1 Enzymatic synthesis of new cap structures

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1.1 SUMMARY

The advent of mRNA-based vaccines has raised interest in synthesis and analysis of messenger RNA and its components including non-canonical structures. We will explore the new synthesis routes for cap structures via enzymatic capping, or incorporation of cap-structured dinucleotides. The expertise in enzymatic synthesis of nucleotide di- and-triphosphates in the Lukavsky lab will be applied to naturally occurring as well as fully synthetic purine nucleoside analogs. After enzymatic synthesis, the resulting di- and triphosphates will be tested for the propensity to be incorporated into mRNA cap structures in Mainz. Stable isotope labeled isomers of these compounds, also obtained by this route, are invaluable internal standards for LC-MS analytics. They will allow to analyze thus generated, biosynthetically capped IVT-mRNAs.

1.2 SCIENTIFIC BACKGROUND

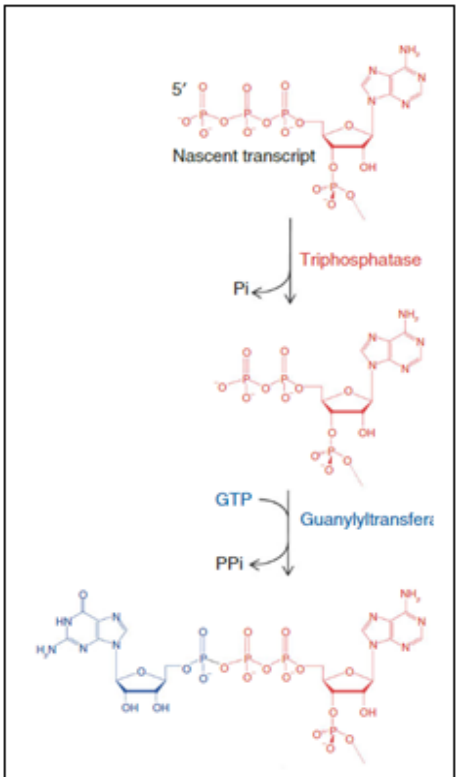

Figure 1: The first two enzymatic steps of typical enzymatic cap synthesis taken from ref.[2]

mRNA cap structures are currently receiving immense attention. In bacteria, new cap structures have been discovered that were thought non-existent. In eukaryotes, more and more enzymes involved in the synthesis of cap structures are being discovered and their role in translation initiation and innate immunity is being characterized. Technologies for analytics of cap structures, in particular by LC-MS, have made significant progress, and thus, the content of mRNA populations in terms of different cap structures, as well as their dynamics under different stress conditions are now accessible to analysis[1]. Most recently, synthetic mRNA in its capacity of antiviral vaccine has gained immense public attention. Here, too, translation efficiency and immunogenicity are key aspects to be investigated, which hold significant stakes in public health3. Two initial enzymatic reactions are required for synthesis of an unmethlyated cap, catalyzed sequentially by the enzymatic activities of RNA triphosphatase and, RNA guanylyl-transferase (Fig. 1). Frequently, both activities are contained in single viral proteins, occasionally even featuring the additional guanine-N7 methyltransferase to complete a so-called “cap 0” structure2. Significantly, certain recombinant viral guanylyl-transferases were shown to accept mononucleotide-diphosphates as substrates 4,5 thereby providing a perspective for the use (or testing) of non-canonical or isotope labeled

diphosphate nucleotides and triphosphate guanosines for the synthesis of a variety of dinucleotide cap structures, as shown in Figure 2.

