Differential Inhibition of mRNA Degradation Pathways by Novel Cap Analogs*

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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 (GppG, m7GpG, or m7,3′-OmpG) and 3′ termini (A3p, A6O, or G3p) on both translation and mRNA degradation in mammalian cells. The results indicated that cap structures that bind elf4E with higher affinity stabilize mRNA to degradation in vivo. mRNA stability depends on the ability of the 5′ terminus to bind elf4E, 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′,5′-OmpCpgG and m2,7,3′-OmpCpgpG, that are predicted to be resistant to cleavage by Dcp1/Dcp2 and Dcp5, respectively. These cap analogs were recognized by elf4E and conferred cap-dependent translation to mRNA both in vitro and in vivo. Oligonucleotides capped with m2,3′,5′-OmpCpgG were resistant to hydrolysis by recombinant human Dcp2 in vitro. mRNAs capped with m2,7,3′-OmpCpgpG, but not m2,3′,5′-OmpCpgpG, 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′,5′-OmpCpgG 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 elf4E (5, 6). Binding of elf4E to the cap occurs during formation of the 48 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).

A second stability element in mRNA is the 3′-terminal poly(A) tract. PABP2,3 binds to poly(A) and is essential for the stability provided by this element, protecting mRNA against exonucleolytic degradation (12–15). PABP also binds to the N terminus of elf4F (16) and stabilizes the elf4F→elf4E complex, enhancing translational reinitiation (17, 18). The stimulation conferred by the cap and poly(A) tract are synergistic rather than additive (19, 20). Thus, for both translation and degradation of mRNA, elements binding to the 5′ and 3′ termini act cooperatively and in close proximity.

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 m2GDP, 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, Dcps (36). Dcps releases m2GMP, 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 m2GMP but not m2GDP 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 elf4E both bind elf4G at nearby sites (16, 43, 44), and PABP increases the affinity of elf4E 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 elf4E inhibits Dcp1/Dcp2 activity in vitro, and this inhibition is thought to be due to elf4E 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 (elf4E, elf4G, elf4A, and elf3) 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 elf4G and PABP as free proteins as well as to the complex of elf4E-EfG-PABP (50). Finally, a temperature-sensitive allele of elf4E suppresses the decapping defect of a dcp1-1 mutant, which argues that dissociation of elf4E 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 elf4E inhibits mRNA degradation in mammalian cells. We used cap analogs that differ in binding affinity for elf4E 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 (m₃,7-β-GppCH₂pG) and one in the β-γ position (m₃,7-β-GpCH₂pG). 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 Gp₂₃-G, m₃Gp₂₃-G, b₃Gp₂₃-G, and m₂,7-β-Gp₂₃-G has been described previously (53–56). Two new cap analogs were designed and synthesized for this study, m₂,7-β-Gp₁₆p₃G and m₂,7-β-Gp₁₆pG. Their synthesis and chemical characterization are described elsewhere (57).

**Construction of Plasmids**—Three plasmids derived from pGEM-6-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 (pLuc-A₃₁) or with 16 G residues (pLuc-G₁₆). For construction of pLuc-A₆₀, two oligonucleotides, 5’-CCCC(A)₉₀CC-GAATTTGG-3’ and 5’-AAACCATTCGG(T)₉₀GGGAGCT-3’, were annealed and inserted into the Hpal and SacI restriction sites of pLuc-A₇. For construction of pLuc-G₁₆, an insert containing G₁₆ was obtained by PCR amplification using linear pluc-A₇ and two oligonucleotides, 5’-AAACCC(G)₉₀CC-GAAT-TGTTAAACATTCTATAGTGTC-3’ and 5’-AGTGCTCATCTAAGGAAACGTTGTTCCTCGGGGCG-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 XmnI and inserted into pluc-A₇. Plasmids pT7-Luc-A₅₀ and pT7-SL₃₁-luc-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 (pLuc-A₇, pLuc-A₆₀, pLuc-G₁₆, or pT7-SL₃₁-luc-A₅₀) with T7 polymerase in the presence of all four nucleoside triphosphates and various cap dinucleotides (58). pluc-A₇, pLuc-A₆₀, and pluc-G₁₆ were digested with Hpal for synthesis of luciferase mRNA and with Ncol for synthesis of capped oligonucleotides. pT7-luc-A₅₀ and pT7-SL₃₁-luc-A₅₀ were digested with Dral 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 RNasey mini kit (Qiagen) using the manufacturer’s protocol. The concentrations of RNAs were determined spectrophotometrically. mRNAs derived from pGEM₆-luc, pluc-A₇, pluc-A₆₀, pT7-luc-A₅₀, or pT7-SL₃₁-luc-A₅₀ with T7 polymerase in the presence of all four nucleoside triphosphates and various cap dinucleotides (58).

