EPH - International Journal of Medical and Health Science
ISSN (Online): 2456-6063
Volume 03 Issue 03 September 2017

DOI: https://doi.org/10.53555/eijmhs.v4i2.36

AN eIF4G-RECRUITING APTAMER INCREASES THE FUNCTIONALITY OF *IN VITRO* TRANSCRIBED mRNA

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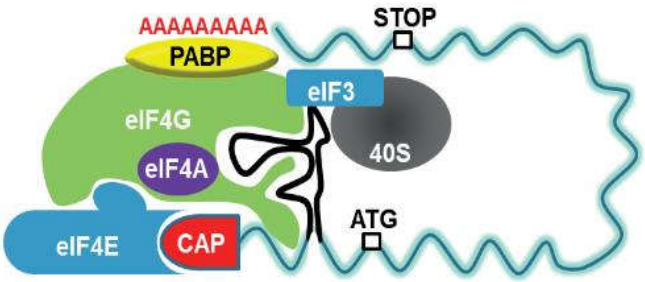


An aptamer (back line) that binds eIF4G is introduced at the 5'end of a mRNA (blue line) in order to help recruiting the subunits of the eukaryotic initiation protein complex.EPH - International Journal of Medical and Health ScienceISSN: 2456 - 6063Volume-4 | Issue-6 | June,201829

Abstract:

Background: *As a versatile and safe vector, in vitro transcribed messenger RNA (ivt mRNA) is currently being intensively evaluated as an active pharmaceutical ingredient. Its therapeutic uses encompass vaccination, cell reprogramming, genome engineering, gene complementation and the expression of protein drugs (e.g., growth factors or antibodies).*
Objectives: *The therapeutic efficacy of ivt mRNA correlates with the efficacy of its translation. Untranslated regions (UTRs) from stable mRNA, such as globin mRNA, and optimized 5' cap structures have been used to improve the functionality of ivt mRNA. However, the recruitment of the eukaryotic initiation factor 4E (eIF4E) protein to the 5' end of transfected ivt mRNA remains a ratelimiting parameter for translation.*
Method: *We added aptamer sequences that bind the eiF4G protein to the 5' UTR of ivt mRNA.*
Results: *One of the tested aptamer sequences produced a several fold increase in ivt mRNA expression (specifically, an increase of threefold to over tenfold depending on mRNA sequence and cell type).*
Conclusion: *This simple modification of the 5' UTR of ivt mRNA may represent an efficacious and general method for improving the therapeutic index of all new mRNA-based therapeutic products.*

Keywords: *mRNA, eIF4G, aptamer, Pseudouridine, 5 methyl cytosine, dendritic cells*

INTRODUCTION

The expression of recombinant genes in vivo can be used for vaccination and gene therapy. Although plasmid DNA and recombinant viruses were initially used for this purpose, in vitro transcribed messenger RNA (ivt mRNA) emerged as a safe alternative in the 1990s and has become a broadly accepted method that has attracted increasing interest during the last ten years 1. With the implementation of the first good manufacturing practice (GMP) and large scale mRNA production facility 2-4, several human clinical studies demonstrating the safety, versatility and efficacy of directly injected ivt mRNA have been published 5-11. Key features of mRNA-based therapies are (i) the intrinsic translatability of the ivt mRNA (the level of protein expression),(ii) the ivt mRNA's immunogenicity (whether innate immune receptors are triggered) and (iii) formulation (site of delivery and expression). With respect to the former consideration, the optimization of the cap structure, untranslated regions (UTRs), open reading frames and poly (A) tail has been reported 1. Due to such optimization, ivt mRNA has become a vector with high expression of the encoded protein. One limiting factor in cellular mRNA translation is eukaryotic initiation factor 4E (eIF4E), which recognizes the 5' cap structure of mRNA 12. EIF4E then recruits the scaffolding protein eIF4G, which, in turn, recruits all other components of the initiation complex to form the eIF4F complex that moves along the mRNA to the AUG start codon. We rationalized that direct recruitment of eIF4G to the 5' UTR could improve the efficacy of translation of ivt mRNA. Aptamers that bind eIF4G have been identified in attempts to block translation in tumor cells 13. Miyakawa et al. demonstrated that three out of eight such aptamers indeed inhibited translation. We took advantage of the aptamers that did not block translation; in particular, we utilized these aptamers as bait to bring eIF4G to the 5' end of ivt mRNA. One of the non-blocking aptamers was most efficient at enhancing the translation of a downstream open reading frame. The addition of this aptamer to ivt mRNA could be proposed as a universal method for improving ivt mRNA-based therapies.

