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SPECIAL TOPIC: Trans-scale RNA regulation and imaging

LETTER TO THE EDITOR

# Enhancement of *in vitro* transcribed (IVT) mRNA translation efficiency through engineered poly(A) tails

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Dear Editor,

*In vitro* transcribed (IVT) mRNA is an emerging class of drug for both therapeutics and vaccines (Hao et al., 2024; Sahin et al., 2014). Enhancing its translation efficiency remains a core challenge for mRNA-based applications (Karikó, 2019). The poly(A) tail is required for mRNA stability, nuclear export and translation (Liu and Lu, 2024). Most engineering efforts have focused on tail length, as the poly(A) tail has been viewed mainly as a structure component and certain length ensures its translation and stability (Sahin et al., 2014). Recent studies show that the 3′ ends of mRNA poly(A) tails can carry non-adenosine extensions that can regulate RNA stability, constituting an additional layer of mRNA stability regulation. We and others has recently identified a novel class of RNA modifications, non-A residues in the body of RNA poly(A) tails (namely U, C and G residues, referred as non-A residues here after), that are widespread in the body of RNA poly(A) tails in mammals (Legnini et al., 2019; Liu et al., 2019; Liu et al., 2023a; Liu et al., 2023b), suggesting an additional layer of RNA post-transcriptional regulation. Here, using IVT mRNAs with poly(A) tail non-A residues, we unexpectedly found that non-A residues can effectively promote the mRNA translation (up to 5-fold in terms of fluorescence intensity and up to 3-fold in terms of proportion of positive cells). Consistent with these results, transcriptome-wide analyses in human cells show that endogenous poly(A) tail non-A residues are associated with enhanced mRNA translation efficiency.

After we and the Rajewsky laboratory identified non-A residues in mouse and human poly(A) tails (Legnini et al., 2019; Liu et al., 2019), we asked the roles of these newly identified non-A residues. We synthesized IVT mRNAs encoding fluorescent reporters that share the same tail length but with different numbers of non-A residues (Figure 1A; Table S1). Equal amounts of each mRNA were transfected into an equal number of HeLa cells, and we monitored both reporter protein and mRNA levels (Figure 1B). Because RNA poly(A) tail is traditionally known as pure-A sequence that supports translation, we initially predicted that mixed incorporation of other nucleotides might impair the role of poly(A) tail in supporting translation, leading to decreased or aborted translation. However, we unexpectedly observed obvious higher levels of EGFP fluorescence in cells transfected with EGFP mRNAs containing non-A residues than those transfected with the pure-A tail control (Figure 1C), which was opposite to our initial prediction. Based on its superior translational enhancement, non-A98-2 was selected for further experiments to validate the role of poly(A) tail internal non-A residues in improving IVT mRNA translation. Fluorescent-activated cell sorting (FACS) analyses confirmed a higher level of EGFP fluorescence for mRNA with the non-A98-2 tail, and the same observation was seen when the mCherry reporter was used instead of EGFP (Figure 1D). These results demonstrate that non-A residues in the poly(A) tail enhance protein production across different IVT mRNA coding sequences.

To determine whether the increase of protein production by non-A residues is through increased translation efficiency or RNA stability, we measured reporter protein and mRNA abundance in the transfected cells by Western blotting and reverse transcription quantitative PCR (RT-qPCR), respectively. Consistent with the observations under microscope and FACS, cells transfected with mRNAs containing non-A residues produced more mCherry and EGFP protein than the pure-A control (Figure 1E). RT-qPCR analysis revealed lower rather than higher mRNA levels in the same samples (Figure 1F), indicating that the increased protein production arises from increased translation efficiency rather than increased mRNA stability. The reduced abundance of highly translated mRNA is consistent with the previous finding that co-translational mRNA degradation is a conserved process (Pelechano et al., 2015). To

