicing (2, 3). 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 (4). In the cytosol, the cap structure is required for efficient translation of mRNA. The cap is specifically recognized by the translational initiation factor eIF4E (5, 6). Binding of eIF4E to the cap occurs during formation of the 43 S initiation complex, which is rate limiting for translation initiation under normal conditions (7, 8). Finally, the cap serves as one determinant of mRNA degradation. Capped mRNAs are more stable than their uncapped counterparts (9). The cap structure helps to protect RNA from degradation by 5′→3′-exonucleases located in the cytosol and nucleus, as demonstrated in both Saccharomyces cerevisiae (10) and mammalian cells (11, 12).

There are two major pathways by which polyadenylated mRNA is degraded in eukaryotic cells, a 5′→3′ pathway and a 3′→5′ pathway, as well as two specialized routes for aberrant mRNA degradation (21). In both the 5′→3′ and 3′→5′ pathways, shortening of the poly(A) tract initiates mRNA decay. There are several mechanisms of deadenylation (21), but one of them involves a poly(A)-specific ribonuclease (22), an enzyme that has affinity for the cap structure (23–25). This provides another link between events at the 5′- and 3′-ends of mRNA. In the 5′→3′ pathway, removal of the cap structure occurs rapidly after poly(A) tract shortening (26). This process is facilitated by the decapping enzyme Dcp1/Dcp2, which in turn exposes the transcripts to digestion by a highly processive 5′→3′-exonuclease Xrn1 (10). Dcp2 plays a catalytic role, whereas Dcp1 stimulates Dcp2 activity (27–29). Both the cap and an oligonucleotide chain of at least 25 nucleotides are required for recognition by the Dcp1/Dcp2 complex (27, 30, 31). Hydrolysis by either the Dcp1/Dcp2 complex or Dcp2 alone releases m7GDP, suggesting that the site of cleavage is between the α- and β-phosphate moieties of the cap but not between the β and γ moieties. In the 3′→5′ pathway, deadenylated mRNA is degraded by the exosome in a 3′→5′ direction, as demonstrated both in vitro (32–35) and in vivo (35). The products are capped oligonucleotides, which are then decapped by a scavenger-decapping enzyme, Dcp6 (36). Dcp6 releases m7GMP, suggesting that cleavage occurs between the β- and γ-phosphate moieties.

In mammalian cells it was initially thought that the 3′→5′ pathway predominates. This conclusion was partially based on the observation of m7GMP but not m7GDP production (35). The use of probes against 5′...
and 3’ sequences also indicated that 3’→5’ degradation was predominant (35). However, the discovery of Dcp2 in mammalian cells opened the possibility that 5’→3’ degradation can also play a significant role. Additionally, it was shown that Dcp5 can efficiently convert m’ GDP to m’GMP in extracts of both yeast and human cells, suggesting that m’GMP production does not allow one to distinguish between the two pathways (37). At present, the relative contributions of 3’→5’ and 5’→3’ pathways in mammalian mRNA degradation are unclear.

Several lines of evidence suggest that the presence of a poly(A) tract inhibits decapping. First, deadenylation precedes decapping regardless of whether the rate of deadenylation is increased or decreased (38, 39). Second, products of the decapping reaction appear only when at least some of the mRNAs have undergone deadenylation (26, 38, 40). Third, mRNAs with poly(A) tracts are resistant to decapping in cell-free extracts, and this effect requires the presence of PABP (41). PABP was also shown to inhibit decapping in yeast (14, 40, 42). Diminished decapping may be due to increased occupancy of the cap by eIF4E, because PABP and eIF4E both bind eIF4G at nearby sites (16, 43, 44), and PABP increases the affinity of eIF4E for the cap (45). Alternatively, PABP may inhibit decapping through a direct and specific association with the 5’-end of capped mRNA (46). PABP also stimulates translation of capped mRNAs (18, 47, 48).

The dual role of PABP in stimulating translation and inhibiting mRNA decay suggests that translation initiation and mRNA decay are linked. This connection is further supported by several observations. Addition of eIF4E inhibits Dcp1/Dcp2 activity in vitro, and this inhibition is thought to be due to eIF4E binding to the cap because m’GTP restores decapping, at least in yeast (49, 50). Inhibition of translational initiation by inserting strong secondary structure in the 5’-UTR of mRNA leads to faster decapping (26), but inhibition of translation elongation by cycloheximide stabilizes mRNA (51). Yeast strains that are defective in several translation initiation factors (eIF4E, eIF4G, eIF4A, and eIF3) show an increase in decapping rate as well as the rate of deadenylation, suggesting that deadenylation may be controlled primarily by the translational status of mRNAs (52). It has been shown that Dcp1 binds to both eIF4G and PABP as free proteins as well as to the complex of eIF4E-eIF4G-PABP (50). Finally, a temperature-sensitive allele of eIF4E suppresses the decapping defect of a dcp1-1 mutant, which argues that dissociation of eIF4E from the cap is required before decapping (49).

In this study, we set out to test directly, by the use of modified mRNA structures, the hypothesis that cap binding by eIF4E inhibits mRNA degradation in mammalian cells. We used cap analogs that differ in binding affinity for eIF4E to determine whether mRNA stability can be affected. Other cap analogs were used to test the relative contribution of the 5’→3’ versus 3’→5’ pathways. If mRNA is degraded by both pathways, selective blockage of one of them should stabilize mRNA. The available evidence suggests that Dcp1/Dcp2 and Dcp5 hydrolyze the cap at different sites. To achieve selective resistance to these two enzymes, we developed novel cap analogs in which each of two bridging pyrophosphate oxygens was separately replaced by a methylene group, one in the α-β position (mGpG and mGpG) and one in the β-γ position (mGpG). The first but not the second of these analogs stabilized mRNA in vivo.

**Experimental Procedures**

**Cell Culture**—The mouse mammary epithelial cell line MM3MG (American Type Culture Collection) was grown as a monolayer at 37 °C in a 5% CO2-humidified atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, minimum Eagle’s medium nonessential amino acid solution, and antibiotics (Invitrogen). Before electroporation, 70% confluent cells were detached using 1× PBS supplemented with 2.5 mM EDTA.

