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Locked Nucleic Acid (LNA)-Modified Dinucleotide mRNA Cap Analogue: Synthesis, Enzymatic Incorporation, and Utilization

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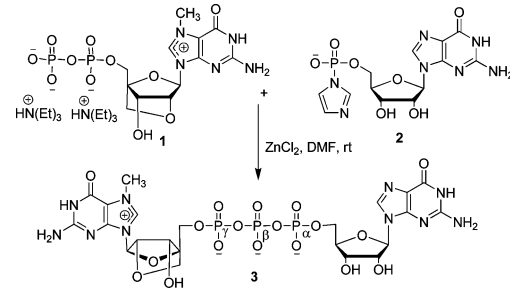
The 5′-end of most of the nuclear-encoded mRNAs in eukaryotes contains a cap structure m^{7}G[5′]ppp[5′]N- (where N is the initiating nucleotide of the encoded sequence), in which a 7-methylguanosine residue is linked to the 5′-end of the transcribed RNA via a 5′−5′ triphosphate bridge.[1,2] In particular, the presence of a cap structure in mRNA is specifically recognized by the protein eukaryotic initiation factor eIF4E, vaccinia virus methyltransferase VP39, and the CBC80/20 nuclear cap-binding complex. The cap plays an important role in many aspects of mRNA metabolism, including mRNA processing, localization, nuclear transport, translation, and the protection of mRNA from premature degradation.[3] The use of RNA as a vehicle for antigen delivery to dendritic cells (DCs) has been the subject of intense interest in view of creating a novel immunotherapeutic approach. Currently, several biotechnology and therapeutic development laboratories utilize 5′-capped mRNA which is transfected into dendritic cells (DCs), where the encoded antigen is expressed allowing endogenous production of the immunogenic protein which is targeted against specific infectious diseases.[4] The disadvantage of using the standard cap, m^{7}G[5′]ppp[5′]G for the *in vitro* synthesis of capped mRNA, involves the incorporation in both the forward (m^{7}G[5′]ppp[5′]G(pN)n) and reverse orientation (G[5′]pppm^{7}G(pN)n) in approximately equal proportions, with the reverse product not properly recognized during translation.[5] This problem has been addressed by the use of antireverse cap analogues (ARCAs) such as 3′-OH[6] and 2′-OH[7] modifications on the m^{7}G moiety in which the cap incorporates exclusively in the forward orientation.

The potential application of locked nucleic acid (LNA) technology[8] in various areas of research such as antisense and antigene strategies, diagnostics, and genotyping and also our continued interest in the design of new cap analogues for various biological applications[9] prompted us to explore the possibility of using LNA in the modification of mRNA cap. Here, we disclose the first example of the synthesis of LNA-modified dinucleotide cap analogue m$^{7(LNA)}$G[5′]ppp[5′]G **3** and its biological evaluation. Also, the molecular modeling interaction of the LNA-modified cap with eIF4E protein is reported.

The required intermediate, m$^{7(LNA)}$GDP **1**, to make the desired **3** for biological evaluation was prepared in four steps from LNA guanosine that involved 5′ monophosphorylation, imidazolide formation, diphosphorylation, and regioselective N^7 methylation reaction.[9] The coupling reaction of m$^{7(LNA)}$GDP **1** with Im-GMP **2** in the presence of $ZnCl_2$ as the catalyst furnished m$^{7(LNA)}$-G[5′]ppp[5′]G **3** in 65% yield (Scheme 1).

The capping efficiency of the newly prepared analogue **3** and standard cap analogue (m^{7}G[5′]ppp[5′]G) **4** was tested in an *in vitro* transcription system by using T7 RNA polymerase on a pTri β Actin template. The capping assay was designed in such a way that during transcription only ATP and GTP were used out of the four NTPs and the resulting transcript was only six nucleotides in

Scheme 1. Synthesis of m$^{7(LNA)}$G[5′]ppp[5′]G **3**



length. All reactions were performed in the presence of [α-^{32}P] ATP to internally label the transcript. The resulting gel shift assay indicates that **4** has a capping efficiency of 59%, while **3** has a 54% capping efficiency (Figure 1A). The capping assay experiment

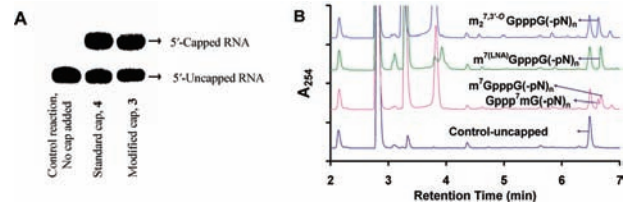


Figure 1. (A) 20% dPAGE gel showing capping efficiency of LNA-modified cap **3** and standard cap **4**. (B) Ion exchange HPLC analysis of forward and reverse orientation of 5′-capped RNA by using standard cap **4**, LNA-modified cap **3**, and ARCA.

reveals that the LNA-modified cap analogue **3** is a substrate for T7 RNA polymerase.

To determine the incorporation orientation of **3**, RNAs were synthesized similar to the capping assay experiment except the addition of radioactive material and the crude reaction mixtures were analyzed by HPLC (Figure 2B). In the case of standard cap

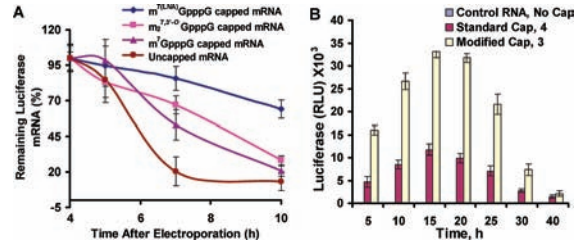


Figure 2. (A) Intracellular stability assay for 5′-capped mRNA poly(A). (B) Translational luciferase data of mRNA poly(A) capped with **3** and **4**.

