Phosphorothioate cap analogs stabilize mRNA and increase translational efficiency in mammalian cells

EWA GRUDZIEN-NOGALSKA,[1] JACEK JEMIELITY,[2] JOANNA KOWALSKA,[2]
EDWARD DARZYNKIEWICZ,[2] and ROBERT E. RHOADS[1]

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

ABSTRACT

Capped RNAs synthesized by in vitro transcription have found wide utility for studying mRNA function and metabolism and for producing proteins of interest. We characterize here a recently synthesized series of cap analogs with improved properties that contain a sulfur substitution for a nonbridging oxygen in either the α-, β-, or γ-phosphate moieties, $m_2^{7,2'\text{-}O}Gppp_SG$, $m_2^{7,2'\text{-}O}Gpp_SpG$, and $m_2^{7,2'\text{-}O}Gp_SppG$, respectively. The new compounds were also modified at the 2′-*O* position of the m^7Guo to make them anti-reverse cap analogs (ARCAs), i.e., they are incorporated exclusively in the correct orientation during in vitro transcription. Each of the S-ARCAs exists in two diastereoisomeric forms (D1 and D2) that can be resolved by reverse-phase HPLC. A major in vivo pathway for mRNA degradation is initiated by removal of the cap by the pyrophosphatase Dcp1/Dcp2, which cleaves between the α- and β-phosphates. Oligonucleotides capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) were completely resistant to hydrolysis by recombinant human Dcp2 in vitro, whereas those capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) and both isomers of $m_2^{7,2'\text{-}O}Gppp_SG$ were partially resistant. Luciferase mRNA capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) had a $t_{1/2}$ of 257 min in cultured HC11 mammary epithelial cells compared with 86 min for m^7Gp_3G-capped mRNA. Luciferase mRNAs capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) and $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) were translated 2.8-fold and 5.1-fold, respectively, more efficiently in HC11 cells than those capped with m^7Gp_3G. The greater yield of protein due to combining higher translational efficiency with longer $t_{1/2}$ of mRNA should benefit applications that utilize RNA transfection such as protein production, anti-cancer immunization, and gene therapy.

Keywords: ARCA; S-ARCA; phosphorothioate cap analogs; hDcp2; translational efficiency; mRNA stability; in vitro transcription

INTRODUCTION

Eukaryotic mRNAs are capped at their 5′-ends by addition of a 7-methylguanosine attached by a 5′–5′ triphosphate bridge to the first transcribed nucleotide of the mRNA chain (Shatkin 1985). The cap plays important roles in all aspects of mRNA metabolism – synthesis, nucleo-cytoplasmic transport, translation, silencing, and turnover. The presence of the cap increases both the accuracy and efficiency of pre-mRNA splicing (Konarska et al. 1984; Edery and Sonenberg 1985). It remains bound to the nuclear cap-binding complex and participates in mRNA processing and export (Visa et al. 1996). The best studied function of the cap is in translation, where it is specifically bound by initiation factor eIF4E (Shatkin 1985). This occurs during formation of the 48S initiation complex, which is rate limiting for translational initiation under normal conditions (Darnbrough et al. 1973). Binding of eIF4E to the cap also plays a role in nucleo-cytoplasmic transport of mRNA (Gorlich and Mattaj 1996) as well as reversible sequestration of mRNA in a nontranslatable state (Richter and Sonenberg 2005). Finally, the cap is one determinant of mRNA decay, protecting it from degradation by 5′→3′ exonucleases in both the cytosol and nucleus (Hsu and Stevens 1993; Sachs 1993; Walther et al. 1998).

mRNA degradation in eukaryotic cells is essential for the control of gene expression and elimination of aberrant transcripts. It occurs predominantly through one of two alternative routes, a 5′→3′ pathway or a 3′→5′ pathway (Parker and Song 2004). The shortening of the 3′-terminal poly(A) tract is the initial step in both these processes (Couttet et al. 1997). In the 5′→3′ pathway, deadenylation

Reprint requests to: Robert E. Rhoads, Department of Biochemistry and Molecular Biology, LA State University Health Sciences Center, Shreveport, LA 71130-3932, USA; e-mail: rrhoad@lsuhsc.edu; fax: (318) 675-5180.

Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.701307.

is followed by rapid decapping by the Dcp1/Dcp2 complex (Muhlrad et al. 1995), which exposes transcripts to degradation by the 5′→3′ exonuclease Xrn1 (Hsu and Stevens 1993). Unlike poly(A) shortening, cap removal cannot be reversed and hence represents the committed step in elimination of an mRNA from the translatable pool. In the 3′→5′ pathway, deadenylated mRNA is degraded by the exosome in the 3′→5′ direction (Wang and Kiledjian 2000; Chen et al. 2001; Wang and Kiledjian 2001; Mukherjee et al. 2002). The products are capped oligonucleotides, which are than decapped by the scavenger decapping enzyme DcpS (Liu et al. 2002).

Dcp2 is the catalytic subunit of the Dcp1/Dcp2 complex, whereas Dcp1 stimulates Dcp2 activity (Lykke-Andersen 2002; van Dijk et al. 2002; Wang et al. 2002). An oligonucleotide chain of at least 25 nucleotides (nt) as well as a cap structure are required for recognition by the Dcp1/Dcp2 complex (Wang et al. 2002; Piccirillo et al. 2003; Steiger et al. 2003). Hydrolysis by either Dcp1/Dcp2 or Dcp2 alone releases m^7GDP, suggesting that cleavage occurs between the α- and β-phosphate moieties of the triphosphate chain rather than the β- and γ-phosphates. Recent studies have reported that the human decapping complex contains three additional proteins: rck/p54, hEdc3, and Hedls (Fenger-Gron et al. 2005). rck/p54 and hEdc3 increase the rate of decapping, whereas Hedls is necessary for the interaction between Dcp1 and Dcp2. Hedls colocalizes with the decapping enzymes in P-bodies (Yu et al. 2005) and functions at a step before decapping, possibly being a target for regulation.

Synthetic cap analogs have provided discriminating tools for elucidating cellular processes occurring during mRNA metabolism and function, both as competitive inhibitors and as alternative structures at the 5′-end of RNAs. In vitro synthesis of capped mRNAs is one of the most important uses for cap analogs. This can be accomplished by transcription of a DNA template with bacterial (Contreras et al. 1982) or bacteriophage polymerases (Konarska et al. 1984; Yisraeli and Melton 1989) in the presence of all four ribonucleoside triphosphates and a cap dinucleotide such as m^7Gp_3G. The products of this reaction are a mixture of mRNAs containing the cap analog incorporated in the correct [m^7G(5′)ppp(5′)GpNp...] and reverse [G(5′)ppp(5′)m^7GpNp...] orientations (Pasquinelli et al. 1995). The latter are not recognized as capped mRNAs by the translational machinery and therefore decrease the translational efficiency of synthetic mRNA preparations.

We and others solved this problem by synthesizing cap analogs that had *O*-methyl or deoxy modifications at either the C2′ or C3′ positions of m^7Guo (Stepinski et al. 2001; Peng et al. 2002; Jemielity et al. 2003). These compounds are incorporated into RNA transcripts exclusively in the correct orientation and are therefore termed "anti-reverse cap analogs" (ARCAs). In a rabbit reticulocyte lysate (RRL) translation system, ARCA-capped mRNAs had translational efficiencies that were twofold higher than transcripts capped with m^7Gp_3G (Stepinski et al. 2001). mRNAs capped with ARCAs were also translated 2- to 2.5-fold more efficiently than those capped with m^7Gp_3G when introduced into cultured mammalian cells (Grudzien et al. 2006). ARCAs consisting of tetraphosphate- and pentaphosphate-containing dinucleotides produced mRNAs of even higher translational efficiency in the RRL system (Jemielity et al. 2003).