Materials and Methods

Production of ivt mRNA

Plasmids containing a wild-type firefly luciferase-coding gene or an optimized firefly luciferase-coding gene (a synthetic gene purchased from Blue Heron Bio with a 5' UTR of human alpha globin, a codon-optimized open reading frame and a double 3' UTR from human beta globin) were used as matrices for PCR amplification with upstream primers that contained a T7 promoter followed by a sequence complementary to the 5' end of the targeted gene, with or without an aptamer sequence between the promoter and the complementary sequence. Similarly, a synthetic codon-optimized gene encoding ZsGreen (a synthetic gene purchased from Blue Heron Bio with a 5' UTR of human alpha globin, a codon-optimized open reading frame and a double 3' UTR from human beta globin) was amplified using PCR. The primer sequences are presented in supplementary Figure 1. The PCR products were analyzed on an agarose gel and purified using a PCR cleanup kit from Qiagen in accordance with the manufacturer's instructions. mRNA was produced by utilizing a HiScribe™ T7 ARCA mRNA Kit (with tailing) (New England Biolabs) to obtain ARCA-capped poly-adenylated mRNA or using GenScript capping and polyadenylation kits on mRNA produced using a T7 RNA polymerase (New England Biolabs) reaction containing all 4 canonical

Results and Discussion

Miyakawa et al. reported the sequences of eight aptamers that could bind to eIF4G 13. Five of these molecules (aptamers 2, 14, 17, 18, and 19) did not inhibit translation. We tested the shortest four of these five molecules (aptamers 2, 14, 17, and 19), which were 41 bases or less in length; aptamer 18, which is 70 residues long, was not tested. Oligonucleotides with a T7 promoter sequence followed by the aptamer sequence and a 20-base sequence that recognizes the wild-type luciferase sequence at its start codon (see supplementary Figure 1 for the primer sequences) were used to generate DNA templates for in vitro transcription. Uncapped, enzymatically capped and ARCA-capped mRNAs were produced using the aforementioned PCR matrices. MRNAs were transfected into human tumor cells (HEK cells) using Lipofectamine 2000, and luciferase activity was recorded. As presented in Figure 1A, uncapped mRNAs, even those with aptamer sequences at their 5' end, were not translated. However, detectable luciferase activity was observed for mRNAs that were enzymatically (Figure 1B) or cotranscriptionally (Figure 1C) capped. Remarkably, mRNAs with the aptamer 17 sequence consistently exhibited several fold increases in luciferase activity compared with mRNAs without a 5' aptamer sequence and mRNAs with the aptamer 2 sequence (aptamers 14 and 19 moderately enhanced translation in certain experiments). Similar results were obtained when mRNAs were transfected into mouse tumor cells (CT26 cells, Figure 1D) or when transfection reagents other than Lipofectamine 2000 (such as MessengerMax, which is a liposome optimized for mRNA transfection, Figure 1E) were used. The addition of the aptamer 17 sequence to different mRNAs, such as codon-optimized luciferase (Figure 1F) or ZsGreen (Figure 1G) with globin stabilization untranslated 5' and 3' sequences, enhanced the expression of reporter proteins. Thus, the aptamer 17 sequence is an optimization element that can function alone or in combination with other mRNA-optimizing sequences, such as stabilizing UTRs. For gene therapy approaches, the mRNA must be deficient in triggering RNA sensors such as Toll-like receptors. This objective can be achieved by substituting modified nucleotides for canonical nucleotides, most notably by replacing canonical uridine with 1methyl-pseudouridine. As shown in Figure 2, the aptamer 17 sequence could increase luciferase expression produced by mRNA molecules containing 1-methyl-pseudouridine instead of uridine, regardless of whether mRNAs are transfected into tumor cells (HEK, cells, Figure 2A) or untransformed human cells (immature dendritic cells, Figure 2B, or mature dendritic cells, Figure 2C).