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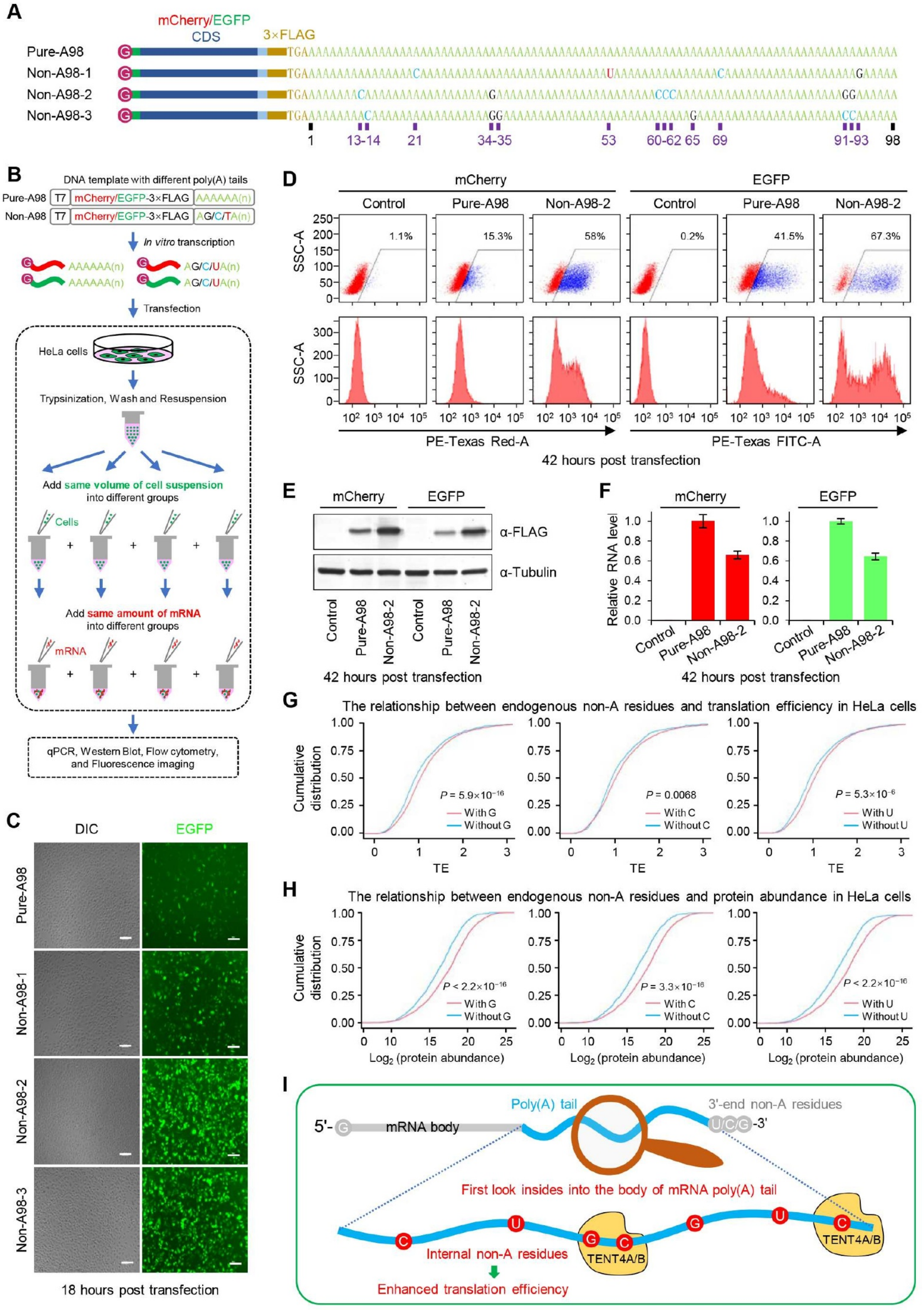

**Figure 1.** Poly(A) tail internal non-A residues promote translation of IVT mRNA. A, Schematic of reporter mRNAs with different non-A residues in poly(A) tails of the same length. The positions of non-A residues in poly(A) tail are indicated as numbers on the bottom. B, Schematic of the translation efficiency reporter system based on mRNA transcripts with different types of poly(A) tails. C, Representative images of HeLa cells 18 h after transfection with reporter mRNAs with different poly(A) tails. Left panels show images of all cells captured by differential interference contrast (DIC) microscope. Right panels show the corresponding fluorescent images of expressed reporters. Scale bar, 100 μm. D, FACS quantification of reporter mRNA expression in HeLa cells 42 h post transfection. The mCherry reporter is shown on the left, and the EGFP reporter is shown on the right. E, Representative image of protein level by Western blot (anti-FLAG antibody was used to detect the FLAG tag for all reporters) at 42 h post transfection of HeLa cells with the reporter mRNA. α-Tubulin is used as an internal control for Western blot analysis. Three biological replicates were performed for Western blot analysis. F, Quantification of mRNA level by RT-qPCR at 42 h post transfection of HeLa cells with the reporter mRNA. *β-Actin* is used as an internal control for RT-qPCR analysis. Three biological replicates were performed for RT-qPCR. Error bars represent standard deviation. G, Cumulative distribution function (CDF) plots of mRNA-normalized TE for genes with or without non-A residues in HeLa cells (Park et al., 2016) (gene number: with G, $n$=2,075; without G, $n$=810; with C, $n$=2,323; without C, $n$=562; with U, $n$=1,884; without U, $n$=1,001). Genes with or without poly(A)+ mRNA containing G, C, or U residues were considered as genes with or without G, C, or U residues. Genes with at least 10 circular consensus sequencing (ccs) reads were included in the analysis. $P$ value was calculated by the Kolmogorov-Smirnov (KS) test. Statistical details were included in Table S2. H, CDF plots of protein abundance quantified by mass spectrometry for genes with or without non-A residues in HeLa cells (Bekker-Jensen et al., 2017) (gene number: with G, $n$=2,398; without G, $n$=1,029; with C, $n$=2,694; without C, $n$=733; with U, $n$=2,166; without U, $n$=1,261). The $P$ value was calculated by the KS test. Statistical details were included in Table S2. I, A model for the full view of a complete single mRNA molecule. The magnified part highlights the non-A residues in the body of poly(A) tails, which are conserved in eukaryotes. The non-A residues in the body of poly(A) tails can enhance the translation of the corresponding mRNA.

