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Efficient preparation and properties of mRNAs containing a fluorescent cap analog: Anthraniloyl-m^{7}GpppG

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Abbreviations: Ant, Anthraniloyl; Ant-m^{7}GTP, Anthraniloyl-7-methyl guanosine triphosphate; Ant-m^{7}GMP, Anthraniloyl-7-methyl guanosine monophosphate; Ant-GTP, Anthraniloyl guanosine triphosphate; IVT, *In vitro* translation; OMP, Outer Membrane Protein; SAM, *S*-Adenosyl Methionine

A method has been developed for synthesising fluorescently labeled capped mRNA. The method incorporates a single fluorescent molecule as part of the 5′-mRNA or oligonucleotide cap site. The fluorescent molecule, Ant-m^{7}GTP is specifically incorporated into the cap site to yield Ant-m^{7}GpppG-capped mRNA or oligonucleotide. Efficient capping was observed with 60–100% of the RNA transcripts capped with the fluorescent molecule. The Ant-m^{7}G derivative, which has been previously shown to interact with the eukaryotic cap binding protein eIF4E, is shown in this paper to be a substrate for the *Vaccinia* capping enzyme and the DCP2 decapping enzyme from *Arabidopsis*. Further, the Ant-m^{7}GTP-capped RNA is readily translated. This Ant-m^{7}GTP-capped RNA provides an important tool for monitoring capping reactions, translation, and biophysical studies.

A distinctive feature found in eukaryotic mRNA and several small RNAs is the presence of a cap structure (m^{7}GpppN, where N is any nucleotide) at the 5′ terminus.[1] The cap structure serves as a multi-purpose modification that is recognized by many cellular proteins involved in pre-mRNA splicing, RNA export, translation initiation and RNA turnover.[2] Proteins that interact with the cap structure include the cap binding complex (CBC) that plays a major role in RNA processing by activating pre-mRNA splicing and nucleocytoplasmic transport of small snRNAs, eukaryotic translation initiation factors (eIFs) that initiate translation of processed mRNAs, as well as the Dcp1/Dcp2 complex which mediates the hydrolysis of the cap structure (decapping) prior to the 5′ → 3′ decay of RNA.[3-7]

Analogs of the mRNA cap have been instrumental in the study of cap-associated processes, in particular, cap-dependent translation.[8-11] It has been demonstrated that 7-methylguanosine, ribose and phosphoryl moieties are the minimal structural requirements for cap analogs.[12] Numerous cap analogs have been synthesized and have been instrumental in biophysical studies of cap binding, in assessing the determinants of translation inhibition, in the purification of a range of eIF4E proteins and in the priming of *in vitro* transcription reactions to synthesize capped RNA.[8,13-17]

In vivo, the cap structure is attached to an mRNA transcript, at the stage of 25–30 nucleotides by consecutive enzymatic reactions catalyzed by triphosphatase, guanylyltransferase, and methyltransferase activities.[1,2,18–24] The reactions are shown below:

ppp5′-r(n)-3′ → pp5′-r(n)-3′ + P1 (Triphosphatase)

pp5′-r(n)-3 + GTP → Gppp5′-r(n)-3′ + PP1(Guanylyltransferase)

Gppp5′-r(n)-3′ + AdoMet → 7Methyl-Gppp5′-r(n)-3′ (Methyltransferase)

This pathway seems to be conserved, although very few examples have been characterized in detail.[25] The consecutive enzymatic activities of capping have been primarily determined using the recombinant *Vaccinia* capping enzyme.[18-20] Capping involves the sequential hydrolysis of a monophosphate group from triphosphorylated RNA, transfer of a GMP moiety to the diphosphorylated RNA transcript and the acceptance of a single methyl group from *S*-adenosylmethionine to the seventh position of guanine moiety.[18,19,25] The recombinant *Vaccinia* capping enzyme has been used to synthesize radiolabeled capped RNA, primarily in studies on the capping and the decapping processes.[6,18]

In this study, we have demonstrated efficient fluorescent labeling at the cap of *in vitro* transcribed RNA, catalyzed by the

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recombinant *Vaccinia* capping enzyme, using Ant-GTP and Ant-m^{7}GTP as the fluorescent GMP donors. To our knowledge, this is the first reported study on synthesising fluorescently-labeled 7-methyl guanosine capped RNA.