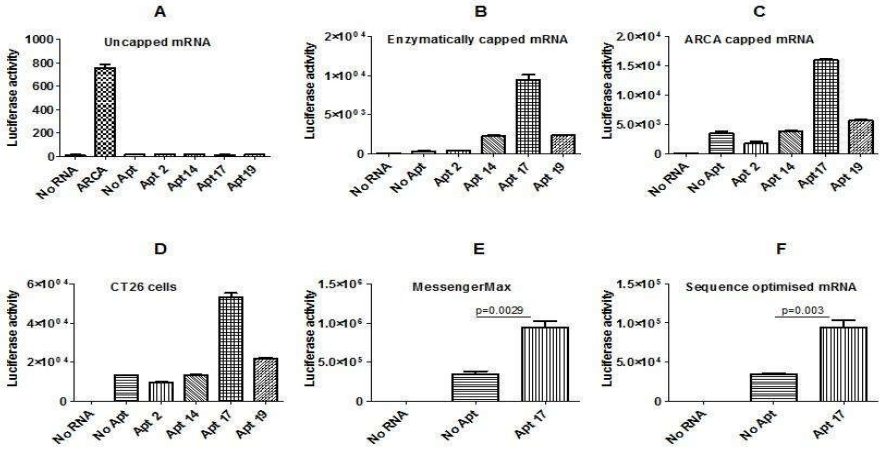
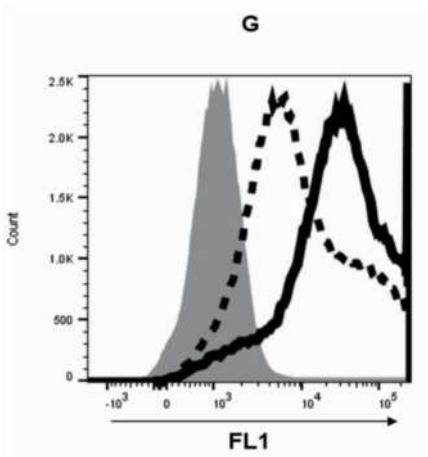


Figure 1:

Effects of eIF4G-binding aptamers on the efficacies of ACGψ ivt mRNAs. Enzymatically capped mRNAs in which uridine residues were replaced by 1-methyl pseudouridine residues and the 5' UTR contained (Apt 17) or lacked (No Apt) the eIF4G-binding aptamer 17 sequence were transfected into HEK cells (A), immature human dendritic cells (B) or mature human dendritic cells (C) using MessengerMax. Twenty-four hours after transfection, luciferase activity was recorded. The means and deviations for experiments performed in triplicate are shown. p values are calculated using unpaired t test.



At that time, fluorescence was measured using FACS (G), with representative results presented: the filled histogram represents untransfected cells, the dash line represents cells transfected with an mRNA without an aptamer in its 5' UTR, and the black line represents cells transfected with an mRNA with the aptamer 17 sequence in its 5' UTR nucleotides or a mixture of A, C, G, and pseudouridine (as triphosphate nucleotides from TriLink). Capped poly-adenylated mRNA was precipitated using LiCl, washed with 75% ethanol, and resuspended in pure water. This mRNA was then quantified using a NanoDrop instrument and analyzed via agarose gel electrophoresis.

Cells and transfection

Human embryonic kidney (HEK) cells and CT26 mouse colon carcinoma cells were maintained in RPMI medium (Thermo Fisher Scientific) containing 10% fetal calf serum (FCS) and 0.2% antimicrobial reagent Normocin (Invivogen). Human dendritic cells were produced from adherent monocytes harvested from peripheral mononuclear blood cells (PBMCs) after 45 minutes of adhesion to plastic and cultured for 6 days in the presence of GM-CSF (800 U/ml) and IL-4 (500 U/ml) from PeproTech (with medium replacement at day 3). They were eventually matured by 24 hours culture in the presence of Protamine-RNA nanoparticles at 5 g/ml 14. Transfections were performed with 200 000 cells per well in 100 microliters of RPMI medium supplemented with 10% FCS and 0.2% antimicrobial reagent Normocin (Invivogen) by adding either 200 ng of mRNA in 12 microliters of Opti-MEM (Thermo Fisher Scientific) and 200 ng of Lipofectamine 2000 (Thermo Fisher Scientific) in 12 microliters of Opti-MEM to each well or 200 ng of mRNA in 5 microliters of Opti-MEM and 400 ng of MessengerMax (Thermo Fisher Scientific) in 5 microliters of Opti-MEM to each well. Luciferase activity was recorded one day after transfection by adding 25 microliters of Bright-Glo (Promega) and measuring activity using GloMax equipment (Promega). ZsGreen signal was recorded by acquiring cells using Fluorescence-activated Cell Sorting (FACS) (Canto, BD Biosciences) and analyzing the results using FlowJo.