**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 monod.
Northern Blotting—Northern blotting was performed by using a ribo-
probe for luciferase mRNA, made by in vitro transcription with SP6
polymerase (Promega) of Ncol-digested pluc-A60. Plasmid was tran-
scribed 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 pro-
tein synthesis was measured by incorporation of [3H]Leu. Calculation of
Kₜ values and normalization of data were performed as described previ-
ously (60). The concentrations of dinucleotide cap analog solutions
were measured by UV absorption at pH 7.0 using λ = 255 nm and εM =
22.6 × 10⁶ M⁻¹ cm⁻¹. 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 chloro-
form, and RNA was precipitated with ethanol. Products of the decap-
ping 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 10SAX/25 column (Whatman). The gradient consisted
of water for 1 min, a linear gradient to 112 mM KH₂PO₄, pH 4.5, for 40
min, a linear gradient of 112–450 mM KH₂PO₄ for 30 min, a linear
gradient of 450 mM to 1.5 M KH₂PO₄ for 30 min, and isotropic elution at
1.5 M of KH₂PO₄ 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 Effi-
ciently 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 incorpo-
rated into mRNA, produced higher translational efficiencies in vitro
than the standard cap analog, m'GpG (56, 58, 62). The first group of
compounds consisted of cap analogs that prevent incorrect incorpora-
tion. One-third to one-half of m'GpG is incorporated into RNA in the
reverse orientation during in vitro transcription (63). "Anti-reverse" cap
analogs (ARCAs) have modifications in either the C-2' or C-3' position
of m'Guo that permit incorporation only in the correct orientation (e.g.
compound 4, m₃β,GpG, in Fig. 1) (56). Because a reversed cap is not
recognized by the protein synthesis machinery, the in vitro translational
efficiency of ARCA-capped mRNAs is roughly twice that of m'GpG-
capped mRNAs. The second group of cap analogs possessed a benzy1
rather than methyl group in the N-7 position (e.g. 3, β,GpG). These also
produce mRNAs that are translated in vitro ~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 latter 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
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' cap and various 3' UTR 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 was present in the untranslated fraction (Fig. 2B, fractions 1–2). Endogenous GAPDH mRNA was more efficiently translated (Fig. 2C, fraction 9), although some was 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 10^7 cells (data not shown). Luc-A31 mRNAs capped with m^7,3'-Gp^6G (4) and b^5'Gp^6G (3) were translated 2.5- and 1.6-fold more efficiently, respectively, than mRNA capped with m^7'Gp^6G (Fig. 3A). This is similar to our previous result with in vitro translation in the RRL system, in which Luc-A31 mRNAs capped with m^7,3'-Gp^6G and b^5'Gp^6G were found to be translated 1.9-fold more efficiently than Luc-A31 mRNA capped with m^7'Gp^6G (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-A31 mRNA transcripts containing either Gp^6G (1), m^7'Gp^6G (2), or m^7,3'-Gp^6G (4) at the 5'-end were electroporated into MM3MG cells. Cells were harvested at various times up to 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-A31 mRNA capped with m^7'Gp^6G was more stable than the same RNA capped with Gp^6G (t_1/2 = 60 versus 45 min). Luc-A31 mRNA capped with m^7,3'-Gp^6G was even more stable (t_1/2 = 90 min). The increase in stability correlates with increased affinity of eIF4E for the cap, because Gp^6G is not recognized by eIF4E, m^7'Gp^6G is incorporated approximately equally in the correct and reversed orientations, the