The anthraniloyl-GTP substrates are ribose-modified cap analogs and have been shown to present negligible alterations to native cap binding properties.[12,17] Ant-m^{7}GTP was previously used as an excellent cap analog to study the binding properties of the cap binding proteins eIF4F, eIF*iso*4F, a single substitution mutant of the human eIF4E protein (K119A) and pokeweed antiviral protein (PAP).[12,17,26]

Cap analogs linked to oligonucleotides have been shown to bind eIF4E with greater efficiency than cap analogs alone.[13] It is expected that fluorescently labeled RNAs synthesized in this paper would prove to be even better ligands for such cap binding proteins. Recently, the synthesis of a second fluorescent cap analog, fluorescein-labeled GMP was reported, where the investigators employed the cap analog to study the binding of eIF4E to the cap structure, and to identify small inhibitor molecules of this interaction.[27] With this cap analog, the fluorescein moiety is coupled to the phosphate group of the GMP moiety,[27] which precludes using it to cap oligonucleotides or mRNA.

In addition to synthesising a fluorescently labeled capped mRNA, we demonstrate that Ant-m^{7}GTP-capped RNA is translated in a wheat germ *in vitro* translation system, and serves as an effective substrate for the DCP2 decapping enzyme. This fluorescent mRNA will have a wide range of applications for studying capping/decapping reactions, translation and biophysical studies.

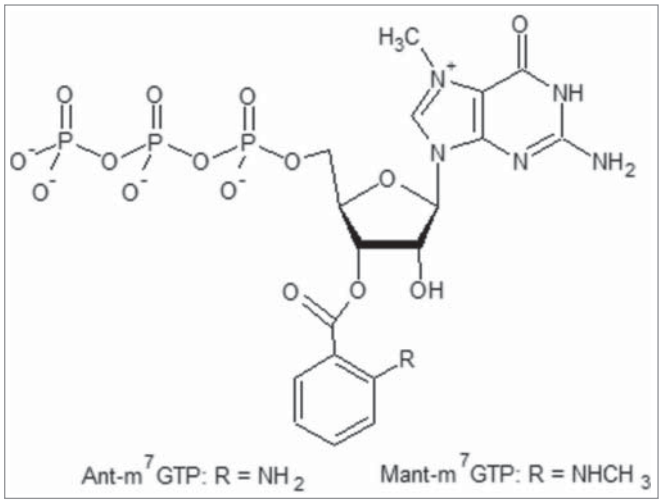

Figure 1. Structure of the substrate Ant-m^{7}GTP used for the capping reactions. The anthraniloyl group is incorporated primarily at the 3′- position of the ribose moiety, with a ratio of 65:35 for incorporation at the 3′- and 2′- positions respectively.[12]

Results

Capping reaction and RNA recovery

Three different fluorescent cap analogs were used in this study, Ant-GTP, Ant-m^{7}GTP and a commercial, Mant-GTP (**Fig. 1**). It has been shown that capping of transcripts of over 25 nucleotides with the human capping enzyme, during transcription, in association with the human transcription complex, is extremely fast with $t_{1/2}$ of less than 15 sec.[23] Even the second methyltransferase step, although considered the lagging step, has been demonstrated to catalyze the methyl transfer reaction with a $t_{1/2}$ of 70 sec.[23] However, the guanylylation of free RNA in the absence of the transcription complex, in the presence of 1 μg of recombinant human capping enzyme, was shown to proceed much slower with a $t_{1/2}$ of 30 min.[23] In the context of transcription and capping in *Vaccinia* virions, it has been demonstrated that the *Vaccinia* capping enzyme associates with the RNA polymerase and functions as a termination factor for elongation to cap nascent nucleotides of at least 25–31 bases in length.[24,28]

Therefore, it was anticipated that in the absence of the RNA polymerase, capping would proceed at much slower rates in the presence of the *Vaccinia* virus capping enzyme. To allow for maximal capping with minimal degradation, an incubation of 4–6 hours was employed. There was >50 % recovery of all transcripts encoding the 1.94 kb *Xenopus* elongation factor 1α (pTRI-Xef) (52–65% recovery), the 1.77 kb luciferase (90–100%) and the 600 bp yeast Outer Membrane Protein (Omp) (87–100% recovery) starting with 176 μg of RNA.