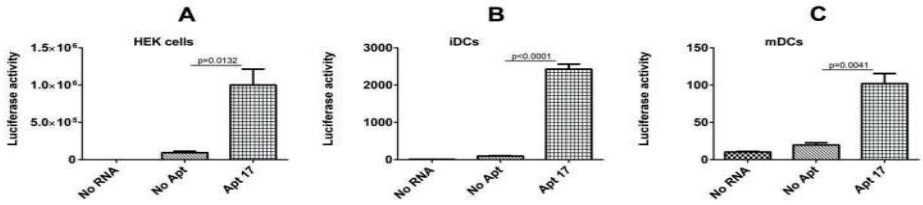


Figure 2: Effects of eIF4G-binding aptamers on the efficacies of ACGψivt mRNAs.
Enzymatically capped mRNAs in which uridine residues were replaced by 1-methyl pseudouridine residues and the 5' UTR contained (Apt 17)or lacked (No Apt) the eIF4G-binding aptamer 17 sequence were transfected into HEK cells (A), immature human dendritic cells (B) or mature human dendritic cells (C) using MessengerMax. Twenty-four hours after transfection, luciferase activity was recorded. The means and deviations for experiments performed in triplicate are shown. p values are calculated using unpaired t test MessengerMax (Thermo Fisher Scientific) in 5 microliters of Opti-MEM to each well. Luciferase activity was recorded one day after transfection by adding 25 microliters of Bright-Glo (Promega) and measuring activity using GloMax equipment (Promega). ZsGreen signal was recorded by acquiring cells using Fluorescence-activated Cell Sorting (FACS) (Canto, BD Biosciences) and analyzing the results using FlowJo. Results and Discussion Miyakawa et al. reported the sequences of eight aptamers that could bind to eIF4G 13. Five of these molecules (aptamers 2, 14, 17, 18, and 19) did not inhibit translation. We tested the shortest four of these five molecules (aptamers 2, 14, 17, and 19), which were 41 bases or less in length; aptamer 18, which is 70 residues long, was not tested. Oligonucleotides with a T7 promoter sequence followed by the aptamer sequence and a 20-base sequence that recognizes the wild-type luciferase sequence at its start codon (see supplementary Figure 1 for the primer sequences) were used to generate DNA templates for in vitro transcription. Uncapped, enzymatically capped and ARCA-capped mRNAs were produced using the aforementioned PCR matrices. MRNAs were transfected into human tumor cells (HEK cells) using Lipofectamine 2000, and luciferase activity was recorded. As presented in Figure 1A, uncapped mRNAs, even those with aptamer sequences at their 5' end, were not translated. However, detectable luciferase activity was observed for mRNAs that were enzymatically (Figure 1B) or co-transcriptionally (Figure 1C) capped. Remarkably, mRNAs with the aptamer 17 sequence consistently exhibited several fold increases in luciferase activity compared with mRNAs without a 5' aptamer sequence and mRNAs with the aptamer 2 sequence (aptamers 14 and 19 moderately enhanced translation in certain experiments). Similar results were obtained when mRNAs were transfected into mouse tumor cells (CT26 cells, Figure 1D) or when transfection reagents other than Lipofectamine 2000 (such as MessengerMax, which is a liposome optimized for mRNA transfection, Figure 1E) were used. The addition of the aptamer 17 sequence to different mRNAs, such as codon-optimized luciferase (Figure 1F) or ZsGreen (Figure 1G) with globin stabilization untranslated 5' and 3' sequences, enhanced the expression of reporter proteins. Thus, the aptamer 17 sequence is an optimization element that can function alone or in combination with other mRNA-optimizing sequences, such as stabilizing UTRs. For gene therapy approaches, the mRNA must be deficient in triggering RNA sensors such as Toll-like receptors. This objective can be achieved by substituting modified nucleotides for canonical nucleotides, most notably by replacing canonical uridine with 1-methyl-pseudouridine. As shown in Figure 2, the aptamer 17 sequence could increase luciferase expression produced by mRNA molecules containing 1-methyl-pseudouridine instead of uridine, regardless of whether mRNAs are transfected into tumor cells (HEK, cells, Figure 2A) or untransformed human cells (immature dendritic cells, Figure 2B, or mature dendritic cells, Figure 2C). In conclusion, the addition of an eIF4G-binding aptamer to the 5' UTR of ivt mRNA can be used as a universal method to increase the quantity of protein produced from recombinant mRNA. The numerous ongoing pre-clinical and clinical studies that involve evaluating the efficacy of mRNA-based therapies should take advantage of the method reported here to increase the functionality of ivt mRNA and thereby achieve a higher therapeutic index with the same conclusion, the addition of an eIF4Gbinding aptamer to the 5' UTR of ivt mRNA can be used as a universal method to increase the quantity of protein produced from recombinant mRNA. The numerous ongoing pre-clinical and clinical studies that involve evaluating the efficacy of mRNAbased therapies should take advantage of the method reported here to increase the functionality of ivt mRNA and thereby achieve a higher therapeutic index with the same dose of mRNA or the same therapeutic index with a lower dose of ivt mRNA.