![Figure 4](image.png)

**TABLE 1**

*In vivo* half-lives of luciferase mRNAs modified at 5' and 3' termini

| No.* | Type of cap and 5'-UTR on luciferase mRNA | Luc-A31 | Luc-A30 | Luc-A60 | Luc-G16 | Luc-A60 |
|------|------------------------------------------|---------|---------|---------|---------|---------|
|      |                                          | t_1/2   | N       | t_1/2   | N       | t_1/2   | N       | t_1/2   | N       | t_1/2   | N       |
| 1    | Gp^6G                                   | 45 ± 3  | 2       | ND      | 120 ± 6 | 3       | 150 ± 13 | 3       | ND      | 174 ± 19 | 4       |
| 2    | m^7'Gp^6G                               | 60 ± 3  | 3       | ND      | 156 ± 6 | 5       | 246 ± 12 | 3       | ND      | 294 ± 9  | 4       |
| 3    | m^7,3'-Gp^6G                            | 90 ± 2  | 4       | 246 ± 9* | 2       | 282 ± 4* | 5       | 474 ± 30 | 4       | ND      | 354 ± 22 | 4       |
| 4    | m^7,3'-Gp^6G-100G                       | 66 ± 5  | 3       | ND      | 180 ± 5 | 3       | ND      | 304 ± 23 | 2       | ND      | ND      |
| 5    | m^7,3'-Gp^6G-200G                       | 126 ± 5 | 3       | ND      | 330 ± 10* | 3      | ND      | 354 ± 22 | 4       | ND      | ND      |
| 6    | m^7,3'-Gp^6G-100G                       | ND      | 228 ± 19* | 3      | ND      | ND      | ND      | ND      | ND      |

* Structures are given in Fig. 1. 
* Degradation of 5'-terminal sequences in Luc-A31, Luc-A30, and Luc-G16 mRNAs capped with the indicated analogs was determined by real time PCR with primers directed against the 5'-end of luciferase mRNA (see “Experimental Procedures”).
* Number of independent assays performed is shown.
* Degradation of 3'-terminal sequences was determined as in Footnote b except a different primer set was used.
* Half-lives that are significantly different (p ≤ 0.05) from that of the m^7'Gp^6G-capped form of the mRNA are indicated.
* ND indicates that an experiment was not done.
latter of which is not recognized by eIF4E (56, 63), and m$_2$G$^7$\,\,\textsuperscript{3',5'-O}\,G$_3$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-A$_{50}$ mRNA than with Luc-A$_{31}$ mRNA. As shown in Fig. 4B and Table 1, the half-lives were lengthened for each of the three caps for Luc-A$_{50}$ compared with its Luc-A$_{31}$ counterparts (t$_{1/2} = 120$ versus $45$ min for Gp$_3$G (1); t$_{1/2} = 156$ versus $60$ min for m$^7$Gp$_3$G (2); and t$_{1/2} = 282$ versus $90$ min for m$_2$G$^7$\,\,\textsuperscript{3',5'-O}\,G$_3$G (4)). However, the ratios of half-lives for Luc-A$_{50}$ compared with Luc-A$_{31}$ were statistically indistinguishable for all three caps (2.7 ± 0.3 for Gp$_3$G; 2.6 ± 0.3 for m$^7$Gp$_3$G; and 3.1 ± 0.2 for m$_2$G$^7$\,\,\textsuperscript{3',5'-O}\,G$_3$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$_3$G, m$^7$Gp$_3$G, and m$_2$G$^7$\,\,\textsuperscript{3',5'-O}\,G$_3$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-A$_{50}$ to Luc-A$_{30}$ can be interpreted in two alternative ways. The first is that decapping follows deadenylation but is slow compared with deadenylation. With Luc-A$_{50}$ the mRNA reaches a deadenylated state sooner than with Luc-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-A$_{50}$ compared with Luc-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 (ΔG$^\circ$ = −21.3 kcal/mol) in the 5'UTR and a 3' terminal 50-nucleotide poly(A) tract (SL$_{13}$-Luc-A$_{50}$) and an identical mRNA lacking the stem-loop (Luc-A$_{30}$). As shown in Fig. 3B, SL$_{13}$-Luc-A$_{50}$ was translated in vivo much less efficiently than Luc-A$_{30}$ 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$_{13}$-Luc-A$_{30}$ and Luc-A$_{50}$ were capped with m$_2$G$^7$\,\,\textsuperscript{3',5'-O}\,G$_3$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$^7$Gp$_3$G-capped Luc-A$_{50}$ by two techniques. In the first, mRNA was detected by Northern blotting at various times after electroporation (Fig. 5A). In the second, $^{32}$P-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 Analogos 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 those obtained with other experimental approaches (21), suggest that binding of the cap by eIF4E inhibits decapping and therefore protects mRNA against degradation. We developed

