Position-Specific Nucleoside Sugar Modifications in mRNA ORF: Enhancing Translational Function through Complete Chemical Synthesis of mRNA

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Contributions

HI, YK, MH, JY, and HA conceptualized and designed the study. MH, KN, KM, TA, KK, and RO performed chemical synthesis. HI, KN, KA, NA, and FH designed and performed the biochemical experiments, including cell experiments. AH, KH, and SS performed LC-MS analysis of the translated peptides, and HI, YK, MH, JY, and HA wrote the manuscript.

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Abstract

In this study, we systematically evaluated the efficacy of chemical modifications in enhancing the translational activity and stability of mRNA. We employed a primarily chemistry-based synthetic approach for the modified mRNA, which is crucial for the position-specific introduction of chemical modifications, enabling an intricate and detailed structure-activity relationship (SAR) study, hitherto unattainable with conventional methods. A pivotal innovation herein was the introduction of 2′-F modification for the nucleosugar in the open reading framework (ORF) at a site-specific position within the codon unit (the first nucleoside in the codon unit, 1[st] NC), which significantly bolstered the biological stability of mRNA without compromising its translational activity. This precise control of chemical modification patterns has emerged as a key to achieving higher peptide expression levels than conventional *in vitro*-transcribed mRNA. Further evaluations of the modifications at the 5′-UTR and poly(A) tail with other types of nucleoside and phosphate analogs also exemplified the importance of terminal modifications on mRNA for improved translational activity. These findings offer a novel framework for molecular design and development of effective mRNA-based therapeutics.

Introduction

Research on the development of messenger RNA (mRNA) therapeutics using mRNA as drug molecules has been greatly accelerated by the successful application of mRNA vaccines to SARS-CoV2.[1-2] mRNA therapeutics[3-4] have a variety of applications, including vaccines against infectious diseases using viral and bacterial antigens,[5] cancer vaccines based on cancer cell-specific antigens,[6-7] and protein replacement therapy for diseases caused by genetic mutations.[8-10] Compared to protein replacement therapy using proteins as drug molecules, mRNA therapeutics offer advantages in terms of molecular design simplicity, predictability of molecular properties, and inherent eukaryotic post-translational modifications vital for protein function. Unlike conventional DNA-based gene therapy, on the other hand, mRNA therapeutics do not require transport to the nucleus for gene expression, have little risk of genome integration, and allow for transient expression of therapeutic effects, enabling better therapeutic control.

The following issues are generally recognized for the future application of mRNA therapeutics as general-purpose medicines[3, 5]: mRNA *in vivo* stability, insufficient translational activity, induction of undesired immune responses, and devising effective delivery methods to target tissues and cells. Among these challenges, introducing non-canonical chemical modifications has shown promise in enhancing mRNA stability and translational activity and reducing immune responses.[11-13] Improvement of mRNA function by introducing chemical modifications has been studied mainly for the 5′-cap, and various cap derivatives have been developed to improve stability by imparting decapping resistance,[14-15 16-19] as well as for tool-oriented applications.[20-22] Although less common, modifications to the poly(A) tail [23-25] and region-specific thiophosphate modifications in the 5′-untranslated region (UTR)[26] have been explored to enhance protein production. In addition, the use of non-canonical nucleobases[27-30] can effectively mitigate undesired immune responses[31-35] triggered by exogenous mRNA. It is well recognized that the use of these modified nucleobases plays a significant role in the success of mRNA vaccine development against SARS-CoV2. While these studies underscore the potential of chemical modifications and the use of non-canonical structures to address the challenges of mRNA therapeutics, it should be noted that the conventional enzyme-based preparative method for mRNA greatly limits the types and patterns of chemical modifications that can be introduced.

On the other hand, our group has also been conducting research to solve issues in mRNA therapeutics from different aspects, including nano-structure design of mRNAs such as circular mRNA, [36-39] introduction of chemical modifications such as specific phosphorothioate modification to 5′-UTR, [26] development of cap analogs for *in vitro* transcription (IVT) that enable purification of capped mRNAs with high-purity and translational activity, [40] and chemical capping reaction on RNA for complete chemical synthesis of mRNA.[41]

In this study, as a new approach for developing effective mRNA, we explored the possibility of introducing chemical modifications into the ribose scaffold of nucleosides, especially those in the open reading frame (ORF) regions. Although modification of the ribose ring would significantly improve biological stability, as exemplified by oligonucleotide therapeutics[42-45] such as ASO and siRNA, there are two significant challenges in applying the same concept to mRNAs: one is that ribose modifications in ORFs, as known

from examples such as 2′-OMe, [30, 46-47] would significantly decrease translational activity. The second issue lies in the synthetic method used for chemically modified mRNA. mRNA is basically synthesized by enzymatic transcription, but even in transcription using engineered RNA polymerases, the types of sugar-modified nucleosides that can be introduced are very limited.[49-53] In addition, when preparing mRNA with a single composition, as in pharmaceutical applications, substitutions with modified nucleosides are inevitably made throughout for the specific nucleobase. *In other words, with conventional transcription-based synthetic methods, it is impossible to introduce position-specific chemical modifications to maximize both mRNA stability and translational activity.* The chemically modified mRNAs (Fig. 1A) developed in this study were prepared by first synthesizing the corresponding RNA oligonucleotides using phosphoramidite chemistry, and then concatenating these RNA fragments by enzymatic or chemical ligation reactions (Fig. 1B) to prepare full-length mRNAs. This method enabled the introduction of many different types of chemical modifications in various patterns in the synthesis of RNA fragments, and to precisely evaluate the effects of the patterns of chemical modifications on the translational activity and stability of the mRNA.