Conclusion

The present manuscript describes a simple method to increase the efficacy of ivt mRNA: the addition of a 5' sequence corresponding to an aptamer that binds the translation factor eIF4G. Enhanced translation induced by this 5' aptamer was observed in both tumor cells and immune cells and using unmodified or modified nucleotides. This simple approach can be used for any ivt mRNA in order to increase expression.

Conflict of Interest Disclosures Steve Pascolo is inventor of a patent on the use of eIF4F-directed aptamers to increase translation of mRNA (EP17198422).

Acknowledgments

This work was supported by the University Research Priority Project (URPP) “Translational Cancer Research”.

Authorship Contributions

MT performed experiments and participate to the redaction of the manuscript TK supported the study and participated to the redaction of the manuscript SP designed the study, performed experiments and wrote the manuscript.

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Supplementary figure 1

PCR primers to generate DNA template for *in vitro* transcription
Underlined: T7 promoter
Bold: Complementary to target gene
Wild type Luciferase
Forward primers
No UTR: TAA TAC GAC TCA CTA TAG GG **CCG CAT CTA GAG GGC C**
Apt 17: TAA TAC GAC TCA CTA TAG GG ACTC ACT ATT TGT TTT CGC GCC CAG TTG CAA AAA GTG TCG **CCG CAT CTA GAG GGC C**
Apt 2: TAA TAC GAC TCA CTA TAG GGT CCA GAC CCC AAC AGA CTC CAT AAC TAA TAT GTC GCA AAA **CCG CAT CTA GAG GGC C**
Apt 14: TAA TAC GAC TCA CTA TAG GGT CCG TAG AAA CGC GTT AAG GTG AAA GTT TGA GGG CTC CTC A**CC GCA TCT AGA GGG CC**
Apt 19: TAA TAC GAC TCA CTA TAG GGT CCG CGG CGC CAT CTC ATG TTT AGT TGT CCT ATG TCG AGC **CCG CAT CTA GAG GGC C**
Reverse primer:
AGC AAG AAA GCG AGC TCT GAA TAA GTT ACA TTT TA
Optimised Luciferase
Forward primers
No UTR-Opt: TAA TAC GAC TCA CTA TAG GG **ATT CTT CTG GTC CCC AC**
Apt 17-Opt: TAA TAC GAC TCA CTA TAG GG ACTC ACT ATT TGT TTT CGC GCC CAG TTG CAA AAA GTG TCG **ATT CTT CTG GTC CCC AC**
Reverse primer
TGT AAT CCA GAG GTT GAT TG