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:

$$\text{ppp5}'\text{-r(n)-3}' \rightarrow \text{pp5}'\text{-r(n)-3}' + \text{P1 (Triphosphatase)}$$

$$\text{pp5}'\text{-r(n)-3} + \text{GTP} \rightarrow \text{Gppp5}'\text{-r(n)-3}' + \text{PP1 (Guanylyltransferase)}$$

$$\text{Gppp5}'\text{-r(n)-3}' + \text{AdoMet} \rightarrow \text{7Methyl-Gppp5}'\text{-r(n)-3}' \text{ (Methyltransferase)}$$

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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