Estimation of capping efficiencies of the Ant-m^{7}G RNA

The rationale for the estimation of capping efficiency was based on the susceptibility to phosphohydrolysis of the 5' terminal triphosphate groups of uncapped transcripts, and the protection conferred by the cap to the triphosphate linkage of capped transcripts, when subjected to alkaline phosphatase (AP) treatment. The capping efficiency was calculated by the percentage of capped transcripts in the total pool of RNA. Estimates of capping efficiency using the Alkaline Phosphatase/ Malachite Green Method (**Table 1**) show capping efficiencies of the RNA transcripts used in this study, and a comparison of identical uncapped Omp transcripts with rGTP and 3 variants of GTP used in this study. Capping estimates of 66–91% were reported using a variety of dinucleotide triphosphate cap analogs in *in vitro* transcription reactions.[9] This suggests that the capping enzyme serves as an efficient alternative to an *in vitro* transcription for obtaining high yields of capped RNA. Interestingly, we were unable to obtain any efficient capping with the Mant-GTP derivative. While there is only a small difference in structure, apparently this disrupts the enzyme interaction. Because we were able to obtain efficient capping with the Ant derivatives, we did not pursue Mant interactions further.

Fluorescence of Ant-RNA

The emission spectra upon excitation at 332 nm of the Ant-m^{7}Omp and of the buffer only prior to addition of the RNA, are shown in **Figure 2**. The spectral properties were similar to the properties of the cap analog Ant-m^{7}GTP[12] with an emission

Table 1. Capping efficiency for RNA transcripts

Type of RNA	Size	Substrate	Capping Efficiency
Yeast Outer Membrane Protein (OMP)	600 bp	rGTP	100%
Yeast Outer Membrane Protein (OMP)	600 bp	Ant-GTP	100%
Yeast Outer Membrane Protein (OMP)	600 bp	Ant-m^{7}GTP *	100%
Yeast Outer Membrane vProtem (OMP)	600 bp	Mant-GTP	Not Detected
Xeonpus elongation Factor 1α (pTRI-Xef)	1.94 kb	Ant-GTP	60-65%
Xeonpus elongation Factor 1α (pTRI-Xef)	1.94 kb	Mant-GTP	Not detected
Luciferase	1.77 kb	Ant-GTP	97-100%

*In the presence of SAM.

maximum of 423 nm. This is indicative that capping with Ant-GTP and Ant-m^{7}GTP has not altered the fluorescence properties of the anthraniloyl group.

In vitro *translation of Ant-m^{7}RNA*

Functional assays also suggest that the synthesized Ant-m^{7}GTP-capped RNA is effectively capped. *In vitro* translation reactions were performed with Ant-m^{7}GTP-capped RNA in wheat germ *in vitro* translation systems. Conditions were chosen using K^+ concentrations that have been previously shown[29] to maximize the difference in translation rates between uncapped and capped transcripts in this system. Weber *et al*, 1977 showed that uncapped transcripts are translated at very low efficiencies in wheat germ IVT systems using K^+ concentrations ranging from 90–100 mM, which are optimal for translation of capped transcripts. Lodish and Rose showed a similar difference in translation efficiency between uncapped and capped vesicular stomatitis virus transcripts.[30]

When assayed in the wheat germ system, there was a significant difference between uncapped transcripts and transcripts capped with Ant-m^{7}GTP/Ant-GTP. Three different sources of RNAs, pTRI-Xef, luciferase and Omp RNA, were used. The results of translation of the Omp RNA are shown in. **Figure 3** Clearly, Ant-m^{7}GTP-capped RNA is readily translated, and is a functional RNA. The translation efficiency of the RNA is shown over a range of RNA concentrations. There was a 7–10-fold higher translation of the Ant-m^{7}GTP-capped RNA as compared to the uncapped counterparts at all concentrations tested. The disparity in translation between capped and uncapped transcripts was substantiated with the longer transcripts, pTRI-Xef and luciferase, as well. In all reactions carried out at 500ng or more of RNA, between 2–16-fold higher rates of translation of anthraniloyl capped transcripts were observed, compared to the uncapped transcripts.

