Exploring the Messenger RNA Capping Code: CleanCap Co-transcriptional Capping Allows Synthesis of Cap 0, Cap 1, and m6A-m Capped RNAs

Dongwon Shin, Kristen A. Azizan, Jordana M. Henderson, Alexandre Lebedev, Richard I. A. Hogrefe, Michael Houston, and Anton P. McCaffrey

Trilink Biotechnologies
San Diego, CA 92121

Abstract

Messenger RNA (mRNA) therapy is an increasingly popular platform technology for expressing proteins in cells or in vivo; since there is minimal risk of insertional mutagenesis, mRNA translation is being utilized to express proteins for genome editing (Cas9, ZFNs and TALENs), protein replacement, vaccines and antibody expression. Sequences mRNAs can be recognized as foreign by the innate immune system and one approach to avoiding innate immune responses is to mimic the structure of endogenous mRNA.

The importance of mRNAs in the T cell stimulation. During capping, Cap 0 (Gppp) is formed as an intermediate. Metaphase of the 2' position of the first capping nucleotide forms Cap 0. Cap 0 is formed by cap modification, frequently found in conjunction with all eukaryotic transcripts. 15-20% of transcription, the 2' position of the second nucleotide maintains its methylation to form Cap 1 (GmGppp). Another frequently found cap modification found in conjunction with Cap 1 is methyltransferase. Cap 1 is important for self-non-self recognition by the innate immune system. Cap 1 is recognized as foreign and ITIF recognizes non-methylated caps. Cap 1 methyltransferase is not required for pattern recognition receptors. Role of Cap 1 is largely unknown because it was not possible to easily generate Cap 1 mRNA in vitro.

CleanCap allows novel cap forms that were not previously accessible such as Cap 2. Cap 2 was found to be a highly efficient reaction without additional purification. Studies in THP-1 Dual monocyte cell (90-99% capping), less expensive than enzymatic capping and is carried out in a “one step” reaction. Co-transcriptional co-capping with CleanCap enhances the efficiency of cap formation, but with cap formation, Cap 1 was found to be inefficient, while Cap 2 was found to be highly efficient. 2.7E+007 Cap 1 CleanCap

Figure 1: Cap 0, Cap 1 and Cap 2 Structures of 5'Ends of mRNAs

Eukaryotic mRNA has a Cap 0 or Cap 2 structure. Eukaryotic mRNA is found in endogenous sequences where it is a cap structure. ITIF: unmethylated cap structures. Recognition of Cap 0 structures is highly efficient, but Cap 1 structures are not.

Figure 2: Function of mRNA Cap Structures

mRNA cap structures are involved in modulating: - Nuclear export - Splicing - Translation - Immunoinhibition - Transcriptional capping

Figure 3: N6-methyladenosine Methylated Caps. Regulate Translation and mRNA Stability

Reversible methylation of α-methyl groups on the 5' cap controls mRNA stability.

Figure 4: mRNA Capping Assay: Enables Quantitation of Multiple Enzymatic Steps

GpppA: transfer reaction - m7Gm: m7G methylation - Cap 1 methylation - Phosphatase step

Figure 5: Anti-Reverse Cap Analog (ARCA) Capping is Inefficient

Co-transcriptional capping with CleanCap™ (Cap 1) helps evade an immune response.

Figure 6: CleanCap Cap Analog Expand the Range of 5' Sequences That Can be Used to Initiate T7 RNA Polymerase

Figure 7: Comparison of Capping Methods

Figure 8: Cap 1 Capping Assay

Cap 0 CleanCap
Cap 1 CleanCap
Cap 2 CleanCap

Figure 9: Cap 1 m6A Capping Assay

Figure 10: Protein Expression for Cap 0, Cap 1 and m6Am Cap 1 Wildtype HPLC Purified Luciferase mRNAs in Mice Polyacrylamide Gel

Figure 11: In Vitro De-Capping with Dcp2 is Decreased with m6A-Capped RNAs. Identity of First and Second Cap-proximal Influence De-capping

Figure 12: Nudt12 and DXO Selectively de-Cap Cap 0 but Not Cap 1 or 2 Cap RNAs

Nudt12
DXO

Contact
Anton McCaffrey
amccaffrey@trilinkbiotech.com

The Modified Nucleic Acid Expert
www.trilinkbiotech.com

Background: Why mRNA Therapeutics?

mRNA is a popular new tool for gene expression - Does not have a risk of insertional mutagenesis - Can correct difficult cells such as non-dividing cells - Can transduce

Applications

Gene editing (Thompson’s, Cam, ZFN, TALENs and CRISPR/Cas9) - Gene replacement - Vaccines

Limitations

Invasive immune response to unmodified mRNA

Solutions

Proper capping - Chemical modification of mRNA can prevent innate immune stimulation - Removal of m6A

Innate immune sensors recognize mRNA

Transfection of cells with unmodified RNAs can lead to cell death due to activation of innate immune pathways

Tox-like receptors 3, 7, 8 and 9 recognize different RNA forms - Found in endosomes where some viruses enter cells

Cytosolic sensors

Protein kinase R (PKR): dsRNA - NOD: long RNA - ITIF: unmethylated cap structures - RIP: 5’-3’ phosphate

Figure 1: Cap 0, Cap 1 and Cap 2 Structures of 5’-Ends of mRNAs

Eukaryotic mRNA have a Cap 0 or Cap 2 structure. Sequencing of primer capping structures is thought to be involved in self/non-self RNA recognition. Cap structure influences activation of PRKRs. RG3 is activated by Cap 0 mRNA, but not Cap 1 mRNA (PRKAR). ITIF: unmethylated cap structures. ITIF: unmethylated cap structures.

Cap structure influences activation of PRKRs. RG3-1 is activated by Cap 0 mRNA, but not Cap 1 mRNA (PRKAR). ITIF: unmethylated cap structures. ITIF: unmethylated cap structures.

Cap structure influences activation of PRKRs. RG3-1 is activated by Cap 0 mRNA, but not Cap 1 mRNA (PRKAR). ITIF: unmethylated cap structures. ITIF: unmethylated cap structures.

Cap structure influences activation of PRKRs. RG3-1 is activated by Cap 0 mRNA, but not Cap 1 mRNA (PRKAR). ITIF: unmethylated cap structures. ITIF: unmethylated cap structures.