**Cap Analogs**—Synthesis of the cap analogs GpG, mGpG, bGpG, and mGpG has been described previously (33–36). Two new cap analogs were designed and synthesized for this study, mGpG and mGpG. Their synthesis and chemical characterization are described elsewhere (57).

**Construction of Plasmids**—Three plasmids derived from pGEM®-luc (Promega) were used as templates for in vitro synthesis of RNA. All of them contained the entire firefly luciferase mRNA coding sequence in pGEM4, but they differed in the 3’-end, producing upon cell-free transcription mRNAs either with no A residues, with 31 A residues (placA+ (58)), with 60 A residues (plac-A60), or with 6 G residues (plac-G16). For construction of plac-A60, two oligonucleotides, 5’-GCC(A)16CC-GAATTTG-3’ and 5’-AACAATTCGG(T)16GGGAGCT-3’, were annealed and inserted into the Hpal and SacI restriction sites of plac-A+. For construction of plac-G16, an insert containing G16 was obtained by PCR amplification using linear plac-A+ and two oligonucleotides, 5’-AAAAACCCG(T)16CC-GAATTTG-3’ and 5’-AGTGTCATCATTTGAAAACGTTCTTCGCGGCGGCG-3’. The incubation conditions were as follows: 2 min at 95°C for polymerase activation; 5 cycles for 45 s each at 95°C, 1 min at 51°C, and 10 min at 72°C; 30 cycles of 45 s at 95°C, 45 s at 65°C, and 1 min 45 s at 72°C; and a final extension at 72°C for 10 min. The resulting product was digested with Smal and Xmn1 and inserted into plac-A+. Plasmids pT7-luc-A50 and pT7-SLlac-A50 (59) were generously provided by Daniel Gallie (University of California, Riverside, CA).

**In Vitro Synthesis of mRNAs**—RNAs containing different cap structures were synthesized by in vitro transcription of luciferase-encoding plasmids (plac-A+, plac-A60, plac-G16, pT7-lac-A50, or pT7-SLlac-A50) with T7 polymerase in the presence of all four nucleoside triphosphates and various cap dinucleotides (58). plac-A+, plac-A60, and plac-G16 were digested with HpaI for synthesis of luciferase mRNA and with Ncol for synthesis of capped oligonucleotides. pT7-lac-A50 and pT7-SLlac-A50 were digested with Drai for synthesis of luciferase mRNA. After incubation, 200-μl reaction mixtures were treated with 3 units of DNase RQ1 (Promega) for 20 min at 37°C, extracted with phenol and chloroform, and the RNA precipitated with ethanol. In some cases, RNA was purified with an RNeasy mini kit (Qiagen) using the manufacturer’s protocol. The concentrations of RNAs were determined spectrophotometrically. RNAs derived from pGEM®-lac, plac-A+, pT7-luc-A50, pT7-SLlac-A50, plac-A60, and plac-G16 contained 3′-terminal tracts of 0A, 31 A, 50 A, 50 A, 60 A, or 6 G residues and are referred to as Luc-A, Luc-A12, Luc-A31, Luc-A50, Luc-A60, and Luc-G50, respectively. 32P-Labeled mRNAs were transcribed in vitro with T7 RNA polymerase in the presence of [α-32P]GTP and cap analog as described above.

**Preparation of Polyosomes**—To separate ribosomal subunits and initiation complexes, 4×106 MM3MG cells were incubated with medium containing 0.1 mg/ml cycloheximide for 5 min at 37°C. The medium was removed, and the cells were treated for 2 min with ice-cold PBS containing 0.1 mg/ml cycloheximide, washed twice with the same medium, and lysed in 700 μl of 0.3 M NaCl, 15 mM Tris-HCl (pH 7.6), 15 mM MgCl2, 1% Triton X-100, 1 mg/ml heparin, and 0.1 mg/ml cycloheximide. After centrifugation at 14,000 × g for 10 min, the supernatant was layered on a 15–50% sucrose gradient in the same buffer but lacking Triton X-100 and centrifuged in a Beckman SW41Ti rotor at 38,000 rpm at 4°C for 2 h. Gradients were fractionated with continuous mon-
itoring of absorbance at 260 nm. RNA from each fraction (1 ml) was isolated and analyzed by real time PCR.

In Vivo Measurement of Translational Efficiency and mRNA Decay—RNA (5 μg) was introduced into 10^7 MM3MG cells by electroporation in a total volume of 400 μl of serum-reduced Opti-MEM® I medium (Invitrogen) in a Gene Pulser cuvette (4 mm gap) by use of a Bio-Rad GenePulser™ set at 0.22 kV and 960 microfarads. All reagents were kept on ice prior to electroporation. Following discharge, the cells were washed twice with cold PBS, centrifuged for 2 min 300 g at room temperature, resuspended in prewarmed complete medium, and placed at 37 °C. When translational efficiency was to be measured, cells were divided into several Eppendorf tubes, placed in a water bath at 37 °C, and shaken. When mRNA stability was to be measured, cells were distributed into 35-mm cell culture dishes and placed at 37 °C in a 5% CO_2 humidified atmosphere. Cells were harvested at various times and washed twice with PBS. For cytoplasmic RNA extraction, 2 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl_2, 0.5% (v/v) Igepal (Sigma), and 1 mM dithiothreitol) RNAs were further purified using the RNeasy mini kit. For protein extraction, 2 × 10^6 cells were lysed in 200 μl of Luciferase Cell Culture Lysis Reagent (Promega). Luciferase activity of cell extracts was measured according to the manufacturer’s protocol (Promega).