Results and Discussions

First, to screen for modification patterns, we designed and prepared a sequence of 91 nucleotides (nt) RNA sequence that could be directly synthesized without ligation and evaluated its translation activity in a cell-free translation system with HeLa lysate (Fig. 2A). The sequence consisted only of 5′-UTR and ORF, and was designed to encode Flag-His_6 peptide to perform a sandwich ELISA for the evaluation of translational activity. As modifications, 2′-fluoro (2′-F), 2′-*O*-methyl (2′-OMe), 2′-*O*-methoxyethyl (2′-*O*-MOE), locked nucleic acid (LNA), DNA, etc., were selected. mRNA modified with 2′-OMe at both ends (NK002) showed 4-fold higher translational activity than unmodified RNA (NK001) (Fig. 2B). Other types of modifications, such as 2′-F, LNA, DNA, 2′-*O*-MOE, and phosphorothioate, were also examined for possible introduction into the mRNA terminus (Fig. S1A). LNA (NK011) modification resulted in a significant loss of translational activity (Fig. S1B), whereas DNA (NK012) and 2′-*O*-MOE (NK009) modifications maintained translational activity, including additional phosphorothioate modifications (NK014 and NK015), as well as for 2′-OMe (NK013) (Fig. S1C).

Next, the introduction of the 2′-F modification into a specific position in the codon unit was tested; the modification was specifically introduced at the codon's first, second, or third nucleoside over the ORF (NK003, NK004, NK005) (Fig. 2A). Interestingly, while the translational activity of mRNA with the modification at all of the second or third nucleoside in the codon unit (2^{nd} NC, 3^{rd} NC) was suppressed by nearly 30-50 % level, the modification at the first nucleoside in the codon unit (1^{st} NC) did not have strong deleterious effects on translational activity. This translational acceptability of the 2′-F modification of the 1^{st} NC was also observed in another sequence (67 nt mRNA coding Flag-His_6). (Fig. S2). In contrast, other types of sugar modification showed a negative effect on translation (Fig. 2C) when all of the 1^{st} NC were modified, translation was suppressed in the case of the 2′-OMe (NK006) and 2′-*O*-MOE (NK007) modifications, and a nearly 50% reduction was observed for the deoxyribose modification (NK008).

Subsequently, screening for the optimum chemical modification patterns of the terminal region (6 nt at the 5′ terminus and 3 nt at the 3′ terminus) was performed with a longer mRNA (145 nt) encoding three repeats of FLAG peptide and His-Tag (Fig. 3A). The mRNAs were prepared by chemical or enzymatic ligation reactions between the 5′ side 80 nt and the 3′ side 65 nt RNA fragments that were prepared using an automated oligonucleotide synthesizer. In the case of RNA prepared by chemical ligation, the target ligated RNA strand was confirmed by LC-MS analysis (Fig. S3). 2′-F modification on the terminus increased peptide production (NK024) to some extent, while LNA and BNA-NC (*N*-Me) modification significantly restrained translation (NK026, NK027) (Fig. 3B). 2′-*O*-MOE modification showed a positive effect on the translation (NK025) with a similar level as 2′-OMe modification (NK023), and the positive effect was further improved by the additional phosphorothioate modification to the terminal region (NK028).

Based on these findings, terminal modification of mRNA with polyadenine (poly(A)) tails was evaluated. To the 145 nt RNA with MOE modification at the terminus, 20 nt of poly(A) with various modification types was also prepared (Fig. 3C). The types of modifications tested on poly(A) were as follows: 2′-F modification for every 2 nt (NK030), cross modification with 2′-F and 2′-OMe (NK031), complete modification with 2′-OMe (NK032), and 2′-*O*-MOE (NK033). The terminus 3 nt of the modified poly(A) was set as 2′-*O*-MOE with a phosphorothioate linkage. Evaluation of the translational activity revealed that the addition of non-modified poly(A) (20 nt) increased the translated peptide amount, and the positive effect was further enhanced by these modifications on poly(A), with 2′-F modification for every 2 nt (NK030) being slightly better than the others (Fig. 3D). In contrast to the ORF cases, various modifications were accepted on poly(A) without loss of translational activity.

To clarify the effect of chemical modification of mRNA at various positions, 5′-UTR, ORF, and poly(A), mRNA samples with different modification sites and patterns were prepared (Fig. 3E) and their translational activities were compared in HeLa cell extracts. The samples tested were as follows: RNA with no modification (NK034), modified only at the 5′-UTR (NK035), ORF (NK036), poly(A) (NK037), modified both at the 5′-UTR and poly(A) (NK038), and modified over all regions (NK039). As shown in Fig. 3F, each modification to either the 5′-UTR, ORF, or poly(A) increased translational activity, and a stronger increment of the translated peptide was observed by the modification of ORF and poly(A) than that for the 5′-UTR. Interestingly, the combination of the 5′-UTR and poly(A) modification (NK038) counteracted the positive effects of each modification, and the additional modification of the ORF enhanced translation (NK039).

After identifying the candidates for optimum modification patterns, four mRNA samples were designed and synthesized with various modification patterns for each region (Fig. 4A). All four samples contained ribose modification in the ORF, 2′-F modification at the 1st NC, and the level of chemical modification on both terminals was changed among these four samples, as shown in Fig. 4A. Evaluation of the translational activity of these modified mRNA revealed that, as the level of terminal modification increased, the amount of translated peptide slightly decreased (NK030 vs. NK031, NK040, NK041) (Fig. 4B). The time course of the translation was also evaluated over 30 min (Fig. 4C). While mRNA without any modification (NK034) showed maximum translation at 5 min after transfection, the modified RNA (NK030, NK031, NK041)

increasingly produced the peptide over 30 min, with moderately modified RNA (NK030) showing the highest translation level at every time point.

The stability of mRNA in HeLa lysates was evaluated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) for the reaction mixture (Fig. 4D). While the unmodified RNA (NK034) showed fast clearance from the mixture, the modified mRNAs (NK030, NK031, and NK041) stably remained in the reaction mixture without degradation for 30 min at 37 °C. While the amount of remaining mRNA was nearly comparable for the three types of modified mRNAs, there was a significant difference in the amount of translation product (Fig. 4C). These results suggest that moderate and high levels of chemical modification at the terminus improved the stability of the mRNA, but the latter negatively influenced its translational activity.