We also synthesized a series of ARCAs with substitutions in the triphosphate chain that were designed to increase the stability of mRNA when introduced into cells. A methylene group was substituted for a bridging oxygen in the α–β linkage ($m_2^{7,3'\text{-}O}Gpp_{CH2}pG$) or the β–γ linkage ($m_2^{7,3'\text{-}O}Gp_{CH2}ppG$) (Kalek et al. 2006). When incorporated into mRNA, $m_2^{7,3'\text{-}O}Gpp_{CH2}pG$ was resistant to hydrolysis by recombinant human Dcp2 (hDcp2) in vitro and increased the stability of RNA in cultured cells (Grudzien et al. 2006). $m_2^{7,3'\text{-}O}Gp_{CH2}ppG$, on the other hand, was resistant to hydrolysis by DcpS (Kalek et al. 2005) but did not stabilize mRNA in cultured cells (Grudzien et al. 2006). Unfortunately the affinity of $m_2^{7,3'\text{-}O}Gpp_{CH2}pG$ for eIF4E was only 60% that of the parent compound, $m_2^{7,3'\text{-}O}Gp_3G$ (Kalek et al. 2005). This resulted in a translational efficiency that was only 68% that of $m_2^{7,3'\text{-}O}Gp_3G$ in the RRL system and 52% in cultured cells (Grudzien et al. 2006).

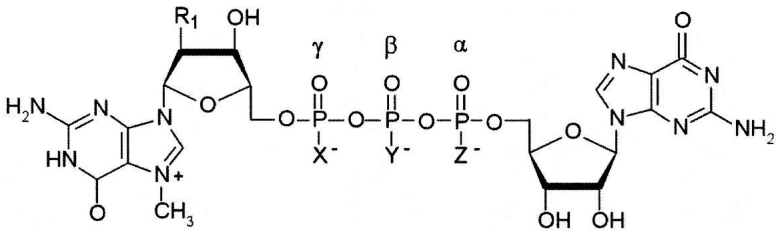
To address this problem, we have explored a different type of modification in the triphosphate chain, the substitution of sulfur atoms for nonbridging oxygen atoms. Previously, it was shown that nucleoside 5′-monophosphorothioates as well as triphosphate analogs such as $Appp_S$, $Gppp_S$, and Gpp_S were stable to phosphatases (Cassel and Selinger 1977; Eckstein et al. 1979). Additionally, polynucleotides containing phosphorothioate internucleolide linkages were found to degrade more slowly than their natural counterparts (Matzura and Eckstein 1968). The new cap analogs contain *O*-methyl groups at the C2′ position of m^7Guo to produce ARCAs, but they also bear phosphorothioate moieties at either the α, β, or γ positions of the triphosphate chain. Due to the presence of stereogenic P-centers, each S-ARCA is obtained as a mixture of two diastereoisomers. These were successfully resolved by reverse-phase HPLC, giving six different compounds that were then tested for their biochemical properties. mRNAs capped with some of the six isomers were resistant to Dcp2 hydrolysis in vitro, were more stable when introduced into cultured cells, and had higher translational efficiencies than any synthetic mRNA produced to date.

RESULTS

Synthesis and biophysical properties of S-ARCAs

A new series of dinucleotide cap analogs containing a phosphorothioate moiety in either the α, β, or γ positions of the 5′,5′-triphosphate chain has recently been synthesized

for the present study: $m_2^{7,2'\text{-}O}Gppp_SG$, $m_2^{7,2'\text{-}O}Gpp_SpG$, and $m_2^{7,2'\text{-}O}Gp_SppG$, respectively (Fig. 1 compounds **3–8**; J. Kowalska, M. Lewdorowicz, J. Zuberek, E. Grudzien-Nogalska, E. Bojarska, J. Stepinski, R.E. Rhoads, E. Darzynkiewicz, R.E. Davis, and J. Jemielity, in prep.). Each of these phosphorothioate cap analogs exists in two diastereoisomeric forms (Rp and Sp). These can be resolved by reverse-phase HPLC and are named D1 and D2 according to their elution order from the reverse-phase HPLC column. In addition to the sulfur substitution, the new compounds were modified at the C2′ position of m^7Guo by a methoxy-for-hydroxy substitution, which ensures that they are ARCAs, i.e., they are incorporated exclusively in the correct orientation during in vitro mRNA synthesis (Stepinski et al. 2001). We chose to use cap analogs modified at the C2′ position rather than the originally modified C3′ position (Stepinski et al. 2001) because substitutions at either position can result in ARCAs (Jemielity et al. 2003) and because the commercially available starting substrate 2′-*O*-methylguanosine is one-tenth the cost of 3′-*O*-methyloguanosine. Unlike members of the methylene series (see Introduction), all six isomers of the S-ARCA series have the same or higher affinity for murine eIF4E compared with the parent compound m^7Gp_3G (**1**), based on quenching of intrinsic Trp fluorescence in eIF4E.



No.	Cap analog	Ref. for synthesis	R_1	X	Y	Z
1	m^7Gp_3G	a	OH	O	O	O
2	$m_2^{7,2'\text{-}O}Gp_3G$	b	OCH_3	O	O	O
3	$m_2^{7,2'\text{-}O}Gppp_SG$ (D1)	c	OCH_3	O	O	S
4	$m_2^{7,2'\text{-}O}Gppp_SG$ (D2)	c	OCH_3	O	O	S
5	$m_2^{7,2'\text{-}O}Gpp_SpG$ (D1)	c	OCH_3	O	S	O
6	$m_2^{7,2'\text{-}O}Gpp_SpG$ (D2)	c	OCH_3	O	S	O
7	$m_2^{7,2'\text{-}O}Gp_SppG$ (D1)	c	OCH_3	S	O	O
8	$m_2^{7,2'\text{-}O}Gp_SppG$ (D2)	c	OCH_3	S	O	O

[a]Darzynkiewicz et al. 1990

[b]Jemielity et al. 2003

[c]Kowalska, J., Lewdorowicz, M., Zuberek, J., Grudzien-Nogalska, E., Bojarska, E., Stepinski, J., Rhoads, R.E., Darzynkiewicz, E., Davis, R.E., and Jemielity, J., manuscript in preparation

FIGURE 1. Structures of cap analogs used in this study. D1 and D2 refer to the two diastereoisomers produced by the phosphorothioate moiety.

mRNAs capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) are resistant to decapping in vitro

As noted in the Introduction, there are two known decapping enzymes: Dcp1/Dcp2, which acts on intact mRNA to initiate 5′→3′ degradation, and DcpS, which acts on short capped oligonucleotides resulting from 3′→5′ degradation. Because Dcp1/Dcp2 (or Dcp2 alone) releases m^7GDP from capped mRNAs (Wang et al. 2002), cleavage is likely to occur between the α- and β-phosphates. One might expect that mRNAs capped with various diastereoisomers of $m_2^{7,2'\text{-}O}Gppp_SG$ or $m_2^{7,2'\text{-}O}Gpp_SpG$ would differ from those capped with the parent ARCA in their susceptibility to cleavage by Dcp1/Dcp2.

We tested oligonucleotides capped with all six S-ARCAs for in vitro hydrolysis by hDcp2 (Piccirillo et al. 2003). The cap analogs used in this study were unlabeled, so to follow products of the digestion reaction, we synthesized capped oligonucleotides in the presence of [α-^{32}P]GTP and a DNA template in which G is the first ribonucleotide specified after the promoter. Products of hDcp2 digestion were further treated with a cocktail of ribonucleases that cleave all phosphodiester bonds to yield 3′-nucleoside monophosphates (3′-NMPs) (RiboShredder; Epicentre). Any nucleotide on the 5′-side of a G residue acquired a ^{32}P-labeled 3′-phosphate group after ribonuclease digestion by nearest-neighbor transfer. Anion exchange chromatography was then applied to resolve the labeled 3′-nucleoside monophosphates (3′-NMP*) resulting from internal positions in the RNA from labeled 5′-terminal products. Uncapped transcripts yield p_3Gp^* as the 5′-terminal product in both the presence and absence of Dcp2, since the m^7Guo moiety is essential for Dcp2 activity (Wang et al. 2002). Capped transcripts yield one of two 5′-terminal products, depending on whether they are sensitive or resistant to Dcp2. An RNA sensitive to Dcp2, for example, one that is capped with $m_2^{7,2'\text{-}O}Gp_3G$ (**2**), will yield pGp^*, whereas an RNA resistant to Dcp2, for example, capped with $m_2^{7,2'\text{-}O}Gpp_{CH_2}pG$ (Grudzien et al. 2006), will yield $m_2^{7,2'\text{-}O}Gpp_{CH_2}pGp^*$.