Real Time PCR—Approximately 2 μg of each total RNA sample isolated from MM3MG cells were treated with DNase RQ1 (Promega) as described above. Reverse transcription was performed on 400 ng of RNA in 20-μl reaction mixtures containing 5.5 mM MgCl_2, 500 μM of each dNTP, 2.5 μM random hexamers, 0.2 units of RNase inhibitor, and 0.8 units of MultiScribe reverse transcriptase (Applied Biosystems). Reaction mixtures were incubated at 25 °C for 10 min, 48 °C for 30 min, and 95 °C for 5 min. Quantitative real time PCR was performed with specific primers designed for each mRNA with the Bacon Designer tool (Bio-Rad). For detecting sequences at the 5' end of luciferase mRNA, the primers were 5'-CGTTCGTTGCGAAGACGATA-3' and 5'-ACGTGTTGACATCTCCATT-3'. For the 3'-end, they were 5'-TTGAGGGTTGCTGATGATCT-3' and 5'-ACATAATTGATCTGGCGATC-3'. Luciferase mRNA from the cell structure to the beginning of the 3'-terminal homopolymer tract consisted of 1714 nucleotides. These primers amplified nucleotides 226–398 and 1093–1183, respectively. Mouse GAPDH mRNA levels were measured by the same method and in the same RNA samples with the use of the primers 5'-CAATGTTTCCGTCTGCAGATC-3' and 5'-GAGAGTGGGATGTTGCTGATGA-3'. Amplification and detection were performed with the iCycler IQ real time PCR detection system in 25-μl reaction mixtures containing 5 μl of the transcription reaction mixture (50 ng of cDNA), 12.5 μl of IQ SYBRgreen Supermix, and 0.3 mM primers (Bio-Rad). The incubation conditions consisted of 3 min at 95 °C for polymerase activation and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Luciferase mRNA levels were calculated using the absolute standard curve method as described in User Bulletin No. 2 for the ABI Prism 7700 Sequence Detection System. After the amount of luciferase mRNA was calculated from a standard curve, it was normalized for the amount of mouse GAPDH mRNA in each sample. Finally, luciferase mRNA remaining at each time point was converted to a percent of the RNA present at zero time, and the results were plotted as log_{10}(RNA) versus time to determine t_{1/2}. For analysis of RNA from polysome gradients, in vitro synthesized green fluorescent protein mRNA was added to each fraction before RNA isolation as an internal standard to control variation in RNA yield. The level of green fluorescent protein mRNA was used to normalize the levels of luciferase and GAPDH mRNA.

Northern Blotting—Northern blotting was performed by using a riboprobe for luciferase mRNA, made by in vitro transcription by SP6 polymerase (Promega) of Ncol-digested pluc-A60. Plasmid was transcribed in a total volume 20 μl in the presence of 5 μCi of [α-32P]GTP (ICN Biochemicals). The membrane was pre-hybridized for 2 h and then hybridized with the probe overnight at 65 °C.

In Vitro Translation—A micrococcal nuclease-treated RRL system was used for in vitro translation as described previously (60). Optimal cap-dependent translation was achieved at 100 mM potassium acetate and 1.4 mM magnesium chloride. For measurement of translational inhibition, the added mRNA was natural rabbit globin mRNA, and protein synthesis was measured by incorporation of [3H]Leu. Calculation of K<sub>v</sub> values and normalization of data were performed as described previously (60). The concentrations of dinucleotide cap analog solutions were measured by UV absorption at pH 7.0 using λ = 255 nm and ε<sub>M</sub> = 22.6 × 10<sup>2</sup> M<sup>-1</sup> cm<sup>-1</sup>. For measurement of translational efficiency, protein synthesis was measured by assaying luciferase activity in a Monolight 2010 luminometer (58). Translational efficiency data were computed and normalized as described previously (58).

In Vitro RNA Decapping Assay—Dcp2 activity was measured with a truncated form of luciferase mRNA (48 nucleotides). The GST-hDcp2 was expressed in Escherichia coli and purified as described previously (61). Capped oligonucleotides were first subjected to digestion with GST-hDcp2 at 37 °C for 2 h (61). The reaction mixture was then extracted once with an equal volume of phenol and twice with chloroform, and RNA was precipitated with ethanol. Products of the decapping reaction were further digested with RNase One (Promega) at 37 °C for 1 h. The products were resolved by anion-exchange HPLC on a 4.6 × 250-mm Partisil 10 SAX/25 column (Whatman). The gradient consisted of water for 1 min, a linear gradient to 112 mM KH<sub>2</sub>PO<sub>4</sub>, pH 4.5, for 40 min, a linear gradient of 112–450 mM KH<sub>2</sub>PO<sub>4</sub> for 30 min, a linear gradient of 450 mM to 1.5 M KH<sub>2</sub>PO<sub>4</sub> for 30 min, and isocratic elution at 1.5 M of KH<sub>2</sub>PO<sub>4</sub> for 5 min, all at a flow rate 0.5 ml/min. Fractions of 2 ml were collected, and Cerenkov radiation was measured.

RESULTS

mRNAs Capped with Modified Analogs Are Translated More Efficiently in Vivo—We took a new approach to investigate the relationship between translational initiation and mRNA decay that utilized mRNAs capped with analogs that modified translational efficiency. Previously we designed and synthesized several cap analogs that, when incorporated into mRNA, produced higher translational efficiencies in vitro than the standard cap analog, m<sub>7</sub>Gp<sub>7</sub>G (56, 58, 62). The first group of compounds consisted of cap analogs that prevent incorrect incorporation. One-third to one-half of m<sub>7</sub>Gp<sub>7</sub>G is incorporated into RNA in the reverse orientation during in vitro transcription (63). “Anti-reverse” cap analogs (ARCA) have modifications in either the C-2 or C-4 position of m<sub>7</sub>Gp<sub>7</sub>G. These also produce mRNAs that are translated 2-fold more efficiently than their 7-methyl counterparts, even without the ARCA modification (62). This is because of a combination of higher % capping (79 versus 69%), higher % correct orientation (76 versus 58%), and higher affinity for eIF4E (62). The later property likely results from more efficient stacking of the benzyl-containing cap with the indole ring of Trp-166 in eIF4E (64, 65). Previously these compounds were tested in vitro with an
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RRL translational system for both inhibition of translation when used as free cap analogs or for stimulation of translation when incorporated into mRNA. However, the RRL system differs in several aspects from intact cells (see "Discussion"). Because mRNA turnover could be studied only in a whole-cell system, it was necessary to test the translational efficiency of mRNAs capped with these new cap analogs in a whole-cell system as well.