The time course of translation was evaluated using HeLa cells over a more extended incubation period of up to 48 h. In the case of lipofection (Fig. 4E), mRNAs that were highly modified in the poly(A) moiety (NK031, NK041) afforded the highest amount of translational product over 48 h compared to mRNAs with the other two types of modification (NK030 and NK040). Notably, the translation level of the unmodified mRNA (NK034) was significantly lower than that of the modified mRNAs, clearly reflecting the results of stability evaluation (Fig. 4D). While the modification level in the 5′-UTR region had little effect on the translation level in the case of mRNAs with a higher level of modification in the poly(A) region (NK031 vs. NK041), a lower level of modification of the 5′-UTR resulted in better translation activity for mRNA with less modification of the poly(A) region (NK030 vs. NK040). In the case of electroporation (Fig. 4F), the trend was somewhat different from that in the lipofection cases; the mRNA with high modification at both termini (NK041) showed the highest level of translation without decreasing the peptide amount over 24 h. Both these experiments suggested that modification of the poly(A) region was more critical for increasing the translation amount than that on the 5′-UTR region in the cellular system.

The amount of mRNA remaining in the HeLa cells after electroporation was quantified by RT-qPCR (Fig. 4G). The result was consistent with the translation level in the electroporation experiment (Fig. 4F); the mRNA was more stable as the level of chemical modification increased.

Various reasons can be considered for the different optimal modification patterns observed in the HeLa cell lysates and cell lines. One possible explanation for this is the varying impact of the mRNA degradation system. Specifically, in the HeLa cell lysate system, where the effect of mRNA degradation was relatively minor, the introduction of extensive chemical modifications at both ends of the mRNA was observed to inhibit translation. In contrast, in the cell system, the significant influence of the mRNA degradation system suggests that resistance to degradation plays a major role in determining the translational activity.

A similar trend in the positive effect of the chemical modification was also observed in other cell lines, where mRNA was administrated to hAoSMCs (human aortic smooth muscle cells) by electroporation, and the time course of the translation was evaluated (Fig. S5A). In addition, the versatility of the design strategy was confirmed by other mRNA sequences, the ones encoding the same hybrid peptide, three repeats of FLAG and HisTag, but with different 5′-UTR sequences (Figs. S5B, C), and the others encoding a different peptide composed of FLAG and Epidermal Growth Factor (EGF) (Fig. S5D). Notably, the latter has a different ORF

sequence, but the 2′-F modification on the 1st NC effectively increased translational activity, demonstrating the broad applicability of the effects of the modification on translational activity.

Finally, the stability and translation activity of chemically modified and canonical mRNA prepared by IVT (NK053) with post-transcriptional capping were compared (Fig. 5A). Based on the above studies, chemically modified RNA (NK052) was designed to have intensive chemical modification at both termini and 2′-F modification at the 1st NC in ORF and was entirely prepared by chemical method; the 5′ side 80 nt RNA fragment with 3′ phosphate terminus was chemically ligated with the 3′-side 85 nt RNA fragment with a 5'-hydroxy terminus. As a control sample, RNA without modification was also prepared by enzymatic ligation of the corresponding RNA fragments that were synthesized using an automatic synthesizer (NK034).

For stability evaluation, mRNA was incubated in 50-fold diluted mouse serum at 37 ˚C, and the amount of remaining mRNA was evaluated by RT-qPCR (Fig. 5B). Chemically synthesized mRNA without a cap (NK034) was degraded rapidly, leaving less than 10 % of the sample after 1 h. Introducing a cap structure to the RNA was effective, as IVT-derived non-modified mRNA (NK053) remained at approximately 25% after 1 h. On the other hand, introducing chemical modification improved stability more significantly; nearly 60% of RNA remained after 1 h of incubation (NK052).

Reflecting this difference in stability under quasi-physiological conditions, while IVT-derived mRNA (NK053) afforded a very low translation level, albeit with a 5 cap, chemically modified mRNA (NK052) stably expressed a much higher level of the peptide in HeLa cells over 24‑48 h in both lipofection and electroporation systems (Figs. 5C, D). These results clearly demonstrate that appropriate chemical modifications enhance peptide production from mRNA in cellular systems. The high serum stability of chemically modified mRNA compared with that of canonical mRNA would ensure the effectiveness of this strategy, even for *in vivo* systems. These results suggested the possibility of administrating mRNA locally without using carrier molecules such as lipid nanoparticles (LNPs), which can cause side effects such as excessive immune responses and undesired widespread distribution *in vivo*.[54-56]

Conclusion

In conclusion, we have systematically demonstrated the effectiveness of chemical modification of the sugar moiety of ribonucleosides in enhancing peptide production from mRNA. The pivotal discovery in this study is that the position-specific 2′-F modification in the 1st NC (first nucleoside in the codon unit) could be accepted without reducing the translational activity and effectively the biological stability of the mRNA. Augmented by the additional chemical modification on both termini, the chemically modified mRNA, even without a 5′ cap, yielded a greater amount of peptide over a longer duration than the IVT-derived canonical mRNA. Site-specific chemical modification for a high level of translation was enabled by the chemical synthesis of the RNA fragments. Ligation reactions with these RNA fragments allowed for the preparation of chemically modified mRNA exceeding 200 nt in length. Building on the SAR insights from this study, we are pursuing the development of highly efficacious mRNA therapeutics. Due to their high and long-lasting activity, chemically modified mRNA might provide novel therapeutic options that are difficult to achieve

with IVT-derived mRNAs in the evolving area of mRNA therapeutics, such as cancer vaccines[7], protein-replacement, and tissue regeneration therapies.

Materials and Methods

RNA fragments and splint DNAs for enzymatic or chemical ligation reactions were purchased from Gene Design or Hokkaido System Science, where they were synthesized using standard phosphoramidite methods. The oligonucleotides used in this study are listed in Tables S1-S3.

Mass spectrometry analysis

The structure of the solid-phase synthesized oligonucleotides was determined by liquid chromatography–mass spectrometry (LC-MS) using an Agilent 6120 series single quadrupole LC/MS system (Agilent Technologies). Liquid chromatography was performed using an ACQUITY BEH C18 column (1.7 mm, 2.1 · 50 mm; Waters) with buffers A (8.6 mM trimethylamine and 100 mM hexafluoroisopropanol in water) and B (methanol).