We chose conditions for treatment with hDcp2 under which an oligonucleotide

capped with $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) was completely digested (Fig. 2A), as indicated by the peak at 38 min, while an RNA capped with $m_2^{7,3'\text{-}O}Gpp_{CH2}pG$ was completely resistant (Fig. 2B), as indicated by the peak at 44 min. For all RNAs, there was a peak corresponding to p_3Gp^* at 55 min resulting from the ~10% of transcripts that are uncapped and therefore not susceptible to hydrolysis by hDcp2. Of the six S-ARCAs, only the D2 isomer of $m_2^{7,2'\text{-}O}Gpp_SpG$ (**6**) produced RNA that was completely resistant to hDcp2 hydrolysis (Fig. 2F). RNAs capped with $m_2^{7,2'\text{-}O}Gppp_SG$ (D1) (**3**), $m_2^{7,2'\text{-}O}Gppp_SG$ (D2) (**4**), or $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) (**5**) were partially resistant (Fig. 2C–E). Oligonucleotides capped with either isomer of $m_2^{7,2'\text{-}O}Gp_SppG$ (**7** and **8**) showed no increase in resistance to hDcp2 compared to the parent ARCA (Fig. 2G,H). The susceptibility of each capped mRNA to hDcp2 hydrolysis is summarized in Table 1.

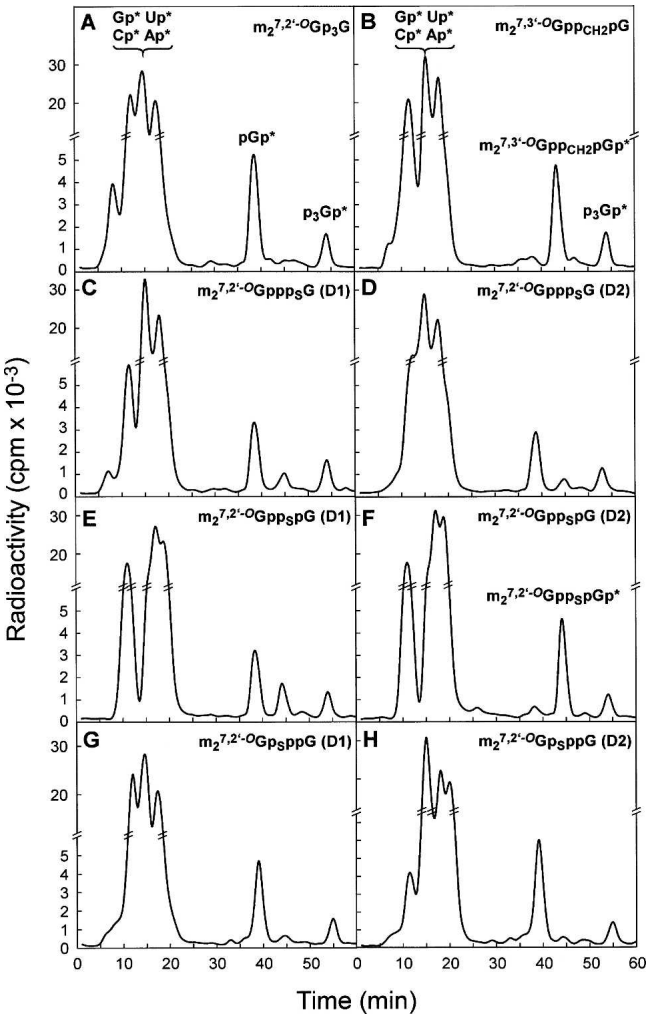


FIGURE 2. $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2)-capped oligonucleotides are resistant to recombinant human Dcp2 in vitro. ^{32}P-Radiolabeled 48-nt oligonucleotides capped with $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) (panel A), $m_2^{7,3'\text{-}O}Gpp_{CH2}pG$ (panel B), or each of the six S-ARCAs (Fig. 1) (panels C–H) were subjected to hDcp2 digestion in vitro, after which the products were further digested with a cocktail of ribonucleases that hydrolyze all phosphodiester bonds. $m_2^{7,3'\text{-}O}Gpp_{CH2}pG$ was included as a negative control for hDcp2 digestion since it had previously been shown to be resistant (Grudzien et al. 2006). Products were resolved by anion-exchange HPLC as described in Materials and Methods. Assignments of radioactive peaks were made from elution times of the following nonradioactive standard compounds, detected by UV absorption: 5′-GMP (17 min), 5′-GDP (37 min), 5′-GTP (47 min), and guanosine-5′-tetraphosphate (55 min).

Total cytoplasmic and polysomal luciferase mRNAs are degraded at the same rate

Because an oligonucleotide capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) was resistant to hDcp2 hydrolysis, we predicted that an mRNA capped with this analog would be more stable in cells. In preliminary experiments, we employed either nucleoporation or electroporation to introduce synthetic luciferase mRNA into HC11 cells and then measured the luciferase mRNA remaining by real-time PCR as a function of time. Both nucleoporation and electroporation permitted measurement of luciferase synthesis and luciferase mRNA concentration in the cells almost immediately after discharge. The two methods yielded similar mRNA decay rates (data not shown), but since nucleoporation produced a higher efficiency of transfection and higher cell viability, it was used for most experiments. Comparable results were obtained with MM3MG cells.

A theoretical possibility was that nucleoporation could produce two populations of cell-associated mRNA, one that was available for recruitment to polysomes and one that was inaccessible, for example, adherent to the outside of cells, encapsulated in membrane vesicles, etc. If a nontranslatable population of mRNA existed, it would likely have a decay rate that was different from that of polysomal mRNA, thus producing misleading results. We therefore compared the decay rates of total and polysomal luciferase mRNA following nucleoporation.

Luciferase mRNA capped with $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) was nucleoporated into HC11 cells. The cells were lysed at various times, and the lysates were layered on sucrose gradients and subjected to ultracentrifugation to isolate polysomes. Fractions containing polysomes were combined, and the RNA was purified. Total cytoplasmic mRNA was isolated from the same cells. Luciferase mRNA concentrations were measured in both samples by real-time PCR. We found that polysomal luciferase mRNA was degraded at the same rate (Fig. 3, filled squares), within experimental error, as cytoplasmic luciferase mRNA (Fig. 3, open squares). This suggests that even if there were both translated and untranslated pools, luciferase mRNA freely exchanges between them. Furthermore, from the relative RNA concentration determined in this experiment, ~70% of total luciferase mRNA was in polysomes, which agrees with the analysis of polysomal distribution presented below. Because of these findings, we subsequently used

TABLE 1. Susceptibility to Dcp2, half-life in HC11 cells, and translational efficiencies of mRNAs capped with S-ARCAs

No.	Type of cap on luciferase mRNA	Dcp2 susceptibility[a]	mRNA $t_{1/2}$ (min)[b]	Relative translational efficiency[c]
1	m^7Gp_3G	ND	86 ± 1**	1.00
2	$m_2^{7,2'\text{-}O}Gp_3G$	100	155 ± 9	2.1 ± 0.2
3	$m_2^{7,2'\text{-}O}Gppp_SG$ (D1)	84	169 ± 19	2.5 ± 0.8
4	$m_2^{7,2'\text{-}O}Gppp_SG$ (D2)	91	164 ± 1	1.8 ± 0.4
5	$m_2^{7,2'\text{-}O}Gpp_SpG$ (D1)	71	185 ± 20*	2.8 ± 0.3
6	$m_2^{7,2'\text{-}O}Gpp_SpG$ (D2)	6	257 ± 4**	5.1 ± 0.5
7	$m_2^{7,2'\text{-}O}Gp_SppG$ (D1)	96	149 ± 9	2.0 ± 0.1
8	$m_2^{7,2'\text{-}O}Gp_SppG$ (D2)	98	139 ± 6	1.9 ± 0.1

[a]The data of Figure 2 were used to estimate susceptibility of oligonucleotides capped with various analogs to hDcp2 hydrolysis. The radioactivities in the peaks eluting at 44 min (undigested cap) and 38 min (pGp*) were corrected for background radioactivity and summed to represent total radioactivity in the cap. Dcp2 susceptibility is given by the radioactivity in pGp* expressed as a percentage of the total. (ND) Not determined.
[b]Degradation rates of luciferase mRNAs capped with the indicated analogs in nucleoporated HC11 cells was determined by real-time PCR as described in Figure 4. The data represent the averages of three to eight experiments. (*) Different from $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) at $p < 0.01$ according to the Student's *t*-test. (**) Different from $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) at $p < 0.001$ according to the Student's *t*-test.
[c]Translational efficiency of luciferase mRNAs capped with the indicated cap analogs in HC11 cells. The luciferase activity was measured and normalized by the amount of luciferase RNA in the cells as described in Materials and Methods. Translational efficiency is expressed relative to m^7Gp_3G-capped mRNA. The data represent the averages of three experiments.

only total cytoplasmic mRNA for determining the stability of S-ARCA-capped mRNAs.