However, as shown in **Figure 3,** translation from Ant-m^{7}Omp RNA was less than that from the native m^{7}Omp RNA at high RNA concentrations. At 500 ng of starting RNA, native capped RNA was translated with 1.2–1.7-fold higher efficiency compared to the anthraniloyl capped counterparts. This reduced translation was only apparent at high RNA concentrations and varied with individual preparations within the range noted above. The reasons for the disparity between the Ant-m^{7}GTP-capped RNA and the native species of capped RNA may be due to a small amount of contamination with the unincorporated cap analog. When Ant m^{7}GTP was used as substrate as compared to Ant-GTP, translation was 1.2–1.4-fold higher at the highest RNA concentrations, depending on the particular preparation. This might be due to slightly less efficient methylation of the cap moiety (although not detected by TLC). Another explanation for the slightly reduced translation may depend on binding of the cap to the eIF4E protein. It has been shown that Ant-m^{7}GTP binds

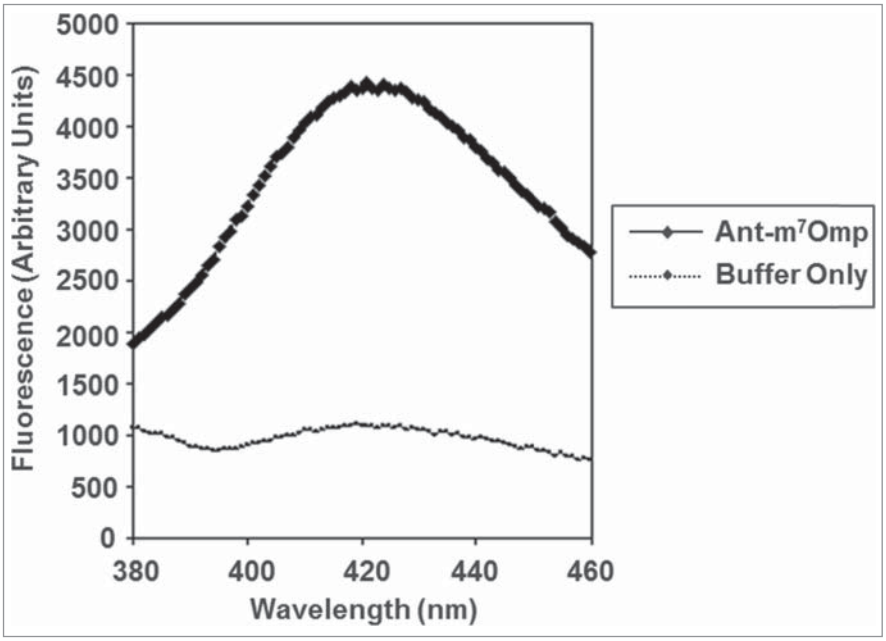

Figure 2. Translation efficiency of Omp transcripts is reported as protein content versus transcript quantity for IVT reactions. Four separate quantities: 0, 100, 250, 500 ng of Omp transcripts (Native (■), Ant-labeled (using Ant-m^{7}GTP) (▲), Ant-labeled (using Ant-GTP) (●) and Uncapped (◆)) were used for identical IVT reactions, as described in the methods section. The RNAs were translated for 150 minutes and the translated products from Omp transcripts were run on a 15% SDS-PAGE, following development in the phosphor-imager.