General procedure for enzymatic ligation reactions

Two RNA fragments were ligated on a 30-nt splint DNA using T4 RNA ligase 2 (New England Biolabs). The ligated RNAs were purified using preparative 5% denaturing PAGE and isolated using the crush-and-soak method to obtain the ligated product.

General procedure for chemical ligation reactions

A mixture containing RNA fragments Nos.56 and 57 (each 100 μM) (Table S2) and splint DNA (200 μM) in 100 mM NaCl was heated at 80 °C for 3 min and gently cooled to room temperature. The reaction mixture was then incubated at 25 °C for 20 h. The ligated RNA was purified using the NAP-10 column and preparative 5% denaturing PAGE, and isolated using the crush-and-soak method to obtain ligated NK052 (4.1 nmol). The yield of each ligated mRNA is shown in Table S4.

Preparation of *in vitro*-transcribed mRNA and its purification by dPAGE

Template DNA for IVT was prepared by PCR using pUC57amp/G-3×FLAG-6×His-DHFR as the template. The plasmid sequences are described in Table S5. The sequences of the PCR primers were as follows:
Forward, 5′-AAGCTAATACGACTCACTATAGGGAGAATACAAGCTACTTGTTCTTTTTGCAGCC ACCATGGACTACAAGGACG -3′;
Reverse, 5′ -TTTTTTTTTTTTTTTTTTTTTCAGTGGTGGTGGTGGTGGTGTTTG -3′.
The PCR mixture consisted of 0.5 μM primers, 0.25 ng/μL pUC57amp/G-3×FLAG-6×His-DHFR vector, 1× PCR buffer of KAPA HiFi HotStart ReadyMixPCR Kit (Toyobo). The mixture was subjected to the following thermal cycling reaction: 95 °C for 5 min, (98 °C for 20 s, 55 °C for 15 s, 72 °C for 30 s) × 25 cycles at 72 °C for 5 min. The reaction mixture was treated with 100 U/μL Dnp I, analyzed by agarose gel electrophoresis, and purified using the QIAquick PCR Purification Kit (QIAGEN).
Transcription reactions were carried out using the MEGAScript T7 Transcription Kit (Invitrogen), according to the manufacturer's protocol. A reaction mixture containing 4 ng/μL DNA (PCR product) and 9 mM NTPs was incubated at 37 °C for 6 h, Turbo DNase I was added, the mixture was further incubated at 37 °C for

15 min, and purified using the Monarch RNA Cleanup Kit (New England Biolabs). Purified RNA was capped with a Vaccinia Capping System (NEB) and ScriptCap 2'-*O*-Methyltransferase Kit (CELLSCRIPT), according to the manufacturer's protocol. The reaction mixture containing 0.5 μg/μL RNA,0.1 mmol/L SAM, 0.5 mmol/L GTP, 0.5 U/μL Vaccinia Capping Enzyme, 2.5 U/μL 2'-*O*-Methyltransferase, 2 U/μL RNase Inhibitor, and 1×Capping Buffer was incubated at 37 °C for 1 h and then purified with Monarch RNA Cleanup Kit. The purified capped-RNA was then treated with Antarctic Phosphatase Enzyme (NEB) and 1×Antarctic Phosphatase Buffer at 37 °C for 1 h. The obtained mRNA was then analyzed by dPAGE and gel-purified mRNA was extracted with buffer. mRNA was recovered by alcohol precipitation. The sequences of the prepared mRNA are listed in Table S6.

***In vitro* translation reaction using HeLa lysate**

Chemically synthesized mRNAs were preheated at 90 °C for 3 min and cooled to 4°C before the translation assay and degradation evaluation. The *in vitro* translation reaction was performed using a 1-Step Human Coupled IVT Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The reaction mixture (10 μL) containing each concentration of mRNA and 0.8 U/μL Murine RNase Inhibitor, and then incubated at 37°C. The reaction was stopped by incubating at 72 °C for 5 min.

***In vitro* transfection of mRNAs for measurement of RNA stability and translation activity using cultured HeLa cells**

The translational activity of each mRNA was evaluated *in vitro* using HeLa cells. In the lipofection experiment, HeLa cells were seeded and cultured at 1.0×10^4 cells/well in RPMI1640 medium containing 10% FBS in 96 well cell culture plates at 37 °C and 5% CO_2. After overnight cultivation, 10 μL/well of transfection reagent containing each concentration of mRNA and 0.3% Lipofectamine MessengerMAX Transfection Reagent diluted with Opti-MEM were added to 40 μL/well of the cultured cells. After 5 h of transfection, the culture supernatant was removed, and then added with RPMI1640 medium containing 10% FBS was added and cultivated. Cultured cells were then washed with ice-cold D-PBS(−) and lysed with 20 μL/well iScript RT-qPCR Sample Preparation Reagent (Bio-Rad) containing 2% Protease Inhibitor Cocktail (EDTA free) (Nacalai Tesque). Electroporation of mRNAs was performed using the Nucleofector 96-well Shuttle System (Lonza) and the SE Cell Line 96-well Nucleofector Kit (Lonza). HeLa cells were resuspended in the Nucleofector Solution and Supplement 1. 20 μL of reaction mixture containing each concentration of mRNA and 200,000 cells of HeLa was pulsed with FF-150 condition, and then seeded and cultivated with 5.0×10^4 cells/well. Cell lysis was performed as previously described.

***In vitro* transfection of mRNAs for measurement of translation activity using cultured human primary Aotic smooth muscle cells**

Electroporation of human primary aortic smooth muscle cells (hAoSMC) (Lonza) was performed using the Nucleofector 96-well Shuttle System and P1 Primary Cell 96-well Nucleofector Kit (Lonza). hAoSMC were pulsed with FF-130 condition. After electroporation, 2.0×10^4 cells/well were seeded and cultivated with

Smooth Muscle Cell Growth Medium 2 (Lonza), and lysed as described above. The translational activity of each mRNA was evaluated as described above.