4, it shows two peaks at 6.63 and 6.68 min indicating the presence of both reverse and forward orientation products. Unlike **4**, the

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LNA-modified cap **3** shows a sharp single peak at 6.67 min showing the presence of an exclusive single orientation product. The results of an identical study with known substrate ARCA $m_2^{7,3'-O}$-G[5′]ppp[5′]G gives strong support for the exclusive single orientation product observed for **3**. Under similar conditions, ARCA shows a single peak at 6.64 min showing the presence of a single product, i.e., a forward orientation product based on the literature data.[6a]

We next measured the effect of LNA-modified cap **3** on the mRNA stability in cultured cells, compared to mRNAs bearing standard cap **4**, $m_2^{7,3'O}$G[5′]ppp[5′]G and an uncapped control of 1.85 kb mRNA poly(A) (see Supporting Information for detailed procedure). The amount of intact intracellular luciferase mRNA poly(A) was measured by real-time PCR using TaqMan assays (Figure 2A). Luciferase mRNA poly(A) with **3** was found to be ∼1.61- and ∼1.28-fold more stable compared to mRNA poly(A) 5′-capped with **4** and $m_2^{7,3'-O}$G[5′]ppp[5′]G cap, respectively, and ∼4.23-fold more stable compared to uncapped control mRNA.

To determine the translational efficiency, the luciferase mRNA poly(A) product generated from the transcription reaction of **3** or **4** was transfected into HeLa cells. Cells were harvested and lysed at different intervals of post-transfection, and the lucirease activity was measured. It is clear from Figure 2B that the RNA capped with LNA-modified cap **3** is translated more efficiently with ∼3.2-fold more activity than the standard cap **4**.

It has been shown that eIF4E is an attractive target for anticancer directed therapy.[10] The ability of a cap analogue as potential inhibitors to target eIF4E means that elevated eIF4E levels in tumor cells might be counteracted by a cap analogue. To understand the specific interaction of LNA-modified cap **3** or standard cap **4** with eIF4E, a molecular modeling study was conducted. Both minimized structures were in line with the previous literature that the phosphate groups of the cap forms a "molecular anchor" by interacting with the amino acids of the eIF4E protein.[11] However, it is to be noted that four amino acids (Lys159, Lys162, Arg157, and Arg112) form H-bonds with the triphosphate bridge of the LNA-modified cap **3**, whereas only three amino acids (Lys 159, Lys162, and Arg157) form H-bonds with standard cap **4** (Figure 3). It is likely that a big

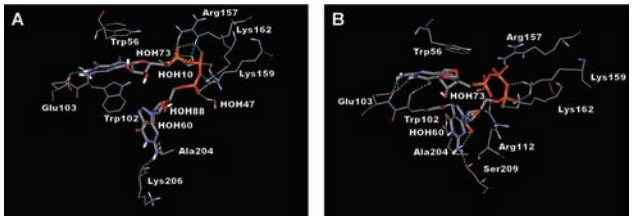

Figure 3. (A) Selected region of eIF4E-standard cap **4**-4E-BP1 peptide complex and (B) eIF4E-LNA modified cap **3**-4E-BP1 peptide. Hydrogen bonds are shown in dotted lines.

conformational change in **3** due to locking the conformation in a C3′-endo pucker forces one of the oxygens of the γ- phosphate group to form an additional H-bond to Arg112.[12] It is noteworthy that the interaction energy difference between these two complexes shows that **3** interacts more efficiently by 47.28 kcal/mol compared to **4** with the eIF4E protein.

In addition to the inherent interest of the unique structural features of LNA that results in increased thermal stability, nuclease resistant, and hybridization specificity,[8] several interesting features merit comment from the present study. First, it appears from both the higher translational data of **3** and HPLC data (Figure 1B) that the LNA-modified cap **3** incorporates exclusively in the forward orientation. Second, the LNA-modified cap analogue **3** (∼3.2-fold) outweighs the standard cap analogue (∼1-fold) and $m_2^{7,3'-O}$-G[5′]ppp[5′]G (∼2.2-fold)[9b] in terms of a translational template. The higher translational data suggest that LNA-modified cap **3** is a potential substrate for mRNA transfection applications such as anticancer immunization, protein production, and gene therapy.[13] Third, the increased intracellular stability data suggest that LNA-modified cap **3** is likely able to produce an immunogenic protein for a longer time span than the other types of templates discussed. All these indicate that this new cap is likely to make a dynamic positive impact on the designing of personalized medicines. Fourth, it seems that the conformational preference of C3′-endo (N-type)[12] in the LNA structure is advantageous for the higher translational and intracellular stability observed for the LNA-modified cap **3**.

In conclusion, we have reported the new antireverse cap analogue **3** and its biological evaluation compared to the standard cap analogue **4**. The remarkable translational properties of new analogue **3** over standard analogue **4** is probably due to the formation of exclusively forward-capped mRNA and the increased cellular stability of the 5′-modified capped mRNA poly(A). The molecular modeling study strongly indicates that the new cap **3** is compatible with the translation initiation factor elF4E and therefore useful for *in vitro* translation experiments as well as *in vivo* studies. Based on the above results, the new LNA-modified cap analogue is likely to be a potential candidate for making transfectable RNAs for antigen delivery to DCs for the purpose of immunotherapy against cancer and infectious diseases. Current biotechnology efforts for *in vitro*, *in cyto*, and potentially *in vivo* protein production will also benefit from these characteristics.

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Supporting Information Available: General experimental procedures, spectral data and ^{1}H NMR spectra for selected compounds, biological assay, and molecular modeling study. This material is available free of charge via the Internet at http://pubs.acs.org.

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