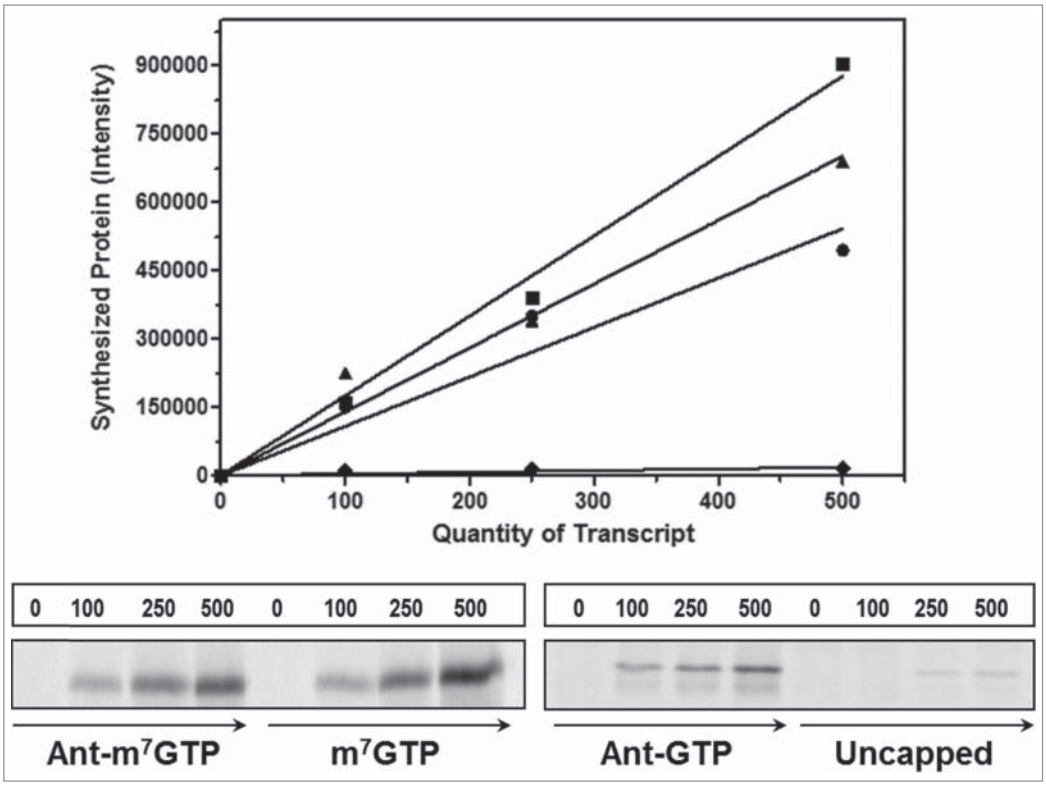

Figure 3. Fluorescence emission spectra of 100 mM Tris buffer, pH 8 prior to and after the addition of Ant-m^{7}Omp RNA upon excitation at 332 nm. The end concentration of Ant-m^{7}Omp is 178 nm.

eIF4F 1.4-fold tighter than the m^{7}GpppG cap analog.[12] This tighter binding could reduce translation by hindering scanning of the initiation complex, which requires release of the cap group. In the X-ray structure of the murine eIF4E protein, both the 2'- and 3'-hydroxyl groups are solvent exposed away from the binding site, suggesting that the presence of the anthraniloyl group will provide minimal steric hindrance to the cap binding events.[31]

Decapping of Ant-labeled RNA by the *Arabidopsis* DCP2 decapping enzyme

As a separate method for assessing the presence of the Ant-labeled cap at the 5' end, as well as for further proof for biological activity, the cap structure was hydrolyzed using a recombinant enzyme specific for the hydrolysis of the cap structure. Decapping enzyme (DCP2) hydrolyses the cap structure prior to the degradation of mRNA in the 5' → 3' direction.[32] The *Arabidopsis* decapping enzyme (AtDCP2) was expressed and purified in a bacterial expression system, and used for decapping of Ant-m^{7}Omp. The resultant decapped transcripts were analyzed in *in vitro* translation reactions, as described in the Materials and Methods section. The decapping reactions produced the expected decrease in *in vitro* translation. Translation of the decapped RNA was 23 +/− 12% of the capped transcripts. These experiments show that RNA capped with the Ant derivative was an effective substrate for the DCP2 decapping enzymes.

Discussion

The incorporation of fluorescent tags or labels to biological molecules plays an important role in the biophysical characterization of macromolecular interactions. The site-specific incorporation of fluorophores into nucleic acids, enzymes, antibodies and receptors has been extensively used to measure binding sensitivities of these macromolecules to DNA/RNA binding proteins, inhibitors, antigens and ligands. Such experiments require a fluorophore with several key features. (**1**) The fluorophore should possess unique excitation and emission wavelengths with minimal overlap with other moieties involved in the binding interactions. (**2**) The fluorescent group must be highly sensitive to small changes in the microenvironment induced by the binding of the interacting partners. (**3**) The fluorophore should not interfere with the biological role of the interacting macromolecule.