Sandwich-ELISA for the evaluation of the translation activity

FLAG-His tag peptides in the translation reaction were measured using sandwich ELISA. The following standard peptides were purchased from COSMO-BIO: 1×FLAG-His_6: NH_2-MDYKDDDDKGGHHHHHH-COOH and 3×FLAG-His_6: NH_2-MDYKDDDDKIIDYKDDDDKGGDYKDDDDKHHHHHH-COOH. 96 well plate was coated with 3 μg/mL of Anti-His-Tag Antibody (Proteintech) in 0.1 mol/L Carbonate buffer (pH 9.4) and then blocked with 3% BSA-TBST. The plate was washed with 1×TBST, and then, each translation reaction and standard peptides in 3% BSA-TBST were added. After 1 h of incubation, a washed plate was added with 0.01 % Monoclonal ANTI-FLAG M2-Peroxidase (HRP) Ab produced in mice (Thermo Fisher Scientific) in 3% BSA-TBST. After washing, the plate was developed with 1-Step Ultra TMB-ELISA and then stopped with 0.5 mol/L sulfuric acid. Abs. 450 – abs. 570 was measured using a spectrophotometer (Bio-Rad).

Evaluation of mRNA stability *in vitro*

The concentration of the remaining mRNA in the translation reaction was measured by RT-qPCR. The sequences and structures of the primers and TaqMan probes used are described below. RT primer: 5' -TCAGTGGTGGTGGTGGTGGTGTTTG -3', FW primer: 5' -GAATACAAGCTACTTGTTCTTTT -3', RV primer: 5' -ATCTTGTCGTCGTCGTCCTT -3', FAM-MGB probe: 5'-FAM-CAGCCACCATG-NFQ-MGB-3'. Reverse transcription of mRNA was performed using the iScript Select cDNA Synthesis Kit (Bio-Rad), according to the manufacturer's protocol. The reaction mixture containing 0.2 μmol/L RT primer, and the translation reaction was diluted with RNase/DNase-free water containing 0.2 U/μL Recombinant RNase Inhibitor. RT-qPCR of obtained cDNAs was performed with Taqman qPCR Master Mix (Applied bio) and s QuantStudio 12K Flex Real-Time PCR System (Applied bio) as described below. 20 μL of reaction mixture containing 4 μL of cDNA, 1.65 μmol/L FW primer, and 1.40 μmol/L RV primer. 0.10 μmol/L FAM-MGB probe and 1×reaction master mix was subjected to the thermal cycling reaction as follows: 50 °C for 2 min, 95 °C for 10 min, (95 °C, 15 s, 60 °C, 1 min) × 50 cycles.

Serum stability assay

The stability of each mRNA in mouse serum was measured as described below. 10 μL of reaction mixture containing 1.6% of mouse serum and 1 μmol/L of each mRNA in THE RNA Storage Solution (Ambion) was incubated at 37 °C for 0, 15, 30, and 60 min, and then 1.2 U/μL of Recombinant RNase inhibitor was added to stop the degradation.

The remaining mRNAs in the reaction mixture were measured using RT-qPCR, as described above.

LC-MS analysis of translated peptides

Chemically synthesized mRNAs were translated with rabbit reticulocyte *in vitro* translation system

(Promega) at 37 °C for 30 min. The translated peptides were purified using an anti-FLAG antibody. The purified peptides and chemically synthesized peptide standards were resuspended in a solution containing [TFA]: [ACN]: [H_2O] = 0.1:2:98 (%vol). These peptides were identified by LC-MS/MS on an Ultimate 3000 RSLCnano LC system (Thermo Fisher Scientific) and a Q Exactive Orbitrap MS/MS system (Thermo Fisher Scientific). Liquid chromatography was performed using a Nano HPLC Capillary Column (particle size; 3.0 µm, column size: 75 µm × 15 cm; Nikkyo Technos, Japan) with buffers A (0.1% formic acid) and B (0.1% formic acid and 90% acetonitrile).

Statistical analysis

No statistical method was used to determine the sample size. No data were excluded from the analysis. The experiments were not randomized. The Investigators were not blinded to the allocation during the experiments and outcome assessment. Statistical analyses were performed using GraphPad Prism9 ver. 9.3.1 (GraphPad Software, USA). The methods used are described in each figure.

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COI

Competing Interest Statement: HA is a cofounder of Crafton Biotechnology. The company focuses on the development of mRNA therapeutics. HI, MH, KM, TA, KA, KK, AH, KH, SS, and JY are employees of Kyowa Kirin Co. Ltd. International patents application covering part of this work has been filed by Nagoya University and Kyowa Kirin Co. Ltd.

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Figures and Tables.

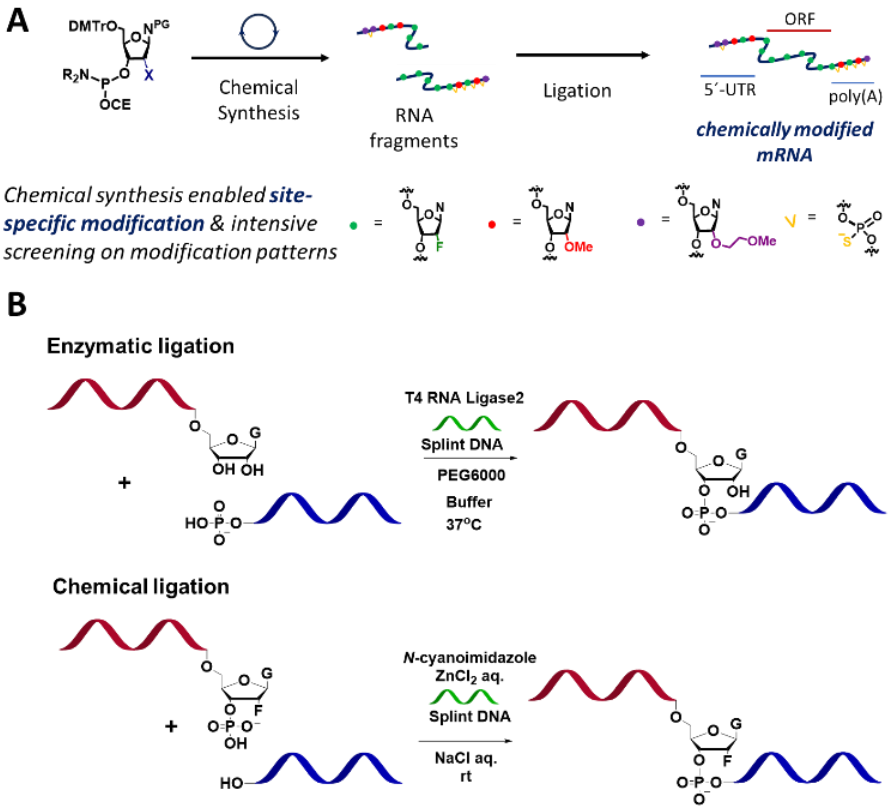


Figure 1. Site-specific nucleosugar-modified mRNA to enhance translation. (A) Concept of the Study. (B) Two ligation methods employed in this study