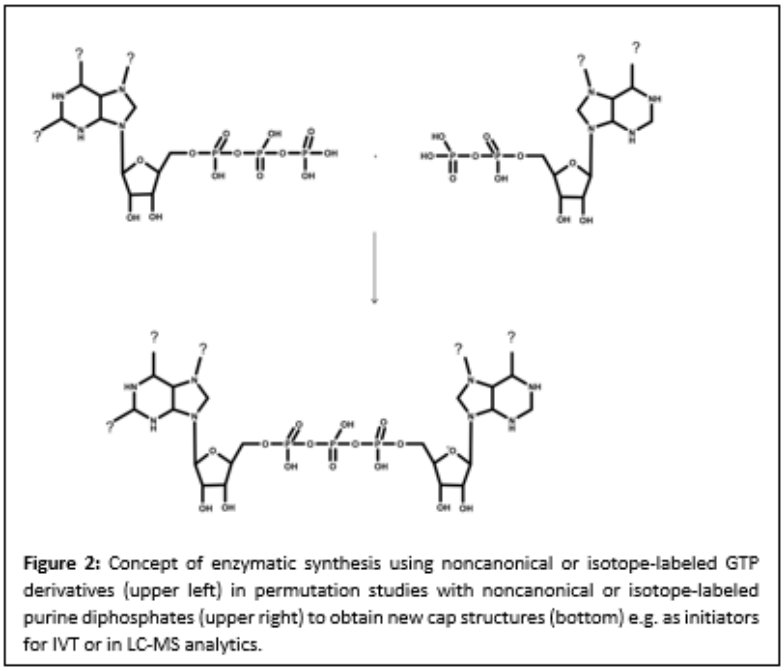

Figure 2: Concept of enzymatic synthesis using noncanonical or isotope-labeled GTP derivatives (upper left) in permutation studies with noncanonical or isotope-labeled purine diphosphates (upper right) to obtain new cap structures (bottom) e.g. as initiators for IVT or in LC-MS analytics.

1.3 AIMS

This project pursues two major aims, both of which capitalize on the preparative application of nucleotide kinases established in the Lukavsky lab for the synthesis of di- and trinucleotides.

Aim 1: Preparative enzymatic synthesis of stable isotope-labeled naturally occurring cap structures.

Stable isotope labelled naturally occurring cap structures are indispensable for accurate quantification of cap structure populations. So far, synthesis requires significant effort in synthetic organic chemistry[1] which is not available to a large section of the RNA community. Our enzymatic *in vitro* approach will make techniques and material generally available, and provide a new access route to hitherto untested structures.

Aim 2: Enzymatic synthesis of new cap-analogues for initiation of transcription by T7 RNA polymerase.

Transcription initiation by T7 RNA polymerase using dinucleotide initiators (e.g. ARCA and related) offers a range of possibilities for the incorporation of noncanonical structures into an mRNA. We will synthesize such structures starting from diphosphates, which can be capped with GTP using different recombinant viral capping enzymes[4,5]. In so doing, we will explore which purine derivatives (e.g. m7G, xanthosine, m2G) are accepted by the various enzymes available.

1.4 WORK PROGRAMME

Make recombinant viral capping enzymes

Viral capping enzymes will be cloned into appropriate expression vectors (e.g. pET28) for expression in E. coli in the Lukavsky lab. For affinity purification and optimal expression yield, we will fuse the viral enzymes with a N-terminal His_6-Strep-His_6-tag, His_6-tag or His_6-lipo-tag

followed by a TEV cleavage site for tag removal using appropriate restriction sites (e.g. NdeI/NheI and XhoI)[6]. Standard nickel affinity chromatography, followed by ion-exchange and size-exclusion chromatography will be employed to obtain pure, active proteins for further experiments.

Synthesize monophosphate-guanosine with substituents at the 7 position for conversion into GDP/GTP.

Stable isotope-labelled GMP will be prepared in the Lukavsky lab. Labelling schemes such as ^{13}C-, ^{15}N-labelling or perdeuteration can be obtained from E. coli cultures grown in minimum medium while deuteration of the 8 position can be achieved in vitro[7]. All these techniques are established in the Lukavsky lab. The resulting, differently isotope-labelled GMP variants will be further processed in the Helm lab, including e.g. alkylation at the N7[8]. The obtained monophosphates with substituents in the 7 position will be enzymatically converted into di/triphosphates in the Lukavsky lab[9,10] to finally incorporate these noncanonical structures into dinucleotides IVT initiators, or directly into mRNAs.

1.4.1 Benefit to RNA research at CEITEC and prospect of future collaborations and joint funding

The proposed research will be conducted in Mainz and at CEITEC. The team members of the Lukavsky lab will benefit from an international perspective and exchange of expertise and knowledge with the partners in Mainz. In preparing the capped mRNAs and testing the translation efficiency and immunogenicity of the novel mRNAs in the Helm lab, they will bring additional knowledge to CEITEC which will benefit their own research in the field of RNA biology. Additionally, the members of the Lukavsky lab will use LC-MS analytics, a valuable tool for analysis of RNA modifications. With the combined preliminary results obtained, we expect to be in a good position to apply for international funding to further pursue the project on a larger scale.

1.5 REFERENCES

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1.6 BUDGET

The Helm lab requests funding for consumables including chemical synthesis reagents (solvents, column material, lab glass), RNA purification reagents (gel electrophoresis, IVT consumables) and LC-MS related (enzymes, solvents, buffers, filters, dedicated column). The Lukavsky lab requests funding for consumables for protein purification (culture media, buffer solutions, concentrators, etc.), enzymes for charging reactions of GMP, travel and daily allowance for secondments of students in Mainz. The budget will be split evenly by both groups.