We therefore developed an in vivo system to measure translational efficiencies of modified mRNAs. This consisted of electroporating synthetic mRNAs into mouse mammary epithelial cells (MM3MG), which have normal eIF4E levels, unlike many mammary gland cell lines (66). RNAs were synthesized in vitro containing various 5'/H11032 cap and various 3'/H11032 termini. Cells were removed at intervals following electroporation and lysed, and luciferase activity in the supernatant was measured by luminometry. Luciferase activity was normalized for the amount of luciferase mRNA that had been delivered into the cells, as measured by real time PCR. The luciferase mRNA concentration did not change appreciably over the period during which luciferase accumulation was measured (~75 min; data not shown). This approach could potentially give false results if luciferase mRNA recovered from electroporated cells consisted of both translated and nontranslated pools. Such a situation could compromise measurement of both translational efficiency and mRNA decay. We therefore tested the polysomal distribution of luciferase mRNA by real time PCR (Fig. 2). The mRNA was predominantly in polysomes (Fig. 2B, fractions 6–9), with disomes containing the most (fraction 6), although some was also present at the sedimentation of initiation complexes (fractions 3–5). More importantly, little luciferase mRNA that had been delivered into the cells, as measured by real time PCR. The luciferase mRNA concentration did not change appreciably over the period during which luciferase accumulation was measured (~75 min; data not shown). This approach could potentially give false results if luciferase mRNA recovered from electroporated cells consisted of both translated and nontranslated pools. Such a situation could compromise measurement of both translational efficiency and mRNA decay. We therefore tested the polysomal distribution of luciferase mRNA by real time PCR (Fig. 2). The mRNA was predominantly in polysomes (Fig. 2B, fractions 6–9), with disomes containing the most (fraction 6), although some was also present at the sedimentation of initiation complexes (fractions 3–5). More importantly, little luciferase mRNA was present in the untranslated fraction (Fig. 2B, fractions 1–2).

Endogenous GAPDH mRNA was more efficiently translated (Fig. 2C, fraction 9), although some also sedimented in the region of initiation complexes. These results suggest that essentially all of the luciferase...
mRNA is actively translated, validating measurements of translational efficiency and rate of degradation.

We found conditions in which accumulation of luciferase was linear with time, after an initial lag period of ~25 min that is required for recruitment of mRNA to ribosomes, completing the polypeptide chain, and release of luciferase into the cytosol (Fig. 3A). Luciferase accumulation was also linear with electroporated mRNA up to 15 μg of mRNA per 10⁷ cells (data not shown). Luc-A₃₃ mRNA capped with m⁷,₃'-⁰Gp₃G (4) and b⁵Gp₃G (3) were translated 2.5- and 1.6-fold more efficiently, respectively, than mRNA capped with m³Gp₃G (Fig. 3A). This is similar to our previous result with in vitro translation in the RRL system, in which Luc-A₃ mRNA capped with m₇,₃'-⁰Gp₃G and b⁵Gp₃G were found to be translated 1.9-fold more efficiently than Luc-A₃ mRNA capped with m⁷Gp₃G (62). It is noteworthy that these two types of cap modifications are completely different (Fig. 1), yet they stimulate translational efficiency in both a nonpoly(A)-dependent in vitro system (RRL) and a poly(A)-dependent in vivo system (MM3MG cells). The increase in translational efficiency is most likely due to more frequent occupancy of the 5' terminus by eIF4E, and recruitment of associated initiation factors.

The Ability to Bind eIF4E, Not Merely the Presence of a Blocking Group at the 5'-End, Stabilizes mRNA against in Vivo Degradation—

We next asked whether the nature of the cap could influence mRNA stability. Luc-A₃₃ mRNA transcripts containing either Gp₃G (1), m⁷Gp₃G (2), or m₇,₃'-⁰Gp₃G (4) at the 5'-end were electroporated into MM3MG cells. Cells were harvested at various times up to 5-6 h after electroporation, and cytoplasmic RNA was extracted. The amount of luciferase mRNA was measured by real time PCR by using primers that amplify sequences near the 5'-end (see “Experimental Procedures”). As shown in Fig. 4A and Table 1, Luc-A₃₃ mRNA capped with m³Gp₃G was more stable than the same RNA capped with Gp₃G (t½ = 60 versus 45 min). Luc-A₃₃ mRNA capped with m₇,₃'-⁰Gp₃G was even more stable (t½ = 90 min). The increase in stability correlates with increased affinity of eIF4E for the cap, because Gp₃G is not recognized by eIF4E, m₇Gp₃G is incorporated approximately equally in the correct and reversed orientations, the

![Image](https://example.com/image.png)
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latter of which is not recognized by eIF4E (56, 63), and m_{2,3\prime,5\prime}-O\text{Gp}_{3}\text{G is incorporated entirely in the correct orientation (56). It is conceivable that differences in stability caused by these cap structures would be observed only on mRNAs with short poly(A) tracts. By using an mRNA with a longer poly(A) tract, we could test the relative contributions of deadenylation and decapping to the overall rate of mRNA decay. For instance, if deadenylation were slow compared with decapping, the differences in mRNA decay due to these cap structures would be less pronounced with Luc-\text{A}_{50} mRNA than with Luc-\text{A}_{31} mRNA. As shown in Fig. 4B and Table 1, the half-lives were lengthened for each of the three caps for Luc-\text{A}_{50} compared with its Luc-\text{A}_{31} counterpart (\(t_\frac{1}{2} = 120\) versus 45 min for Gp\text{G} (1); \(t_\frac{1}{2} = 156\) versus 60 min for m\text{G}p\text{G} (2); and \(t_\frac{1}{2} = 282\) versus 90 min for m_{2,3\prime,5\prime}-O\text{Gp}_{3}\text{G} (4)). However, the ratios of half-lives for Luc-\text{A}_{50} compared with Luc-\text{A}_{31} were statistically indistinguishable for all three caps (2.7 ± 0.3 for Gp\text{G}; 2.6 ± 0.3 for m\text{G}p\text{G}; and 3.1 ± 0.2 for m_{2,3\prime,5\prime}-O\text{Gp}_{3}\text{G}). Thus, the effects of these cap analogs on mRNA stability are the same for mRNAs with short and long poly(A) tracts.