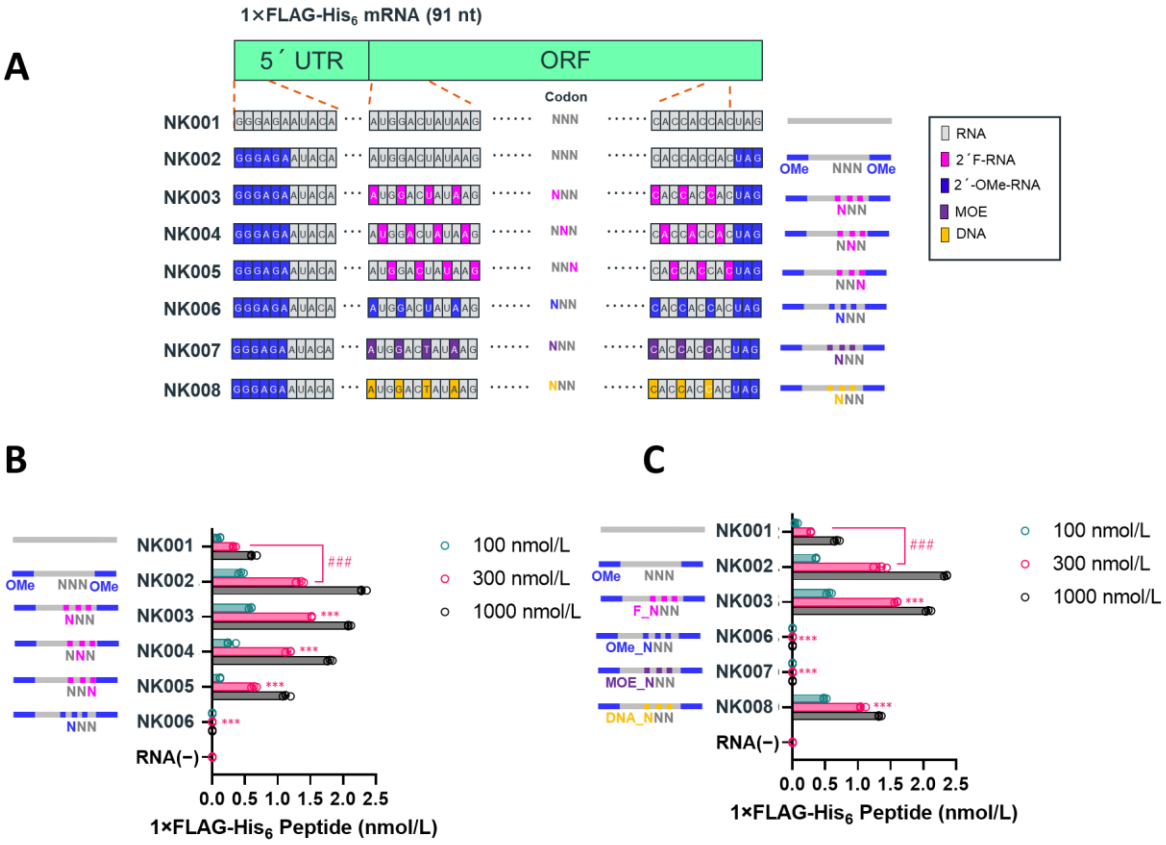


Figure 2. Effect of sugar modification of the ORF of mRNA on translation activity.

(A) Design and sequence of sugar modifications of chemically synthesized mRNAs. (B, C) The concentration of encoded peptides from each mRNA obtained after 30 min of reaction of the HeLa cell lysate in the *in vitro* translation system. (n = 3). Data are presented as mean ± standard error. The statistically significant differences between "NK001" and "NK002" (B, C) in Student's t-test are marked as follows. n.s., $p > 0.05$; #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$. Statistically significant differences for each mRNA from "NK002" (B, C) in one-way ANOVA followed by Dunnett's test are marked as follows. n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