Stability of mRNAs capped with S-ARCAs

We next asked whether capping with S-ARCAs could influence mRNA stability in cultured cells. Luciferase mRNAs were synthesized in vitro containing various 5′-terminal caps and a 60-nt 3′-terminal poly(A) tract. Following nucleoporation, cells were removed at intervals up to 8 h. The amount of luciferase mRNA was measured by real-time PCR using primers that amplify sequences near the 5′-end in order to accentuate detection of 5′→3′ degradation. Luciferase mRNA remaining at each time point was plotted as $\log_{10}$([RNA]) versus time to determine $t_{1/2}$ (Fig. 4; Table 1).

Luciferase mRNA capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) was significantly more stable ($t_{1/2}$ = 257 ± 4 min) than mRNA capped with either natural cap, m^7Gp_3G (**1**) ($t_{1/2}$ = 86 ± 1 min) or the parent ARCA, $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) ($t_{1/2}$ = 155 ± 9 min). This is the analog that produced RNA with the greatest resistance to hDcp2 (Table 1). The transcript capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) (**5**) showed a modest increase in $t_{1/2}$ compared to $m_2^{7,2'\text{-}O}Gp_3G$ (**2**), which correlates with its intermediate susceptibility to hDcp2. The two isomers of $m_2^{7,2'\text{-}O}Gppp_SG$ (**3**, **4**) had $t_{1/2}$ slightly longer than $m_2^{7,2'\text{-}O}Gp_3G$ (**2**), which correlates with a slight decrease in hDcp2 susceptibility, whereas the two isomers of $m_2^{7,2'\text{-}O}Gp_SppG$ (**7**, **8**) were essentially the same as $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) with regard to both parameters.

Translational efficiency of luciferase mRNAs capped with S-ARCAs in HC11 cells

Determination of translational efficiencies in cultured cells of luciferase mRNAs capped with the six S-ARCAs involved two measurements: the increase in luciferase activity following nucleoporation, measured in cleared cell lysates by luminometry, and the concentration of luciferase mRNA measured by real-time PCR. Translational efficiency for each type of mRNA is given by the rate of luciferase accumulation per unit of luciferase mRNA. We developed conditions under which accumulation of luciferase was linear with time, after an initial lag period of ~30 min that is required for recruitment of mRNA to ribosomes, completion of the first polypeptide chain, and release of luciferase into the cytosol (Fig. 5). The rate of luciferase accumulation was normalized for the concentration of luciferase mRNA present in the same cells immediately after nucleoporation. To determine the latter, it was necessary to determine the $t_{1/2}$ for mRNA decay as shown in Figure 4 and then extrapolate back to zero time.

Luciferase mRNAs capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) (**5**) and $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) were translated 2.8- and 5.1-fold more efficiently than m^7Gp_3G-capped mRNA,

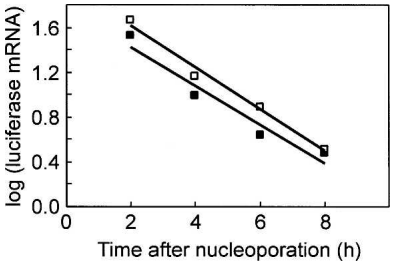


FIGURE 3. Total cytoplasmic and polysomal luciferase mRNAs are degraded at the same rate in HC11 cells. Luciferase mRNA capped with $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) was introduced into HC11 cells by nucleoporation, and the cells were lysed at the indicated time points. (■) Cytoplasmic or (□) polysomal luciferase mRNA was isolated and measured by real-time PCR, as described in Materials and Methods. The data are plotted as $\log_{10}$([RNA]) versus time after nucleoporation. The slopes are −0.173 ± 0.029 for cytoplasmic mRNA and −0.188 ± 0.015 for polysomal mRNA.

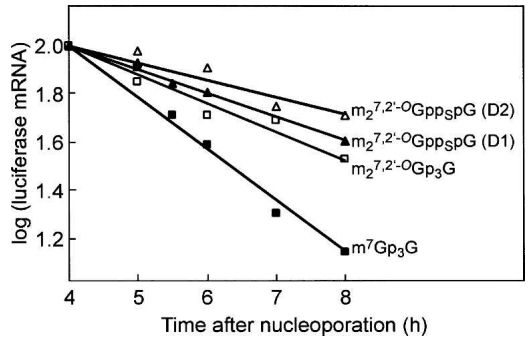


FIGURE 4. Semi-log plot showing decay of luciferase mRNA in HC11 cells as a function of cap structure. Luciferase mRNAs capped with (■) m^7Gp_3G (**1**), (□) $m_2^{7,2'\text{-}O}Gp_3G$ (**2**), (▲) $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) (**5**), and (△) $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) were nucleoporated into HC11 cells. RNA was isolated at the indicated times and measured by real-time PCR, and $t_{1/2}$ was determined as described in Materials and Methods. The results of replicate experiments and statistical treatments are given in Table 1.

respectively (Fig. 5; Table 1). None of the isomers of $m_2^{7,2'\text{-}O}Gppp_SG$ (**3**, **4**) or $m_2^{7,2'\text{-}O}Gp_Sppp G$ (**7**, **8**) conferred higher translational efficiency compared to the parent ARCA, $m_2^{7,2'\text{-}O}Gp_3G$ (**2**). For cell-free translation in the RRL system, luciferase mRNAs capped with the various S-ARCAs were translated with similar rates as those capped with $m_2^{7,2'\text{-}O}Gp_3G$ (**2**) (data not shown).

Luciferase mRNA capped with S-ARCAs is more efficiently recruited to polysomes in HC11 cells

The measurement of translational efficiency described above is somewhat indirect because it involves independent measurements of luciferase accumulation, luciferase mRNA concentration, and luciferase mRNA $t_{1/2}$. Luciferase protein itself is subject to degradation with a $t_{1/2}$ of ~3 h (Thompson et al. 1991), which could further complicate this analysis. We therefore sought an independent means to verify that luciferase mRNA capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) is initiated more rapidly than luciferase mRNA with the other capped analogs. An increase in the rate of initiation relative to elongation or termination results in a shift of the mRNA from lighter to heavier polysomes (Lodish 1971). The type of cap structure is not expected to affect the rate of elongation. Thus, a shift to heavier polysomes indicates faster initiation. We compared the steady-state polysomal distribution of luciferase mRNA capped with m^7Gp_3G (**1**), $m_2^{7,2'\text{-}O}Gp_3G$ (**2**), and $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) (Fig. 6).

Luciferase mRNA was electroporated into HC11 cells. The cells were lysed 4 h later, and the cleared supernatants were layered on sucrose gradients and subjected to ultracentrifugation. Both endogenous GAPDH and exogenous luciferase mRNA were predominantly present in polysomes (Fig. 6, fractions 6–11), although some were also found in the region of untranslated messenger ribonucleoprotein complexes (mRNP) (Fig. 6, fractions 1–2) and initiation complexes (Fig. 6, fractions 3–5). Luciferase mRNA capped with the parent ARCA, $m_2^{7,2'\text{-}O}Gp_3G$ (**2**), was shifted to heavier polysomes compared to transcripts capped with m^7Gp_3G (**1**) (Fig. 6, cf. C and D). Luciferase mRNA capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) was shifted to even heavier polysomes and was simultaneously lost from the mRNP region (Fig. 6E). These results suggest that luciferase mRNA capped with $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) is initiated faster than the other mRNAs, confirming the results based on accumulation of luciferase activity.