If the progressive increase in stability caused by capping with Gp\text{G}, m\text{G}p\text{G}, and m_{2,3\prime,5\prime}-O\text{Gp}_{3}\text{G} is indeed because of increased occupancy of the cap by eIF4E, which in turn inhibits decapping by Dcp1/Dcp2, the data comparing stability of Luc-\text{A}_{50} to Luc-\text{A}_{31} can be interpreted in two alternative ways. The first is that decapping follows deadenylation but is slow compared with deadenylation. With Luc-\text{A}_{50}, the mRNA reaches a deadenylated state sooner than with Luc-\text{A}_{31}. Despite this, the three caps have the same relative effect on mRNA stability. If decapping had been fast compared with deadenylation, the difference between the three cap structures would have been ameliorated for Luc-\text{A}_{50} compared with Luc-\text{A}_{31}. The other interpretation is that decapping and deadenylation occur independently of each other, i.e. deadenylation is not a prerequisite for decapping.

Our working model is that eIF4E and Dcp1/Dcp2 compete for the cap. High affinity for eIF4E reduces decapping and vice versa. An alternative interpretation is that binding of eIF4E to the cap promotes efficient initiation but that it is high translational efficiency per se that protects against degradation. To test this, we electroporated two forms of luciferase mRNA, one containing a hairpin loop (\(\Delta G° = -21.3\) kcal/mol in the 5′-UTR and a 3′-terminal 50-nucleotide poly(A) tract (SL_{13}\text{-Luc-}\text{A}_{50}) and an identical mRNA lacking the stem-loop (Luc-\text{A}_{50}). As shown in Fig. 3B, SL_{13}\text{-Luc-}\text{A}_{50} was translated in vivo much less efficiently than Luc-\text{A}_{50} as shown previously for these mRNAs (59). However, the rates of in vivo decay were the same (\(p < 0.05\); Fig. 4, C versus D; Table 1). Thus, the rate of decay is correlated with the type of cap (both SL_{13}\text{-Luc-}\text{A}_{50} and Luc-\text{A}_{50} were capped with m_{2,3\prime,5\prime}-O\text{Gp}_{3}\text{G} (4)) but not the translational efficiency.

Measurement of mRNA levels by real time PCR provides quantitative results, but it does not indicate whether the mRNA is intact. If a stable intermediary breakdown product of mRNA were to accumulate, it would give misleading results on the rate of mRNA degradation. We therefore examined the quality of m\text{G}p\text{G}-capped Luc-\text{A}_{50} by two techniques. In the first, m\text{G}p\text{G} mRNA was detected by Northern blotting at various times after electroporation (Fig. 5A). In the second, 32P-labeled mRNA was introduced into cells by electroporation and detected by PhosphorImager at various times (Fig. 5B). In both cases, the predominant form migrated as the intact mRNA. Furthermore, the rate of decay measured with either of these two methods was similar to that measured by real time PCR.

mRNAs Capped with Novel Methylene-Containing Analogs Are Resistant to Decapping in Vitro—As noted in the Introduction, current models hold that 5′ → 3′ exonucleolytic decay follows decapping. The results presented in Fig. 4, as well as these obtained with other experimental approaches (21), suggest that binding of the cap by eIF4E inhibits decapping and therefore protects mRNA against degradation. We developed

![Figure 5. Integrity of luciferase mRNAs after electroporation into MM3MG cells. A. m\text{G}p\text{G}-Luc-\text{A}_{50} was electroporated into MM3MG cells, and the RNAs were isolated at the indicated time points. The mRNA was detected by Northern blotting as described under “Experimental Procedures.” B. 32P-labeled m\text{G}p\text{G} capped Luc-\text{A}_{50} was electroporated into MM3MG cells. RNA was isolated at the indicated times, resolved by electrophoresis on a 1.25% formaldehyde agarose gel, and visualized on a PhosphorImager.](https://example.com/figure5)

**TABLE 2**

Biophysical and biochemical properties of methylene-containing cap analogs

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<thead>
<tr>
<th>Compound</th>
<th>Cap analog</th>
<th>(K_{\text{As}} \times 10^{-6} )</th>
<th>(K^{\text{mu}} )</th>
<th>Relative translational efficiency in vitro</th>
<th>Relative translational efficiency in vivo</th>
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<td>2</td>
<td>m\text{G}p\text{G}</td>
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<td>17.1 ± 1.0</td>
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<td>4</td>
<td>m_{2,3\prime,5\prime}-O\text{Gp}_{3}\text{G}</td>
<td>7.4 ± 0.1</td>
<td>14.3 ± 1.3</td>
<td>1.9 ± 0.4</td>
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<tr>
<td>5</td>
<td>m_{2,3\prime,5\prime}-O\text{P}<em>{\text{P}</em>{1}}\text{PPG}</td>
<td>4.7 ± 0.03</td>
<td>29.3 ± 2.3</td>
<td>1.1 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>m_{2,3\prime,5\prime}-O\text{P}<em>{\text{P}</em>{1}}\text{PPG}</td>
<td>4.4 ± 0.2</td>
<td>33.5 ± 4.5</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

\(^a\) Equilibrium association constants for interaction of mouse eIF4E (amino acids 28–217) with methylene-containing cap analogs at 20 °C are shown. Data are from Ref. 57.

\(^b\) Inhibitory constants (\(K_{i}\)) for inhibition of rabbit globin mRNA (60) translation in a rabbit reticulocyte lysate system are shown. Data are from Ref. 57.

\(^c\) Translational efficiency of luciferase mRNAs capped with methylene-containing cap analogs in a RRL system are shown. Two syntheses of capped RNA and three translation reactions were performed for each cap analog. The relative translational efficiency was calculated as described previously (58). The data represent the averages from experiments similar to that shown in Fig. 6B.

\(^d\) Translational efficiency of Luc-\text{A}_{50} capped with methylene-containing cap analogs in MM3MG cells are shown. Luciferase activity was normalized by the amount of luciferase RNA in the cells. Relative translational efficiency was calculated as above. The data represent the averages from experiments similar to that shown in Fig. 6B.

\(^e\) Data are from Ref. 58.
were synthesized that were capped with each of the 4 values obtained for mGpG (data summarized in Table 2). Both of the methylene cap analogs were 2-fold less effective than mGpG for inhibition of β-globin synthesis. This is in good agreement with the K_{AS} values obtained for direct binding of these compounds to elf4E.