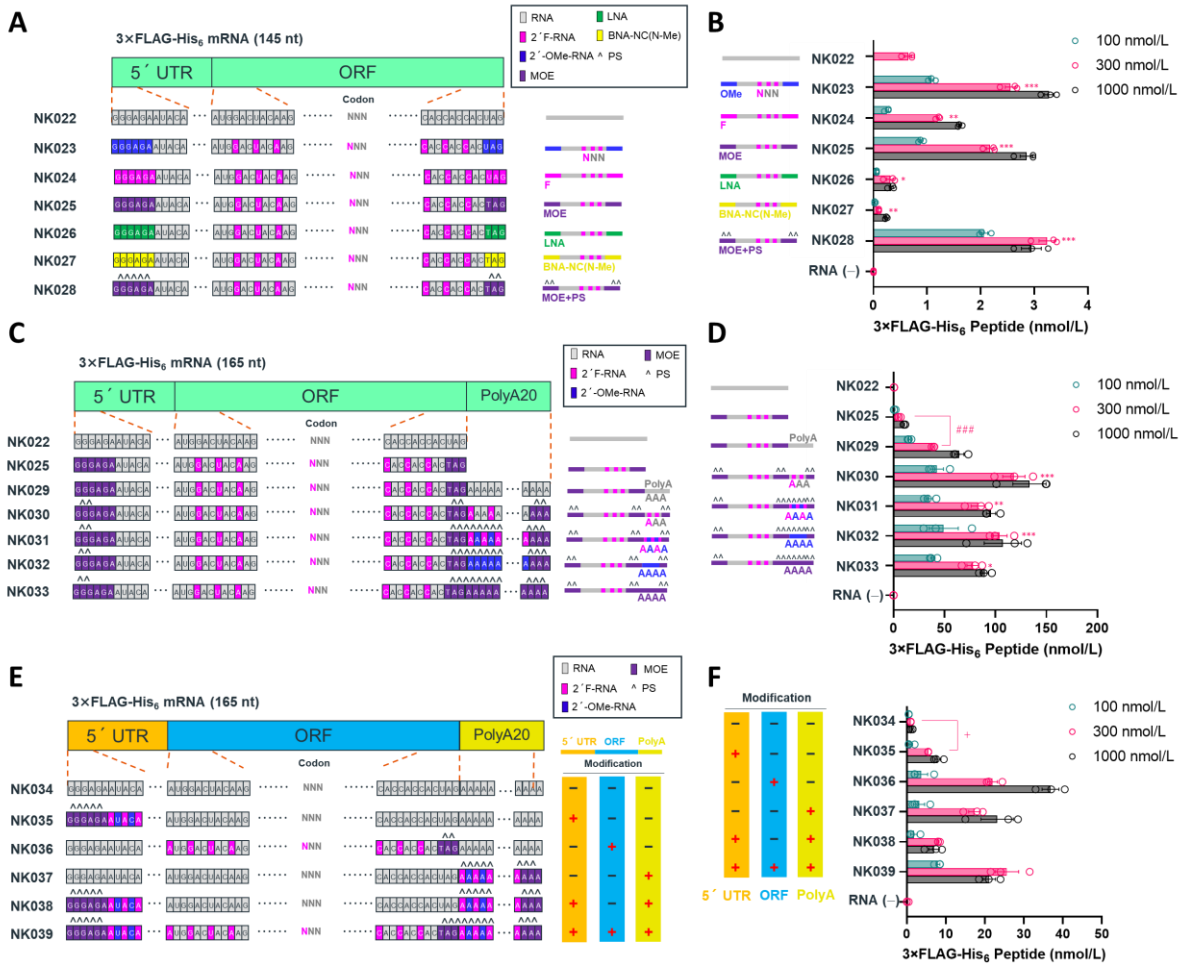


Figure 3. Effect of sugar and backbone modification of the 5' UTR and 3' PolyA of mRNA on translation activity.

(A, C, E) Design and sequencing of sugars and backbone modifications of chemically synthesized mRNAs. (B, D, F) The concentration of encoded peptides from each mRNA obtained after 30 min of reaction with the HeLa cell lysate *in vitro* translation system (n = 3). Data are presented as the mean ± standard error. The statistically significant differences between "NK025" and "NK029" (D) in Student's t-test are marked as follows. n.s., $p > 0.05$; #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$. Both standard errors of "NK034" and "NK035" (D) were 0 and described as "+". The statistically significant differences for each mRNA from "NK022" (B) or "NK029" (D) in one-way ANOVA followed by Dunnett's test are marked as follows. n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