DISCUSSION

The series of new S-ARCAs modified both at the C2′ of m^7Guo and at one of the phosphate moieties of the triphosphate chain represents the next step in the search for more stable and more efficiently translated mRNAs. Although sulfur substitution for oxygen is considered conservative because the van der Waals radius, P–S bond length (Liang and Allen 1987), and anionic character of parent and modified oligonucleotides are all very similar (Frey and Sammons 1985; Chang et al. 1986), there are nonetheless important differences between phosphate and phosphorothioate groups, including the existence of diastereoisomers. Interestingly, these isomers can exhibit different sensitivities to nucleases. Nuclease P1 hydrolyzes the Sp more rapidly than the Rp diastereoisomer (Potter et al. 1983), whereas ribonuclease T1 and snake venom phosphodiesterase hydrolyze the Rp but not the Sp diastereoisomer (Eckstein et al. 1972; Burgers and Eckstein 1978). Based on these observations, one would expect that mRNAs capped with various diastereoisomers of $m_2^{7,2'\text{-}O}Gppp_SG$ or

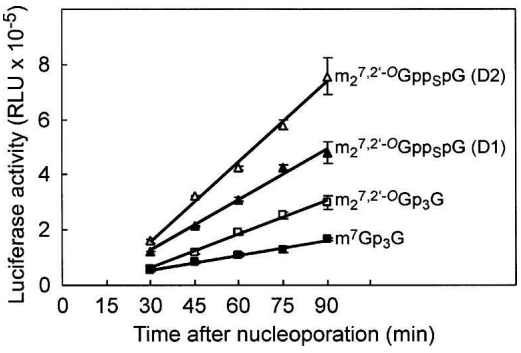


FIGURE 5. Translational efficiency of luciferase mRNA in HC11 cells as a function of cap structure. Luciferase mRNAs capped with (■) m^7Gp_3G (**1**), (□) $m_2^{7,2'\text{-}O}Gp_3G$ (**2**), (▲) $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) (**5**), and (△) $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) were nucleoporated into HC11 cells, which were then lysed at the indicated time points. Equal amounts of total protein were assayed for relative light units (RLUs) as an indicator of luciferase activity as described in Materials and Methods. Error bars refer to a single experiment. The results of replicate experiments and statistical treatments are given in Table 1.

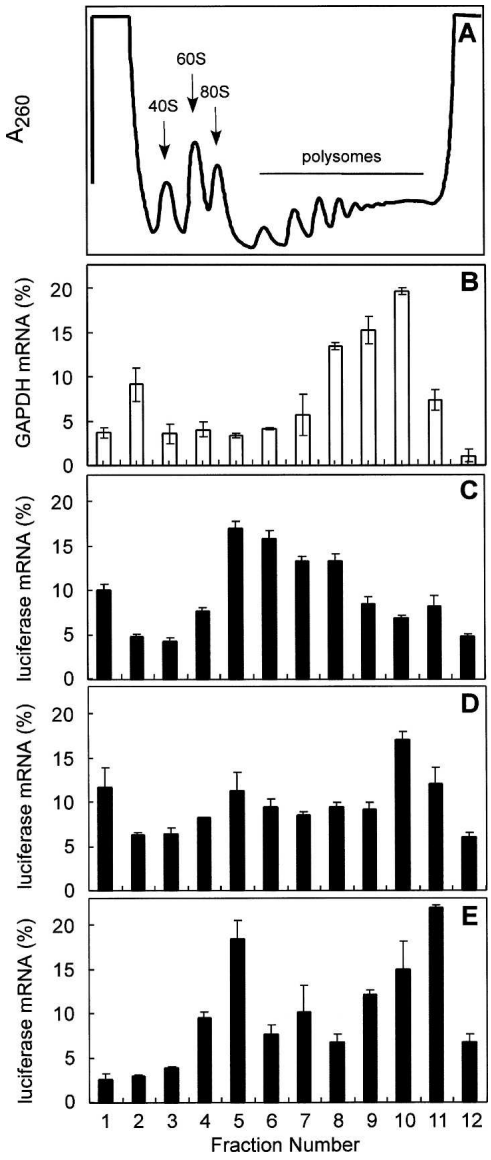


FIGURE 6. Polysomal distribution of luciferase and GAPDH mRNAs in HC11 cells. Luciferase mRNAs capped with (*C*) m^7Gp_3G (**1**), (*D*) $m_2^{7,2'\text{-}O}Gp_3G$ (**2**), and (*E*) $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) were electroporated into HC11 cells. The cells were lysed 4 h later, the lysates were subjected to ultracentrifugation on 15%–45% sucrose gradients, and 1 mL were fractions collected. (*A*) Ribosomal subunits (40S, 60S), monosomes (80S), and polysomes were detected by absorbance at 260. The distribution of (*B*) GAPDH and (*C–E*) luciferase mRNA was determined by real-time PCR as described in Materials and Methods.

$m_2^{7,2'\text{-}O}Gpp_SpG$ might differ in their sensitivities to cleavage by Dcp1/Dcp2. In fact, we found that only one of six tested compounds, $m_2^{7,2'\text{-}O}Gpp_SpG$ D2 (**6**), was completely resistant to hDcp2, while $m_2^{7,2'\text{-}O}Gpp_SpG$ D1 (**5**) and both isomers of $m_2^{7,2'\text{-}O}Gppp_SG$ (**3**, **4**) were partially resistant.

These findings provide some insight into the catalytic mechanism of human Dcp2. This enzyme belongs to a hydrolase family in which all members contain the signature Nudix motif and require a divalent cation for activity, Mg^{2+} being preferred over Mn^{2+} (Bessman et al. 1996; Mildvan et al. 2005). Although the detailed mechanisms of various Nudix hydrolases differ, all contain three conserved glutamate residues that coordinate the divalent cation (Mildvan et al. 2005). Mutation of one of these residues in Dcp2 completely abolishes its enzymatic activity (Dunckley and Parker 1999; Lykke-Andersen 2002; Wang et al. 2002; Steiger et al. 2003). Previously, it was shown that a truncated form of hDcp2 lacking 94 amino acid residues preceding the Nudix fold generated m^7GMP in addition to m^7GDP, suggesting nucleophilic attack at the α- as well as β-phosphate of the cap structure (Piccirillo et al. 2003). Also, it has been speculated that the mechanism of hDcp2 involves attack at the α-phosphate because the reaction catalyzed by hDcp2 closely resembles that catalyzed by the *Escherichia coli* hydrolase Orf186 (O'Handley et al. 1998). Our results suggest that the nucleophilic attack occurs at the β- rather than the α-phosphate, because the analogs substituted at the β-position were more resistant to cleavage than any of the α- or γ-substituted analogs. Interestingly, the sensitivities of D1 and D2 isomers of $m_2^{7,2'\text{-}O}Gpp_SpG$ (**5** and **6**) were quite different. This is presumably a reflection of the orientation of catalytic amino acid residues and/or the divalent cation in the active site of Dcp2 with respect to the β-phosphate. Like many Nudix hydrolases, hDcp2 requires Mg^{2+} for activity, and its activity is greatly enhanced by Mn^{2+} (Piccirillo et al. 2003; Steiger et al. 2003) One possible explanation for the resistance of $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2)-capped RNA is the vastly weaker interaction of S-Mg^{2+} compared with O-Mg^{2+}, or S-Mn^{2+} compared with O-Mn^{2+} (Eckstein 1985).

We observed a correlation between the $t_{1/2}$ of the various S-ARCA-capped mRNAs and their enzymatic susceptibilities to hDcp2. $m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**) was the least susceptible to hDcp2 and also showed the greatest increase in transcript stability. This was followed in order by $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) (**5**), $m_2^{7,2'\text{-}O}Gppp_SG$ (D1) (**3**), and $m_2^{7,2'\text{-}O}Gppp_SG$ (D2) (**4**). Luciferase mRNAs capped with $m_2^{7,2'\text{-}O}Gp_SppG$ (D1) (**7**) and $m_2^{7,2'\text{-}O}Gp_SppG$ (D2) (**8**) had the same susceptibility to Dcp2 hydrolysis, as well as the same $t_{1/2}$, as mRNA capped with the parent compound, $m_2^{7,2'\text{-}O}Gp_3G$ (**2**). This suggests that susceptibility to hDcp2 is a strong determinant of mRNA stability. Previously we observed that mRNAs capped with Gp_3G, m^7Gp_3G, and $m_2^{7,3'\text{-}O}Gp_3G$ exhibited increasing $t_{1/2}$ values in electroporated mammalian cells (Grudzien et al. 2006). This finding, along with data from other laboratories, supported the hypothesis that the rate of decapping is partially determined by competition for cap binding between Dcp2 and eIF4E. However, it is unlikely that the different affinities of S-ARCAs for eIF4E are responsible for the differences in $t_{1/2}$ we report here. The various S-ARCAs have affinities for eIF4E (J. Kowalska, M. Lewdorowicz, J. Zuberek, E. Grudzien-Nogalska, E. Bojarska, J. Stepinski, R.E. Rhoads, E. Darzynkiewicz, R.E. Davis, and J. Jemielity,

in prep.) that are in the order of $m_2^{7,2'\text{-}O}Gpp_SpG$ (D1) (**5**)>$m_2^{7,2'\text{-}O}Gppp_SG$ (D1) (**3**)>$m_2^{7,2'\text{-}O}Gp_SppG$ (D1) (**7**)>$m_2^{7,2'\text{-}O}Gpp_SpG$ (D2) (**6**)>$m_2^{7,2'\text{-}O}Gp_SppG$ (D2) (**8**)>$m_2^{7,2'\text{-}O}Gppp_SG$ (D2) (**4**), which bears no resemblance to the order of $t_{1/2}$ values (Table 1). It is possible that the affinities of different cap analogs for eIF4E influence mRNA $t_{1/2}$, but susceptibility to Dcp2 hydrolysis predominates.