Anthraniloyl groups (**Fig. 1**) have been shown to be excellent fluorophores. First, the fluorescent properties of the anthraniloyl group, primarily the excitation and emission maxima of 320 nm and 410 nm, respectively, and a fluorescence lifetime of 8 ns, are distinct from the equivalent properties of aromatic amino acids and pyrimidine or purine of nucleic acids.[33] Second, the anthraniloyl group in Ant-m^{7}GTP can serve as an excellent cap analog, having been successfully used in the characterization of binding properties of wheat germ eIF4F isoforms[12] and other eIF4E mutant variants,[26] with a significant increase in fluorescence induced upon cap binding. Thirdly, in this publication we have shown that the presence of the anthraniloyl group at the cap structure does not inhibit or significantly diminish the translation capacity of Ant-m^{7}GTP-capped RNA and serves satisfactorily as a substrate for the capping and decapping enzymes.

Native post-transcriptional capping efficiencies have been reported in prior studies. One such study demonstrated that nearly 100% of 57 pmol of transcripts were capped in the presence of 25 μM [α-^{32}P]-GTP, 50 μM SAM, and 1.9 pmol of capping enzyme.[18] Further, the same study reported that during the same time period only 45% of the transcripts were capped in the absence of SAM. The high capping efficiency observed in this study is comparable to the reported maximal capping in the presence of unmodified GTP. The transfer of a guanylate moiety containing an anthraniloyl group at either the 2'- or 3'- position

of the ribose to uncapped RNA does not serve as a steric barrier for the capping mechanism. This is the first reported case of the synthesis of capped transcripts consisting of a cap structure with unique fluorescent properties amenable for biophysical cap-interaction translation studies.

A rather unexpected finding from this study involves the Ant-m^7GMP strong donor capacity of the Ant-m^7GTP substrate. This was surprising because it has been demonstrated that m^7GTP is unable to serve as a donor for the guanylylation. The extent of capping observed with Ant-m^7GTP demonstrates efficient transfer of Ant-m^7GMP. It is unlikely that there is contamination of Ant-GTP within Ant-m^7GTP, since the synthesis of the cap analog uses commercially available m^7GTP. It is further of interest that in our hands we were unable to obtain efficient capping with the only slightly different Mant-GTP analog.

The Ant-m^7GTP-capped RNA showed an emission maximum at 423 nm analogous to Ant-7-methyl-GTP upon excitation at 332 nm. Both, the 538 bp Ant-m^7Omp and the 1.77 kb Ant-m^7Luciferase, gave similar emission spectra although the emission maximum of Ant-m^7Luciferase was slightly lower at 413 nm. Therefore, capping did not quench the fluorescence of the anthraniloyl group in any of the RNA species tested.

Prior studies have demonstrated that naturally-capped transcripts such as reovirus RNA, VSV RNA and globin RNA are translated at much higher efficiencies compared to their uncapped counterparts.[34] The data obtained in this study, demonstrating the several fold increase in *in vitro* translation, is consistent with the previous comparisons of uncapped and capped RNA. The reactions employed in this study in a wheat germ IVT system that is primarily dependent on the presence of the cap structure, using optimal concentrations of K^+ for maximal disparity between capped and uncapped transcripts, not only confirmed the presence of the cap at the 5' extremity but, also, showed that Ant-m^7GTP-capped RNA is a biologically active fluorescent RNA species. These fluorescent RNAs will be of use for future biophysical studies, and will aid in increasing our understanding of mRNA translation and degradation.

Materials and Methods

Synthesis of Ant-guanosine derivatives

Ant-GTP and Ant-m^7GTP were synthesized as described in Ren and Goss.[12] Briefly, a 10 mg sample of m^7GTP or GTP was dissolved in 0.5 ml water and the pH adjusted to 9.5 with NaOH. To this solution, isatoic anhydride (5 mg) was added with stirring, and the pH maintained at 9.6 with NaOH. The reaction was allowed to proceed for 3 hours at 37°C. The pH was adjusted to 7.0 with HCl. The resulting Ant-derivative was purified using Sephadex G-25 column chromatography and fractions analyzed by TLC, as described.[12] Samples were freeze-dried and stored at −80°C.