**TABLE 2**

| Compound | Cap analog | $K_{d}$, $10^{-6}$a | $K_{d}^b$ | Relative translational efficiency in vitroc | Relative translational efficiency in vivod |
|----------|------------|---------------------|-----------|------------------------------------------|------------------------------------------|
| 2        | m$^7$Gp$_3$G | 11.5 ± 0.3          | 17.1 ± 1.0 | 1.0                                      | 1.0                                      |
| 4        | m$_2$G$^7$\,\,\textsuperscript{3',5'-O}\,G$_3$G | 7.4 ± 0.1           | 14.3 ± 1.3 | 1.9 ± 0.4                                 | 2.5 ± 0.1                                 |
| 5        | m$_2$G$^7$\,\,\textsuperscript{3',5'-O}\,G$_{3PP}$ | 4.7 ± 0.03          | 29.3 ± 2.3 | 1.1 ± 0.3                                 | 1.2 ± 0.2                                 |
| 6        | m$_2$G$^7$\,\,\textsuperscript{3',5'-O}\,G$_{32P}$ | 4.4 ± 0.2           | 33.5 ± 4.5 | 1.3 ± 0.3                                 | 1.3 ± 0.1                                 |

$a$ 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-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.

![Figure 5](image_url)
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\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}G\textsubscript{pp}\textsubscript{C}\textsubscript{ppG}). Because of the high stability of the P–C bond, mRNAs capped with m\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}G\textsubscript{pp}\textsubscript{C}\textsubscript{ppG} are pre- dicted to resist cleavage by DcpS, because a product of its reaction is m\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}Gp* (Fig. 7A). The decrease in \(K_{\text{AS}}\) 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 eIF4E. The methylene-containing cap analogs have also been assayed for inhibition of cap-de- pendent 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 m\textsubscript{2}Gp\textsubscript{G} for inhibition of β-globin synthesis. This is in good agreement with the \(K_{\text{AS}}\) values obtained for direct binding of these compounds to eIF4E.