Next we measured the efficiency with which transcripts capped with the methylene-containing compounds are translated in vitro and in vivo, a measure of interaction with the entire protein synthesis machinery. Forms of Luc-A_{A0} were synthesized that were capped with each of the new cap analogs as well as with mGpG and GpG as controls. mRNAs capped with m_{2-3′}Gpp(CH2)2G (6) and m_{2-3′}Gpp(CH2)2ppG (5) were translated in vitro slightly better than those capped with mGpG (2) (1.3- and 1.1-fold, respectively; see Fig. 6A and Table 2). They were also translated 26-fold more efficiently than GpG-capped mRNA, which indicates the cap dependence of the translational system. The methylene-containing caps did not confer as much of an increase in translational efficiency over mGpG-capped mRNA as the corresponding ARCA not containing the methylene substitutions (4). All three cap analogs (compounds 4–6) are incorporated into RNA only in the correct orientation because of the ARCA modification, but the expected 2-fold increase in translational efficiency was partially offset by the lower affinity for elf4E of the methylene-containing analogs (Table 2). To measure protein synthesis in vivo, we used the approach described above for Fig. 3A. As observed in vitro, translational efficiencies in vivo of mRNAs capped with m_{2-3′}Gpp(CH2)2ppG (5) and m_{2-3′}Gpp(CH2)2G (6) were slightly higher than those capped with mGpG (2) (Fig. 6B and Table 2).

Although it is important that the methylene-containing caps are recognized by the translational machinery, their most important property should be resistance to decapping. For this, we utilized recombiant human Dcp2 and capped oligonucleotides, because this enzyme only recognizes mRNA fragments of ≥25 nucleotide residues (30). Radio-labeled oligonucleotides capped with either m_{2-3′}Gpp(CH2)2G (6) or m_{2-3′}Gpp* (4) were synthesized in vitro from the same luciferase template used above except that it was truncated with Ncol to produce a 48-nucleotide RNA. These capped oligonucleotides were subjected to Dcp2 digestion in vitro (15, 27), after which the products were digested with a nonspecific endoribonuclease (RNase One) and subjected to anion-exchange HPLC (Fig. 7). The presence of [α-32P]GTP in the initial transcription reaction results in transfer of 32P to any nucleotide immediately 5′ to a G residue, which includes one nucleotide moiety of the cap structure (nearest neighbor transfer). Labeled nucleoside 3′-monophosphates (15–40 min), resulting from internal position in the RNA, were resolved from 5′-terminal products (80–110 min). Some transcripts remain uncapped (~10%) under the particular conditions of in vitro transcription employed. These yield labeled guanosine-5′-triphosphate 3′-monophosphate (pGp*; in which * indicates the labeled phosphate group) (Fig. 7A, 108 min). Uncapped RNA is not a substrate for Dcp2, so pGp* remains after Dcp2 digestion (Fig. 7, A versus C and B versus D). RNAs capped with ARCA, unlike mGpG, yield a single type of 5′-terminal structure after RNase One digestion, because the orientation is always correct. In the case of mRNAs capped with m_{2-3′}Gpp(CH2)2G (4), this is m_{2-3′}pGp* (Fig. 7A, 82

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FIGURE 6. Translational efficiency of methylene-containing cap analogs. A, in vitro translational efficiency of mRNAs capped with methylene-containing compounds. Luc-A_0, mRNAs capped with GpG (filled squares), m_{2′}-GpG (filled triangles), m_{2-3′}Gpp(CH2)2ppG (open squares), or m_{2-3′}Gpp(CH2)2G (open triangles) were translated in vitro in the RRL system, and relative light units (RLU) were determined in triplicate as described under “Experimental Procedures.” B, in vivo translational efficiency of mRNAs capped with methylene-containing compounds. Luciferase activity produced by Luc-A_0, mRNAs capped with mGpG (filled triangles), m_{2-3′}Gpp(CH2)2ppG (open squares), and m_{2-3′}Gpp(CH2)2G (open triangles) were determined by quenching of intrinsic Trp fluorescence (57) (data summarized above for Fig. 3A). As observed in vitro, translational efficiencies in vivo of mRNAs capped with m_{2-3′}Gpp(CH2)2ppG (5) and m_{2-3′}Gpp(CH2)2G (6) were slightly higher than those capped with mGpG (2) (Fig. 6B and Table 2).

A different approach to test this directly: introduction of an mRNA that is resistant to decapping. To achieve this, we designed and synthesized two novel cap analogs with substitutions for bridging pyrophosphate oxygens. In one analog, a methylene group was substituted for oxygen between the α- and β-phosphate moieties (m_{2-3′}Gpp(CH2)2G), whereas in the other, the substitution was between the β- and γ-phosphate moieties (m_{2-3′}Gpp(CH2)2ppG) (compounds 6 and 5, respectively, in Fig. 1). Both cap analogs are ARCA, which ensures that they would be incorporated exclusively in the correct orientation during in vitro mRNA synthesis. Because Dcp1/Dcp2 releases mGpG from capped mRNA (27), hydrolysis is likely to occur between the α- and β-phosphates. An alternative route leading to mGpG could be cleavage of the ester bond between C-5′ and the α-phosphate followed by phosphatase action. Because of the high stability of the P–C bond, mRNAs capped with m_{2-3′}Gpp(CH2)2G are predicted to resist cleavage by Dcp1/Dcp2. On the other hand, mRNA capped with m_{2-3′}Gpp(CH2)2ppG are predicted to resist cleavage by DcpS, because a product of its reaction is mGMP (35).

The binding affinity of the new cap analogs to murine elf4E has been determined by quenching of intrinsic Trp fluorescence (57) (data summarized in Table 2). Binding affinities for the methylene-containing cap analogs are lower than for mGpG (Table 2; 2.6-fold for m_{2-3′}Gpp(CH2)2G (6); 2.4-fold for m_{2-3′}Gpp(CH2)2ppG (5)). They are more similar to the parent compound m_{2-3′}GppG (4) but are still reduced in comparison (1.7-fold for m_{2-3′}Gpp(CH2)2G; 1.6-fold for m_{2-3′}Gpp(CH2)2ppG). The decrease in K_{AS} may occur because replacement of these oxygen atoms with methylene groups would be expected to change the geometry of the polyphosphate chain and charge distribution of cap analogs. This may also eliminate hydrogen bonds or diminish electrostatic interactions with positively charged amino acid residues at the entrance to cap binding slot of elf4E. The methylene-containing cap analogs have also been assayed for inhibition of cap-dependent translation using the RRL system containing native β-globin mRNA (57) (data summarized in Table 2). Both of the methylene cap analogs were 2-fold less effective than mGpG for inhibition of β-globin synthesis. This is in good agreement with the K_{AS} values obtained for direct binding of these compounds to elf4E.