Previously, we demonstrated a correlation between affinity of a free cap analog for eIF4E and the translational efficiency of mRNA capped with that analog in the RRL translation system (Grudzien et al. 2004). All of the S-ARCAs have higher affinity for eIF4E than the parent ARCA, yet S-ARCA-capped mRNAs do not differ in cell-free translational efficiency, at least under the conditions we employed. This may indicate that there is an upper limit beyond which high affinity for eIF4E cannot accelerate overall translation in the RRL. Thus, when the rate of cap binding becomes sufficiently high, some other step in protein synthesis initiation becomes rate limiting. mRNA recruitment may be abnormally fast in the RRL system because initiation factors are present at approximately fivefold higher levels than in more normal cells (Rau et al. 1996). In contrast to the cell-free translation results, mRNAs capped with the $m_2^{7,2'\text{-}O}Gpp_SpG$ isomers (**5** and **6**) had higher translational efficiencies in cultured cells than mRNA capped with the parent compound $m_2^{7,2'\text{-}O}GpppG$ (**2**). These higher translational efficiencies correlated with resistance to hDcp2 and longer mRNA $t_{1/2}$. Current models for initiation of protein synthesis and mRNA decapping do not explain this correlation.

Synthetic mRNAs have unique applications for protein expression, for example, in cases in which the protein of interest requires post-translational modifications that occur only in specific cell types. Such RNAs can be introduced not only into cultured cells but also into living animals through delivery systems that protect the RNA against degradation (Anderson et al. 2003; Zohra et al. 2005). RNA has certain advantages over DNA for applications such as immunization against autologous tumors with autologous mRNA-transfected dendritic cells (Mitchell and Nair 2000). This approach has been successfully tested in preclinical trials to develop melanoma vaccines (Kyte et al. 2005). Since RNAs do not carry the risk of insertional mutagenesis, another promising application for synthetic mRNAs is gene therapy, for example, introducing a chimeric immune receptor into T-lymphocytes (Rabinovich et al. 2006). These observations provide strong motivation to increase protein yields from transfected mRNAs. For applications involving development of an immune response, increasing the duration of protein expression by stabilizing the mRNA is particularly desirable. The original ARCAs improved both translational efficiency and mRNA stability compared with conventional caps (Stepinski et al. 2001; Jemielity et al. 2003; Grudzien et al. 2006). The use of ARCA-capped mRNA together with a long poly(A) tract was reported to enhance protein expression in mouse dendritic cells by 700-fold (Mockey et al. 2006). We have demonstrated here that S-ARCAs confer even greater translational efficiency and stability to transfected mRNAs, making them a preferred choice for mRNA transfection applications.

MATERIALS AND METHODS

Cell culture

HC11 is a line of mammary epithelial cells that were clonally derived from the COMMA-1D line (Danielson et al. 1984). The cells were grown in RPMI 1640 medium containing 10% Bovine Growth Serum (HyClone), 5 μg/mL bovine insulin (Sigma), and 10 ng/mL recombinant EGF (BD Biosciences).

In vitro synthesis of mRNAs

Capped RNAs were synthesized by in vitro transcription of a luciferase-encoding plasmid (p*luc*-A_{60}) with T7 polymerase in the presence of all four nucleoside triphosphates and various cap dinucleotides (Jemielity et al. 2003). A typical transcription reaction contained 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 0.1 mg/mL BSA, 1 U/μL RNasin (Promega), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 0.1 mM GTP, 1 mM cap analog, 15 μg/mL DNA, and 1 U/μL T7 polymerase (Promega). p*luc*-A_{60}, which contains an entire firefly luciferase mRNA sequence in pGEM4 (Promega), and a 3′-terminal 60-nt poly(A) tract (Grudzien et al. 2006), was digested with NcoI for synthesis of capped oligonucleotides and with HpaI for synthesis of luciferase mRNA.

Capped oligonucleotides of 48 nt were synthesized in the presence of 10 μCi/μL [α-^{32}P]GTP (ICN) in 50 μL reaction mixtures for 45 min at 37°C. Reaction mixtures were extracted with phenol and chloroform, and then RNAs were separated from unincorporated nucleotides with spin columns, according to the manufacturer's protocol (Ambion). The concentrations of RNAs were determined by Cerenkov counting, the specific radioactivity of [α-^{32}P]GTP in the final transcription reaction mixture being used for conversion of counts per minute to picomoles.

mRNAs were synthesized in 200 μL reaction mixtures for 45 min at 37°C. Reaction mixtures were treated with 3 units of DNase RQ1 (Promega) for 20 min at 37°C, and RNA was purified with an RNeasy mini kit (QIAGEN) using the manufacturer's protocol. The concentrations of RNAs were determined spectrophotometrically.

In vitro RNA decapping assay

Dcp2 activity was measured with capped 48-nt oligonucleotides as substrates. GST-hDcp2 was expressed in *E. coli* and purified as described previously (Wang et al. 2002). Capped oligonucleotides were first subjected to digestion with GST-hDcp2 for 2 h at 37°C in 10 mM Tris-HCl at pH 7.5, 100 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM $MnCl_2$, 2 mM dithiothreitol, and 0.1 mM spermine (Piccirillo et al. 2003). The reaction mixture was then extracted once with an equal volume of phenol and twice with chloroform, after which RNA was precipitated with ethanol. Products of the decapping reaction were further digested with a

cocktail of ribonucleases (RiboShredder; Epicentre) for 1 h at 37°C. 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 KH_2PO_4 (pH 4.5) for 20 min, a linear gradient of 112–450 mM KH_2PO_4 for 15 min, a linear gradient of 450 mM to 1.5 M KH_2PO_4 for 15 min, and isocratic elution at 1.5 M of KH_2PO_4 for 9 min, all at a flow rate of 1 mL/min.

Real-time PCR

For measurement of mRNA stability, ~2 μg of each total RNA sample were isolated from HC11 cells. RNA was purified with an RNeasy mini kit, treated with 3 units of DNase RQ1 (Promega) for 20 min at 37°C, and further incubated for 10 min at 65°C to inactivate the enzyme. For measurement of RNA distribution in polysomes, 100 μL of each 1 mL fraction were used to isolate RNA using an RNeasy 96 kit (QIAGEN). 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 for 10 min at 25°C, for 30 min at 48°C, and for 5 min at 95°C. Quantitative real-time PCR was performed with specific primers designed for each mRNA with the Beacon Designer tool (Bio-Rad). Luciferase mRNA levels were measured with two primers, 5′-CGTTCGGTTGGCAGAAGCTA-3′ and 5′-ACTGTTGAGCAATTCACGTTCATT-3′, which amplify nucleotides 226–398 from the 5′-end. Mouse GAPDH mRNA levels were measured by the same method and in the same RNA samples with primers 5′-CAATGTGTCCGTCGTGGATCT-3′ and 5′-GAAGAGTGGGAGTTGCTGTTGA-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, each of 15 sec 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. Luciferase mRNA was then normalized for the amount of mouse GAPDH mRNA in each sample, an indicator of total cellular RNA purified from each cell extract.

Measurement of translational efficiency and mRNA decay in HC11 cells

Two methods, electroporation and nucleoporation, were used to deliver RNA into HC11 cells. For electroporation, 5 μg of RNA were introduced into 10^7 cells in a total volume of 400 μL of serum-reduced RPMI 1640 medium in a Genepulser cuvette (4 mm gap) with a Bio-Rad Genepulser set at 0.22 kV and 960 μF. Following discharge, cells were washed twice with PBS, centrifuged at 300*g* for 2 min at room temperature, resuspended in prewarmed complete medium, and placed at 37°C. Nucleoporation was performed with a Nucleofector II (Amaxa Biosystems) using the manufacture's protocol. One microgram of RNA was introduced into 10^6 cells in Nucleofector Solution V using program T-024.