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\textsubscript{40} were synthesized that were capped with each of the new cap analogs as well as with m\textsubscript{2}Gp\textsubscript{G} and Gp\textsubscript{G} as controls. mRNAs capped with m\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}G\textsubscript{pp}\textsubscript{C}\textsubscript{ppG} (6) and m\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}G\textsubscript{pp}\textsubscript{C}\textsubscript{ppP} (5) were translated in vitro slightly better than those capped with m\textsubscript{2}Gp\textsubscript{G} (2) (1.3- and 1.1-fold, respectively; see Fig. 6A and Table 2). They were also translated ~26-fold more efficiently than Gp\textsubscript{G}-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 m\textsubscript{2}Gp\textsubscript{G}-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 eIF4E 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\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}G\textsubscript{pp}\textsubscript{C}\textsubscript{ppP} (5) and m\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}G\textsubscript{pp}\textsubscript{C}\textsubscript{ppG} (6) were slightly higher than those capped with m\textsubscript{2}Gp\textsubscript{G} (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\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}G\textsubscript{pp}\textsubscript{C}\textsubscript{ppG} (6) or m\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}G\textsubscript{pp}\textsubscript{C}\textsubscript{ppP} (4) were synthesized in vitro from the same luciferase template used above except that it was truncated with NcoI 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 [\textsubscript{\alpha-\textsuperscript{32}P}]GTP in the initial transcription reaction results in transfer of \textsubscript{\alpha-\textsuperscript{32}P}GTP 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 (p\textsubscript{5}Gp\textsubscript{G*} in which * indicates the labeled phosphate group) (Fig. 7A, 108 min). Uncapped RNA is not a substrate for Dcp2, so p\textsubscript{5}Gp\textsubscript{G*} remains after Dcp2 digestion (Fig. 7, A versus C and B versus D). RNAs capped with ARCAS, unlike m\textsubscript{2}Gp\textsubscript{G}, 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\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}Gp\textsubscript{G} (4), this is m\textsubscript{2}7,3′-O\textsubscript{CH}\textsubscript{2}p\textsubscript{5}Gp\textsubscript{G*} (Fig. 7A, 82
FIGURE 7. m$_2$7,3'-GppCH$_2$pG-capped oligonucleotides are resistant to human Dcp2 in vitro. 
$^{32}$P-Radiolabeled RNAs (48 nucleotides) capped with m$_2$7,3'-Gp$_3$G (A and C) or m$_2$7,3'-Gpp$_3$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'-GMP (32 min), 5'-GDP (64 min), 5'-GTP (90 min), and guanosine-5'-tetraphosphate (108 min).

FIGURE 8. In vivo decay of luciferase mRNA having methylene-containing caps. Luc-A$_{31}$ (A), Luc-A$_{60}$ (B), and Luc-G$_{16}$ (C) mRNAs capped with Gp$_3$G (filled circles), m$_7$Gp$_3$G (open squares), m$_2$7,3'-Gpp$_3$pG (filled triangles), m$_7$7,3'-Gp$_3$G (open circles), or m$_2$7,3'-Gpp$_3$pG (open triangles) were electroporated into MM3MG cells. Decay of 5'-terminal sequences was determined as in Fig. 4. D, half-lives of Luc-A$_{60}$ capped with m$_7$Gp$_3$G (filled squares), m$_2$7,3'-Gp$_3$G (filled triangles), or m$_2$7,3'-Gpp$_3$pG (open 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 m$_7$Gp$_3$G, $-0.015 \pm 0.001$ for m$_2$7,3'-Gp$_3$G, and $0.026 \pm 0.001$ for m$_2$7,3'-Gpp$_3$pG. These slopes are all significantly different from each other ($p \leq 0.05$). The averages of three experiments ± S.E. are shown.
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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. Thus,
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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$^7$Gp$\alpha$ moiety to eIF4E or Dcp1/Dcp2 and one presenting G$\beta$. 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 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-follower 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$^7$Gp$\gamma$-Gp$\alpha$- and bGp$\beta$-G-capped mRNA are translated more efficiently than m$^7$Gp$\beta$-G-capped mRNAs. Furthermore, these differences are observed regardless of whether the mRNA contains a long, short, or no poly(A) tract. Thus, in the in vivo 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 Gp$\alpha$, m$^7$Gp$\alpha$, and m$^7$G$\beta$. The most straightforward interpretation is that cap occupancy by eIF4E protects mRNA against decapping by Dcp1/Dcp2, although the types of assays we 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$^7$Gp$\gamma$-Gp$\alpha$-G (6) cannot be decapped by Dcp2 and therefore cannot enter the 5'→3' degradation pathway. The other methylene-containing analog, m$^7$Gp$\gamma$-Gp$\gamma$ppG (5), cannot be hydrolyzed by Dcp5 (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 Gp$\beta$-capped Luc-G$\gamma$ had a shorter half-life than m$^7$Gp$\alpha$-capped Luc-G$\gamma$ was a surprise. It has been demonstrated that Gp$\beta$-capped mRNAs are not decapped by Dcp2 (27). Thus, the Gp$\beta$ 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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