Synthesis of RNA transcripts

All transcripts were synthesized by *in vitro* transcription using the SP6 polymerase. *Xenopus* elongation factor 1α (pTRI-Xef) linearized template, used for the synthesis of pTRI-Xef RNA, was supplied with the SP6 *in vitro* transcription kits (Ambion®, USA). The pSP-*luc*+NF fusion vector for the transcription of a luciferase transcript was obtained from Promega, Omp (outer membrane protein) gene (*S. cerevisiae* YBR230C open reading frame) cloned into the pSP73 vector, for the SP6 driven transcription, was donated by Dr. Lena Burri (personal communication).

Reactions to transcribe uncapped transcripts contained 0.1 – 1 μg of linearized template and 5 mM concentration of each NTP, as suggested in the Megascript *in vitro* transcription kit (Ambion®, USA). All reactions were carried out for 2–4 h, in a buffer supplied by the manufacturer, in the presence of varying quantities of the SP6 polymerase mix. Luciferase transcripts were also synthesized with the Ribomax™ large scale RNA production system – SP6 (Promega, Co., USA), as instructed by the manufacturer, using linearized pSP-*luc*+NF.

Capping reactions

The capping reaction mixture contained 50 mM Tris-HCl, pH 7.9, 6 mM KCl, 2.5 mM DTT, 1.25 mM $MgCl_2$, 0.1 mg/ml BSA, 40 U RNaseOUT ribonuclease inhibitor, 0.5 mM SAM, up to 176 μg of uncapped RNA, 90–150 μM of the Ant-GTP analog, and 10 U of *Vaccinia* capping enzyme (Ambion®, USA) in a final volume of 50–60 μl. The reactions were incubated at 37 °C for 4–6 hours, and the total RNA was precipitated with 3.25 M lithium chloride. The precipitated RNA was resuspended in 200 μl of RNase-free water prior to use.

Measurement of RNA quantity

The quantity of total RNA was calculated from the A_{260} of 200-fold diluted aliquots of all RNA species in UV cuvettes (Eppendorf, Int.) using a Biophotometer (Eppendorf, Int.). The concentrations were calculated under assumption that 1.0 A_{260} of ssRNA corresponded to 40 μg/ml of RNA.

Determination of capping efficiency

The capping efficiency was determined using the Malachite Green phosphatase assay, as described in Harder *et al.*[35,36] Both, capped and uncapped RNAs were treated with calf alkaline phosphatase (Promega, Co., USA), and incubated at 37 °C for 1 hour to hydrolyze the exposed phosphate groups prior to the assay with the Malachite Green reagent. The capping efficiency was reported from the percentage of capped transcripts in a total pool of RNA species that has undergone capping, assuming that alkaline phosphatase treatment released all 3 5' terminal phosphate groups on the uncapped RNA.

In vitro *translation*

The *in vitro* translation reactions were assembled according to the manufacturer's instructions supplied with the Wheat Germ Extract (Promega, Co, USA). The 20 μl reaction consisted of wheat germ extract, 100 mM KAc, 1 mM amino acid mixture (minus methionine), 40 U RNaseOUT ribonuclease inhibitor (Invitrogen, Co.), [^{35}S]-methionine (1,175 C_i/mmol) and 0–560 ng of an *in vitro* transcribed RNA. The reactions were

incubated for 2.5 hours at 25 °C, and the protein products were analyzed by 16% Tris-tricine (and/or 15% Tris-glycine) PAGE. The gels were dried, visualized by phosphor-imaging, and the intensities were measured using Imagescan 5.2 software.

Decapping reactions

The At5g13570 gene from *Arabidopsis*, which encodes a homolog of the human DCP2 decapping enzyme as characterized by Piccirillo *et al*,[6] expressed as a recombinant protein in an *E. coli* expression system, was used in decapping assays. Decapping reactions were run overnight on 15.4 μg of Ant-labeled RNA with 8 μg of the AtDCP2 decapping protein in 50 mM Tris, pH 8 containing 5 mM $MnCl_2$ (metal ion cofactor) in a final volume of 120 μl. The RNA from the reactions was precipitated with 3.25 M lithium chloride, and dissolved in 50 μl of dH_20. The effect of decapping on translation was assayed in standard IVT reactions with 900 ng of Ant-m^7Omp RNA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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