For measurement of luciferase synthesis, cells were divided into several Eppendorf tubes, placed in a water bath at 37°C, and shaken. For protein extraction, 2 × 10^5 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).

For measurement of mRNA stability, 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 × 10^5 cells were lysed in 175 μL of 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 and analyzed by real-time PCR as described above. 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 $t_{1/2}$.

The translational efficiencies of various luciferase mRNAs were determined by normalizing the rate of luciferase synthesis with the concentration of luciferase mRNA present in cells at zero time.

Measurements of mRNA distribution in polysomes

HC11 cells (4 × 10^6) were treated for 2 min with ice-cold PBS containing 0.1 mg/mL cycloheximide, washed twice with the same medium, and lysed in 600 μL of 0.3 M NaCl, 15 mM Tris-HCl (pH 7.6), 15 mM $MgCl_2$, 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%–45% sucrose gradient in the same buffer but lacking Triton X-100 and centrifuged in a Beckman SW41Ti rotor at 38,000 rpm for 2 h at 4°C. Gradients were fractionated with continuous monitoring of absorbance at 260 nm. RNA from each fraction (1 mL) was isolated using an RNeasy 96 kit and analyzed by real-time PCR. For analysis of RNA from polysome gradients, 100 pg of in vitro-synthesized GFP mRNA were added to each fraction before RNA isolation as an internal control for variations in RNA yield. The level of GFP mRNA was used to normalize the levels of luciferase and GAPDH mRNA.

ACKNOWLEDGMENTS

This project was supported by Grant No. 2R01GM020818 from the National Institute of General Medical Sciences (NIH), Grant No. IS-3837-06 from the U.S.–Israel Binational Agricultural Research and Development Fund, Grant No. 2P04A00628 from the Polish Ministry of Science and Higher Education, and Grant No. 55005604 from the Howard Hughes Medical Institute (to E.D.). The authors express their gratitude to Megerditch Kiledjian (Rutgers University) for the pGEX-hDcp2 plasmid, Lynn Harrison (LSUHSC-S) for help with nucleoporation, and the LSUHSC-S Research Core Facility for instrumentation.

Received June 24, 2007; accepted July 23, 2007.

REFERENCES

Anderson, D.M., Hall, L.L., Ayyalapu, A.R., Irion, V.R., Nantz, M.H., and Hecker, J.G. 2003. Stability of mRNA/cationic lipid lipoplexes

in human and rat cerebrospinal fluid: Methods and evidence for nonviral mRNA gene delivery to the central nervous system. *Hum. Gene Ther.* **14:** 191–202.

Bessman, M.J., Frick, D.N., and O'Handley, S.F. 1996. The MutT proteins or "Nudix" hydrolases, a family of versatile, widely distributed, "housecleaning" enzymes. *J. Biol. Chem.* **271:** 25059–25062.

Burgers, P.M.J. and Eckstein, F. 1978. Absolute configuration of the diastereomers of adenosine 5′-*O*-(1-thiotriphosphate): Consequences for the stereochemistry of polymerization by DNA-dependent RNA polymerase from *Escherichia coli*. *Proc. Natl. Acad. Sci.* **75:** 4798–4800.

Cassel, D. and Selinger, Z. 1977. Activation of turkey erythrocyte adenylate cyclase and blocking of the catecholamine-stimulated GTPase by guanosine 5′-(γ-thio) triphosphate. *Biochem. Biophys. Res. Commun.* **77:** 868–873.

Chang, S.B., Alben, J.O., Wisner, D.A., and Tsai, M.D. 1986. Phospholipids chiral at phosphorus: Fourier-transform infrared study on the gel–liquid crystalline transition of chiral thiophosphatidylcholine. *Biochemistry* **25:** 3435–3440.

Chen, C., Gherzi, R., Ong, S., Chan, E., Raijmakers, R., Pruijn, G., Stoecklin, G., Moroni, C., Mann, M., and Karin, M. 2001. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107:** 451–464.

Contreras, R., Cheroutre, H., Degrave, W., and Fiers, W. 1982. Simple, efficient in vitro synthesis of capped RNA useful for direct expression of cloned eukaryotic genes. *Nucleic Acids Res.* **10:** 6353–6362.

Couttet, P., Fromont-Racine, M., Steel, D., Pictet, R., and Grange, T. 1997. Messenger RNA deadenylylation precedes decapping in mammalian cells. *Proc. Natl. Acad. Sci.* **94:** 5628–5633.

Danielson, K.G., Oborn, C.J., Durban, E.M., Butel, J.S., and Medina, D. 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. *Proc. Natl. Acad. Sci.* **81:** 3756–3760.

Darnbrough, C., Legon, S., Hunt, T., and Jackson, R.J. 1973. Initiation of protein synthesis: Evidence for messenger RNA-independent binding of methionyl-transfer RNA to the 40S ribosomal subunit. *J. Mol. Biol.* **76:** 379–403.

Darzynkiewicz, E., Stepinski, J., Tahara, S.M., Stolarski, R., Ekiel, I., Haber, D., Neuvonen, K., Lehikoinen, P., Labadi, I., and Lönnberg, H. 1990. Synthesis, conformation, and hydrolytic stability of P1, P3-dinucleoside triphosphates related to mRNA 5′-cap, and comparative kinetic studies on their nucleoside and nucleoside monophosphate analogs. *Nucleosides Nucleotides* **9:** 599–618.

Dunckley, T. and Parker, R. 1999. The DCP2 protein is required for mRNA decapping in *Saccharomyces cerevisiae* and contains a functional MutT motif. *EMBO J.* **18:** 5411–5422.

Eckstein, F. 1985. Nucleoside phosphorothioates. *Annu. Rev. Biochem.* **54:** 367–402.

Eckstein, F., Schulz, H.H., Ruterjans, H., Haar, W., and Maurer, W. 1972. Stereochemistry of the transesterification step of ribonuclease T 1. *Biochemistry* **11:** 3507–3512.

Eckstein, F., Cassel, D., Levkovitz, H., Lowe, M., and Selinger, Z. 1979. Guanosine 5′-*O*-(2-thiodiphosphate). An inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. *J. Biol. Chem.* **254:** 9829–9834.

Edery, I. and Sonenberg, N. 1985. Cap-dependent RNA splicing in a HeLa nuclear extract. *Proc. Natl. Acad. Sci.* **82:** 7590–7594.

Fenger-Gron, M., Fillman, C., Norrild, B., and Lykke-Andersen, J. 2005. Multiple processing body factors and the ARE binding protein TTP activate mRNA decapping. *Mol. Cell* **20:** 905–915.

Frey, P.A. and Sammons, R.D. 1985. Bond order and charge localization in nucleoside phosphorothioates. *Science* **228:** 541–545.

Görlich, D. and Mattaj, I.W. 1996. Nucleocytoplasmic transport. *Science* **271:** 1513–1518.

Grudzien, E., Stepinski, J., Jankowska-Anyszka, M., Stolarski, R., Darzynkiewicz, E., and Rhoads, R.E. 2004. Novel cap analogs for in vitro synthesis of mRNAs with high translational efficiency. *RNA* **10:** 1479–1487.

Grudzien, E., Kalek, M., Jemielity, J., Darzynkiewicz, E., and Rhoads, R.E. 2006. Differential inhibition of mRNA degradation pathways by novel cap analogs. *J. Biol. Chem.* **281:** 1857–1867.

Hsu, C. and Stevens, A. 1993. Yeast cells lacking 5′→3′ exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5′ cap structure. *Mol. Cell. Biol.* **13:** 4826–4835.

Jemielity, J., Fowler, T., Zuberek, J., Stepinski, J., Lewdorowicz, M., Niedzwiecka, A., Stolarski, R., Darzynkiewicz, E., and Rhoads, R.E. 2003. Novel "anti-reverse" cap analogues with superior translational properties. *RNA* **9:** 1108–1122.

Kalek, M., Jemielity, J., Grudzien, E., Zuberek, J., Bojarska, E., Cohen, L.S., Stepinski, J., Stolarski, R., Davis, R.E., Rhoads, R.E., et al. 2005. Synthesis and biochemical properties of novel mRNA 5′ cap analogs resistant to enzymatic hydrolysis. *Nucleosides Nucleotides Nucleic Acids* **24:** 615–621.