The luciferase activity produced by Luc-A_0, mRNAs capped with GpG, mGpG, m_{2-3′}Gpp(CH2)2ppG, and m_{2-3′}Gpp(CH2)2G (open triangles) are translated in vitro in the RRL system, and relative light units (RLU) were determined in triplicate as described under “Experimental Procedures.” B, in vivo translational efficiency of mRNAs capped with methylene-containing compounds. Luciferase activity produced by Luc-A_0, mRNAs capped with mGpG (filled triangles), m_{2-3′}Gpp(CH2)2ppG (open squares), and m_{2-3′}Gpp(CH2)2G (open triangles) were determined by quenching of intrinsic Trp fluorescence (57) (data summarized above for Fig. 3A). As observed in vitro, translational efficiencies in vivo of mRNAs capped with m_{2-3′}Gpp(CH2)2ppG (5) and m_{2-3′}Gpp(CH2)2G (6) were slightly higher than those capped with mGpG (2) (Fig. 6B and Table 2).

Although it is important that the methylene-containing caps are recognized by the translational machinery, their most important property should be resistance to decapping. For this, we utilized recombinant human Dcp2 and capped oligonucleotides, because this enzyme only recognizes mRNA fragments of ≥25 nucleotide residues (30). Radio-labeled oligonucleotides capped with either m_{2-3′}Gpp(CH2)2G (6) or m_{2-3′}Gpp* (4) were synthesized in vitro from the same luciferase template used above except that it was truncated with Ncol to produce a 48-nucleotide RNA. These capped oligonucleotides were subjected to Dcp2 digestion in vitro (15, 27), after which the products were digested with a nonspecific endoribonuclease (RNase One) and subjected to anion-exchange HPLC (Fig. 7). The presence of [α-32P]GTP in the initial transcription reaction results in transfer of 32P to any nucleotide immediately 5′ to a G residue, which includes one nucleotide moiety of the cap structure (nearest neighbor transfer). Labeled nucleoside 3′-monophosphates (15–40 min), resulting from internal position in the RNA, were resolved from 5′-terminal products (80–110 min). Some transcripts remain uncapped (~10%) under the particular conditions of in vitro transcription employed. These yield labeled guanosine-5′-triphosphate 3′-monophosphate (pGp*; in which * indicates the labeled phosphate group) (Fig. 7A, 108 min). Uncapped RNA is not a substrate for Dcp2, so pGp* remains after Dcp2 digestion (Fig. 7, A versus C and B versus D). RNAs capped with ARCA, unlike mGpG, yield a single type of 5′-terminal structure after RNase One digestion, because the orientation is always correct. In the case of mRNAs capped with m_{2-3′}Gpp(CH2)2G (4), this is m_{2-3′}pGp* (Fig. 7A, 82
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**FIGURE 7.** \(\text{m}_2^7,3^7\text{-GppCH}_2\text{pG}-\text{capped oligonucleotides are resistant to human Dcp2 in vitro.}^{27}\) \(^{\text{P}}\)-Radiolabeled RNAs (48 nucleotides) capped with \(\text{m}_2^7,3^7\text{-Gpp}_2\text{pG} (A\) and \(C\) or \(\text{m}_2^7,3^7\text{-GppCH}_2\text{pG} (B\) and \(D\)) were digested with either RNase One (\(A\) and \(B\)) or recombinant human Dcp2 plus RNase One (\(C\) and \(D\)) followed by anion-exchange HPLC as described under "Experimental Procedures." Assignments of radioactive peaks were made from elution times of the following nonradioactive standard compounds, detected by UV absorption: \(5^\text{'-GMP} (32\text{ min}), 5^\text{'-GDP} (64\text{ min}), 5^\text{'-GTP} (90\text{ min}),\) and guanosine-5'-tetraphosphate (108 min).

**FIGURE 8.** In vivo decay of luciferase mRNA having methylene-containing caps. \(\text{Luc-A}_{31} (A), \text{Luc-A}_{60} (B),\) and \(\text{Luc-G}_{16} (C)\) mRNAs capped with \(\text{Gp}_3\text{G} (\text{filled circles}), \text{m}_7^7\text{-Gp}_3\text{G} (\text{open squares}), \text{m}_2^7,3^7\text{-GppCH}_2\text{pG} (\text{filled triangles}), \text{m}_2^7,3^7\text{-GppCH}_2\text{pG} (\text{open circles}),\) or \(\text{m}_2^7,3^7\text{-GppCH}_2\text{pG} (\text{filled squares})\) were electroporated into MM3MG cells. Decay of 5'-terminal sequences was determined as in Fig. 4. \(D,\) half-lives of \(\text{Luc-A}_{60}\) capped with \(\text{m}_7^7\text{-Gp}_3\text{G} (\text{filled squares}), \text{m}_2^7,3^7\text{-GppCH}_2\text{pG} (\text{filled triangles}), \text{m}_2^7,3^7\text{-GppCH}_2\text{pG} (\text{open circles}),\) or \(\text{m}_2^7,3^7\text{-GppCH}_2\text{pG} (\text{filled squares})\) were determined as in \(A\) except that the PCR primers were directed against 3'-terminal sequences. \(E,\) the same RNA preparations used in \(D\) were also quantified by real time PCR but with a primer set directed against 5'-terminal sequences. The ratio of 5'-terminal to 3'-terminal sequences was computed at each time point. The slopes are \(-0.015 \pm 0.001\) for \(\text{m}_7^7\text{-Gp}_3\text{G}, 0.015 \pm 0.001\) for \(\text{m}_2^7,3^7\text{-GppCH}_2\text{pG},\) and \(0.026 \pm 0.001\) for \(\text{m}_2^7,3^7\text{-GppCH}_2\text{pG}.\) These slopes are all significantly different from each other (\(p \leq 0.05).\) The averages of three experiments \pm S.E. are shown.
DISCUSSION