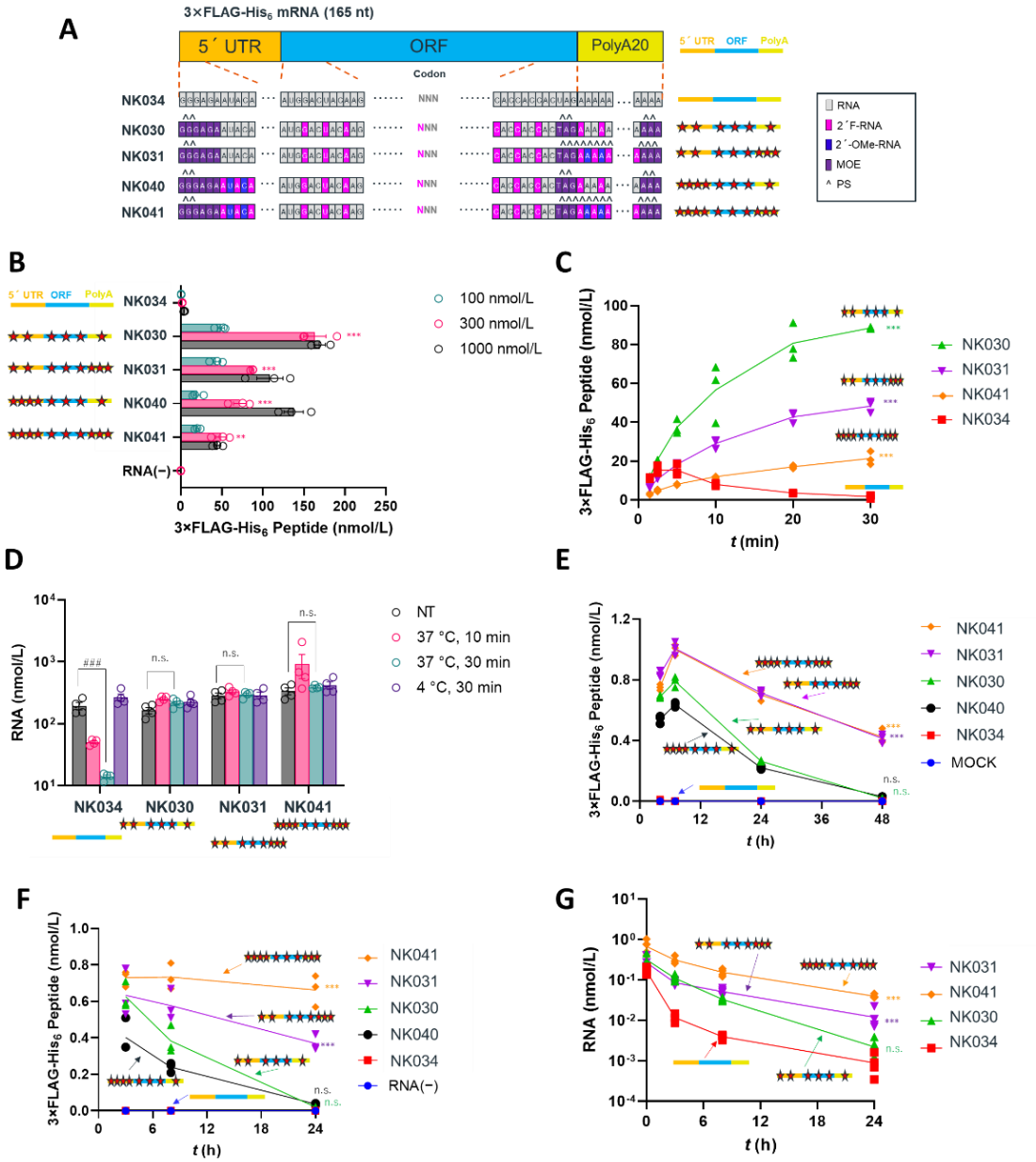


Figure 4. Effects of sugar and backbone modifications on mRNA stability and translation activity

(A) Design and sequencing of sugars and backbone modifications of chemically synthesized mRNAs. (B) The concentration of encoded peptides from each mRNA obtained after 30 min of reaction with the HeLa cell lysate *in vitro* translation system. (n = 3). (C, D) The time course of the concentration of translated peptides (C, n = 3) and remaining RNA (D, n = 4) obtained 30 min reaction of HeLa cell lysate in an *in vitro* translation system. (E, F) Time course of the concentration of encoded peptide from HeLa cells after lipofection using messengerMAX (E) and electroporation (C) of each mRNA (n = 3). (G) Time course of the remaining RNA after electroporation in HeLa cells (n = 4). Data are presented as the mean ± standard error. The statistically significant differences between "NT" and "37 °C, 30 min" (D) in student's t-tests were marked as follows. n.s., $p > 0.05$; #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$. Statistically significant differences for each mRNA from "NK034" (B, C, E, F, G) in one-way ANOVA followed by Dunnett's test are marked as follows. n.s., $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

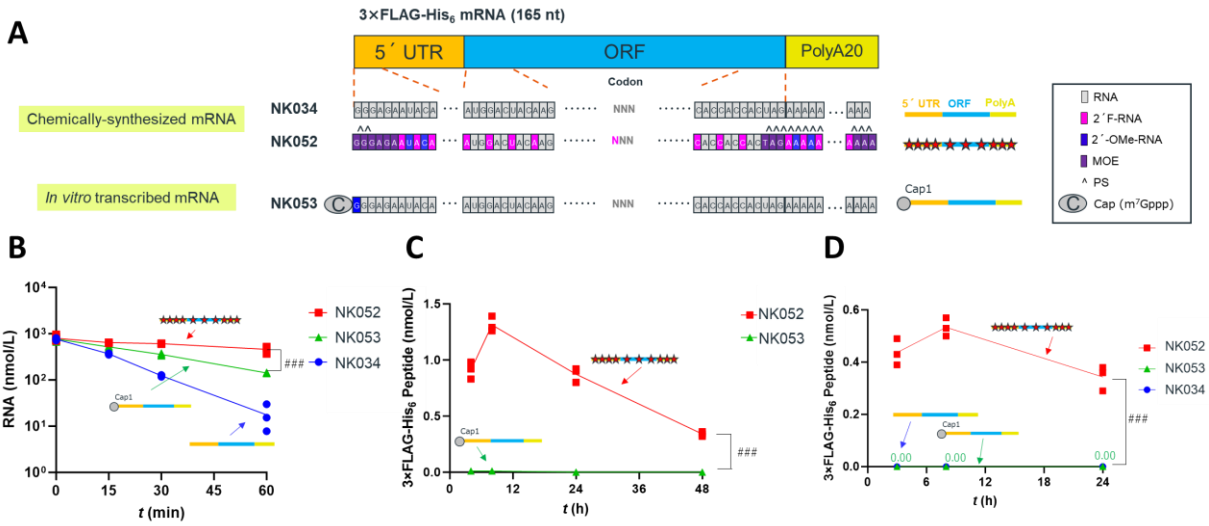
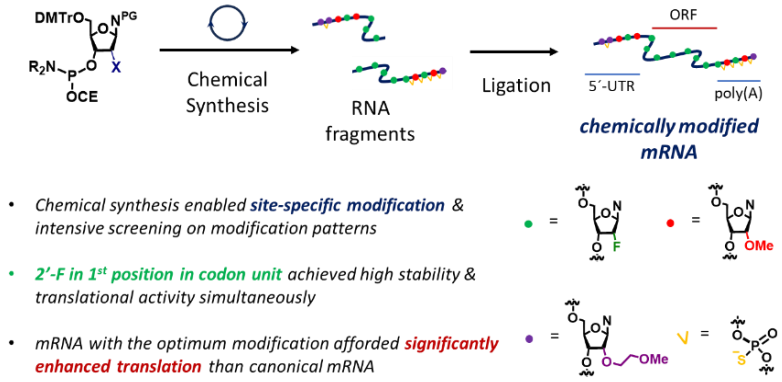


Figure 5. Improvement of mRNA stability and translation activity by sugar and backbone modifications. (A) Design and sequence of sugar and backbone modification of chemically synthesized mRNAs. (B) Time course of the concentration of the remaining RNA after incubation with mouse serum (n = 3). (C, D) Time course of the concentration of encoded peptide from HeLa cells after lipofection using messengerMAX (C) and electroporation (D) of each mRNA (n = 3). Data are presented as the mean ± standard error. The statistically significant differences between "NK052" and "NK053" in Student's t-test are marked as follows. n.s., $p > 0.05$; #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$.

Graphical Abstract



We systematically evaluated the efficacy of chemical modifications in enhancing the translational activity and stability of mRNA, with a notable innovation being the introduction of a 2′-F modification at specific sites, which significantly improved the biological stability of mRNA without compromising its translational activity. These findings provide a new framework for molecular design and development of effective mRNA-based therapeutics.