Kalek, M., Jemielity, J., Darzynkiewicz, Z.M., Bojarska, E., Stepinski, J., Stolarski, R., Davis, R.E., and Darzynkiewicz, E. 2006. Enzymatically stable 5′ mRNA cap analogs: Synthesis and binding studies with human DcpS decapping enzyme. *Bioorg. Med. Chem.* **14:** 3223–3230.

Konarska, M.M., Padgett, R.A., and Sharp, P.A. 1984. Recognition of a cap structure in splicing in vitro of mRNA precursors. *Cell* **38:** 731–736.

Kyte, J.A., Kvalheim, G., Aamdal, S., Saeboe-Larssen, S., and Gaudernack, G. 2005. Preclinical full-scale evaluation of dendritic cells transfected with autologous tumor-mRNA for melanoma vaccination. *Cancer Gene Ther.* **12:** 579–591.

Liang, C. and Allen, L.C. 1987. Sulfur does not form double bonds in phosphorothioate anions. *J. Am. Chem. Soc.* **109:** 6449–6453.

Liu, H., Rodgers, N.D., Jiao, X., and Kiledjian, M. 2002. The scavenger mRNA decapping enzyme DcpS is a member of the HIT family of pyrophosphatases. *EMBO J.* **21:** 4699–4708.

Lodish, H.F. 1971. α and β globin messenger ribonucleic acid. *J. Biol. Chem.* **246:** 7131–7138.

Lykke-Andersen, J. 2002. Identification of a human decapping complex associated with hUpf proteins in nonsense-mediated decay. *Mol. Cell. Biol.* **22:** 8114–8121.

Matzura, H. and Eckstein, F. 1968. A polyribonucleotide containing alternation P=O and P=S linkages. *Eur. J. Biochem.* **3:** 448–452.

Mildvan, A., Xia, Z., Azurmendi, H., Saraswat, V., Legler, P., Massiah, M., Gabelli, S., Bianchet, M., Kang, L., and Amzel, L. 2005. Structures and mechanisms of Nudix hydrolases. *Arch. Biochem. Biophys.* **433:** 129–143.

Mitchell, D.A. and Nair, S.K. 2000. RNA transfected dendritic cells as cancer vaccines. *Curr. Opin. Mol. Ther.* **2:** 176–181.

Mockey, M., Goncalves, C., Dupuy, F.P., Lemoine, F.M., Pichon, C., and Midoux, P. 2006. mRNA transfection of dendritic cells: Synergistic effect of ARCA mRNA capping with poly(A) chains *in cis* and *in trans* for a high protein expression level. *Biochem. Biophys. Res. Commun.* **340:** 1062–1068.

Muhlrad, D., Decker, C.J., and Parker, R. 1995. Turnover mechanisms of the stable yeast PGK1 mRNA. *Mol. Cell. Biol.* **15:** 2145–2156.

Mukherjee, D., Gao, M., O'Connor, J.P., Raijmakers, R., Pruijn, G., Lutz, C.S., and Wilusz, J. 2002. The mammalian exosome mediates the efficient degradation of mRNAs that contain AU-rich elements. *EMBO J.* **21:** 165–174.

O'Handley, S.F., Frick, D.N., Dunn, C.A., and Bessman, M.J. 1998. Orf186 represents a new member of the Nudix hydrolases, active on adenosine(5′)triphospho(5′)adenosine, ADP-ribose, and NADH. *J. Biol. Chem.* **273:** 3192–3197.

Parker, R. and Song, H. 2004. The enzymes and control of eukaryotic mRNA turnover. *Nat. Struct. Mol. Biol.* **11:** 121–127.

Pasquinelli, A.E., Dahlberg, J.E., and Lund, E. 1995. Reverse 5′ caps in RNAs made in vitro by phage RNA polymerases. *RNA* **1:** 957–967.

Peng, Z.-H., Sharma, V., Singleton, S.F., and Gershon, P.D. 2002. Synthesis and application of a chain-terminating dinucleotide mRNA cap analog. *Org. Lett.* **4:** 161–164.

Piccirillo, C., Khanna, R., and Kiledjian, M. 2003. Functional characterization of the mammalian mRNA decapping enzyme hDcp2. *RNA* **9:** 1138–1147.

Potter, B.V., Connolly, B.A., and Eckstein, F. 1983. Synthesis and configurational analysis of a dinucleoside phosphate isotopically chiral at phosphorus. Stereochemical course of *Penicillium citrum* nuclease P1 reaction. *Biochemistry* **22:** 1369–1377.

Rabinovich, P.M., Komarovskaya, M.E., Ye, Z.J., Imai, C., Campana, D., Bahceci, E., and Weissman, S.M. 2006. Synthetic messenger RNA as a tool for gene therapy. *Hum. Gene Ther.* **17:** 1027–1035.

Rau, M., Ohlmann, T., Morley, S.J., and Pain, V.M. 1996. A reevaluation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate. *J. Biol. Chem.* **271:** 8983–8990.

Richter, J. and Sonenberg, N. 2005. Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* **433:** 477–480.

Sachs, A.B. 1993. Messenger RNA degradation in eukaryotes. *Cell* **74:** 413–421.

Shatkin, A.J. 1985. mRNA cap binding proteins: Essential factors for initiating translation. *Cell* **40:** 223–224.

Steiger, M., Carr-Schmid, A., Schwartz, D.C., Kiledjian, M., and Parker, R. 2003. Analysis of recombinant yeast decapping enzyme. *RNA* **9:** 231–238.

Stepinski, J., Waddell, C., Stolarski, R., Darzynkiewicz, E., and Rhoads, R.E. 2001. Synthesis and properties of mRNAs containing the novel "anti-reverse" cap analogues 7-methyl(3′-*O*-methyl) GpppG and 7-methyl(3′-deoxy)GpppG. *RNA* **7:** 1486–1495.

Thompson, J.F., Hayes, L.S., and Lloyd, D.B. 1991. Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene* **103:** 171–177.

van Dijk, E., Cougot, N., Meyer, S., Babajko, S., Wahle, E., and Seraphin, B. 2002. Human Dcp2: A catalytically active mRNA decapping enzyme located in specific cytoplasmic structures. *EMBO J.* **21:** 6915–6924.

Visa, N., Izaurralde, E., Ferreira, J., Daneholt, B., and Mattaj, I. 1996. A nuclear cap-binding complex binds Balbiani ring pre-mRNA cotranscriptionally and accompanies the ribonucleoprotein particle during nuclear export. *J. Cell Biol.* **133:** 5–14.

Walther, T.N., Wittop Koning, T.H., Schumperli, D., and Müller, B. 1998. A 5′–3′ exonuclease activity involved in forming the 3′ products of histone pre-mRNA processing in vitro. *RNA* **4:** 1034–1046.

Wang, Z. and Kiledjian, M. 2000. Identification of an erythroid-enriched endoribonuclease activity involved in specific mRNA cleavage. *EMBO J.* **19:** 295–305.

Wang, Z. and Kiledjian, M. 2001. Functional link between the mammalian exosome and mRNA decapping. *Cell* **107:** 751–762.

Wang, Z., Jiao, X., Carr-Schmid, A., and Kiledjian, M. 2002. The hDcp2 protein is a mammalian mRNA decapping enzyme. *Proc. Natl. Acad. Sci.* **99:** 12663–12668.

Yisraeli, J.K. and Melton, D.A. 1989. Synthesis of long, capped transcripts in vitro by SP6 and T7 RNA polymerases. In *Methods in enzymology* (eds. J.E. Dahlberg and J.N. Abelson), pp. 42–50. Academic Press, San Diego.

Yu, J.H., Yang, W.-H., Gulick, T.O.D., Bloch, K.D., and Bloch, D.B. 2005. Ge-1 is a central component of the mammalian cytoplasmic mRNA processing body. *RNA* **11:** 1795–1802.

Zohra, F.T., Chowdhury, E.H., Nagaoka, M., and Akaike, T. 2005. Drastic effect of nanoapatite particles on liposome-mediated mRNA delivery to mammalian cells. *Anal. Biochem.* **345:** 164–166.

Phosphorothioate cap analogs stabilize mRNA and increase translational efficiency in mammalian cells

Ewa Grudzien-Nogalska, Jacek Jemielity, Joanna Kowalska, et al.

RNA 2007 13: 1745-1755 originally published online August 24, 2007
Access the most recent version at doi:10.1261/rna.701307