The process of mRNA degradation in vivo is extraordinarily complex, involving at least four distinct pathways and dozens of proteins (21). Despite the fact that major strides have been made over the past decade in our understanding of these processes, there are still many unanswered questions, not only in the mechanisms of individual pathways but also in the relationships between, and relative contributions of, alternative pathways. An inverse relationship between mRNA translation and mRNA degradation has been demonstrated by a variety of techniques, including translational inhibitors, variant forms of initiation factors, and introducing AUGs with poor context or high 5′-UTR secondary structure into mRNAs (21). In this study we have developed a new approach to study the relationships between translation and degradation: the use of modified cap structures. Cap analogs that differ in affinity for eIF4E affect a very specific, but critical, step in translational initiation, the cap-eIF4E interaction, and hence could potentially yield more interpretable results than less targeted interventions. For instance, the use of eIF4E sequence variants with reduced cap affinity may indeed cause diminished cap occupancy but also decrease steady-state levels of eIF4E or alter the distribution of eIF4E between free and eIF4G-complexed states. This would in turn lead to a smaller fraction of the N terminus of eIF4E existing in an ordered structure (67, 68) and likely affect PABP (16, 44) or Dcp1 (50) binding to eIF4G.

The six cap analogs in this study differ in their affinity for eIF4E when incorporated into mRNA in vitro, which allows us to compare their effects on both translational efficiency and rate of mRNA degradation. These and other cap analogs could also be used in assays for individual steps occurring during mRNA degradation, e.g. deadenylation.
even though we have shown that mRNAs are more stable if capped by a structure that promotes higher binding to eIF4E, we do not know whether stabilization occurs because Dcp1/Dcp2 activity is directly antagonized or because cap binding by eIF4E inhibits deadenylation, which precedes decapping. We also recognize that the greater binding of ARCA-capped mRNAs by eIF4E occurs because conventional in vitro-synthesized mRNAs consist of two populations of mRNA, one presenting an m'7GpG moiety to eIF4E or Dcp1/Dcp2 and one presenting GpG. The translational initiation and degradation rates we observe represent the average rates for these two populations, only one of which is present in ARCA-capped mRNAs. There are other ARCA's that have higher intrinsic affinities for eIF4E, e.g. those containing 7-benzyl moieties (62) or tetra- rather than triphosphate bridges (58). mRNAs synthesized with these analogs consist of a single population and yet are translated more efficiently than those capped with the standard ARCA (4) (62). Thus, further studies of mRNA translation and degradation that utilized these cap analogs, as well as assays of specific steps, could provide additional insight into the relationships between mRNA translation and mRNA degradation.

In previous studies of the translational properties of modified cap analogs, we and others have always used in vitro translation systems (56, 58, 60, 62, 69, 70). The most common and best characterized of these is the RRL system, from which has come a great deal of fundamental information about eukaryotic protein synthesis, e.g. identification of the canonical initiation factors (71–73), translation of heterologous mRNA (74), and regulation of initiation factor activity (75). Yet the RRL system has been criticized as being a poor model for in vivo translation, partly because initiation factors are present at ~5-fold higher levels than in more typical cells (76). Furthermore, dependence of translation on poly(A) is difficult to demonstrate except under special conditions (77). Obviously, any experiments investigating how translation and mRNA degradation are interrelated must take into account the poly(A) tract, because it is the single strongest determinant of mRNA degradation (21). A comparison of mRNA translation in vitro to mRNA degradation in vivo would be highly suspect. For this reason, we developed a quantitative, in vitro assay to measure the translational efficiencies of in vitro-synthesized mRNAs. Even though we had previously shown both ARCA- and 7-benzyl-containing mRNAs are more efficiently translated in the RRL system (62), it was not clear that the advantage conferred by these novel cap analogs would persist in a poly(A)-dependent system. The results presented here, however, indicate that both m'7,5-CpG3G- and b’GpG-capped mRNAs are translated more efficiently than m'7GpG-capped mRNAs. Furthermore, these differences are observed regardless of whether the mRNA contains a long, short, or no poly(A) tract. Thus, the in vitro system is suitable for studying factors that simultaneously affect mRNA translation and mRNA stability. The results indicate an inverse relationship between cap binding and mRNA degradation for RNAs capped with GpG, m'7GpG, and m'7,5-CpG3G. The most straightforward interpretation is that cap occupancy by eIF4E protects mRNA against decapping by Dcp1/Dcp2, although the types of assays performed do not distinguish between a direct and indirect effect (see below), nor have we demonstrated changes in cap occupancy by eIF4E in vivo.

Of the two predominant pathways for mRNA degradation, the 5'–3' pathway is faster for those yeast mRNAs that have been studied (21). As noted in the Introduction, some experiments in mammalian cells suggest that the 5'–3' predominates, whereas others favor the 3'–5' pathway. Because of these uncertainties in assessing the contributions of the two pathways, and because this may change as a function of such factors as mRNA type, cellular stress conditions, and global translation rate, it could be informative to selectively block one of these pathways. The evidence presented in this work supports the view that mRNAs capped with m'7,5-CpG3G (6) cannot be decapped by Dcp2 and therefore cannot enter the 5'–3' degradation pathway. The other methylene-containing analog, m'7,5-CpG3G (5), cannot be hydrolyzed by DcpS (57), but because this step occurs only after 3'–5' degradation, mRNAs capped with this compound will still be degraded by both 5'–3' and 3'–5' pathways.

A poly(G) tract when placed at the 3'-end of RNA is known to inhibit the 3'–5' degradation pathway. Therefore, our observation that GpG-capped Luc-G46 had a shorter half-life than m'7GpG-capped Luc-G46 was a surprise. It has been demonstrated that GpG-capped RNAs are not decapped by Dcp2 (27). Thus, the GpG cap should have prevented 5'–3' degradation, whereas the 3'–terminal poly(G) tract should have prevented 3'–5' degradation. Yet instead of a more stable mRNA, we observed one that was less stable. This suggests that a different pathway is responsible for the degradation of this type of mRNA.

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