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further confirm this effect of non-A residues in promoting translation, we also tested different time points after mRNA transfection and found consistently stronger fluorescence from 6 to 120 h after transfection. To quantify fluorescence in transfected cells, we defined two gates for the fluorescent-positive population. A permissive gate (P2) was set to include all cells with fluorescence above the untransfected control, while a more stringent gate (P3) was set to isolate only the cells with a strong positive signal. At 24 h after mRNA transfection, we observed 1.2-fold fluorescence positive cells while 5-fold relative fluorescence intensity as measured by the P2 gate, and we observed 3-fold fluorescence positive cells together with 2-fold relative fluorescence intensity as measured by the P3 gate (Figure S1). Together, these results demonstrate that non-A residues markedly enhance the translation efficiency of IVT mRNA.

The above findings demonstrate that non-A residues enhance the translation efficiency of IVT mRNAs. To test whether this effect also applies to endogenous transcripts, we asked whether non-A residues presented in endogenous cellular mRNAs correlate with higher translation efficiency. Therefore, we investigated the relationship between translation efficiency and non-A residues using the available ribosome engagement data and the poly(A) tail non-A residue data for human cells (Legnini et al., 2019; Park et al., 2016).

We compared cumulative distribution plots of translation efficiency for genes whose mRNAs do or do not contain non-A residues, since cumulative distribution plots have been widely used in analyzing translation efficiency and non-A residues in poly(A) tails (Lim et al., 2018). Interestingly, genes whose mRNAs bear G, C or U residues showed significantly higher translation efficiencies in HeLa cells (Figure 1G; Table S2). Given that mRNA translation ultimately results in the accumulation of proteins, we next investigated the relationship between non-A residues and protein abundance in HeLa cells, for which the mass-spectrometry-based proteomic data were available (Bekker-Jensen et al., 2017). The results showed that proteins encoded by genes whose mRNAs harbor non-A residues accumulated to significantly higher abundance than those encoded by genes whose mRNAs do not in HeLa cells (Figure 1H; Table S2). This correlation is consistent with that seen in the ribosome engagement data. These results suggest that the non-A residues are positively associated with translation efficiency, which may serve as regulators of translation of endogenous mRNAs, demonstrating that poly(A) tails can act as active regulators of mRNA translation rather than simple structural components. Therefore, these unbiased transcriptome-wide analyses of endogenous mRNA non-A residues and translation strongly support our finding that non-A residues enhance the translation of IVT mRNA.

Tremendous success has been achieved in mRNA vaccine against the global COVID-19 pandemic. Improving translation efficiency has been one of the main technical challenges in mRNA-based therapeutics and vaccines (Sahin et al., 2014). In this study, we demonstrate that non-A residues are conserved novel RNA modifications in diverse eukaryotes and that they can enhance mRNA translation (Figure 1I). The discovery that non-A residues enhance translation of IVT mRNA provides an entirely new strategy to promote the development of mRNA-based therapeutics and vaccines. The molecular mechanism by which poly(A) tail internal non-A residues enhance translation remains unclear. We hypothesize that these residues either recruit specific RNA-binding proteins or alter the affinity of poly(A)-binding protein (PABP). These changes may modulate the interaction between the poly(A) tail and the 5′-cap, influencing the assembly of the translation initiation machinery. Additionally, the specific dose, position, and combination of non-A residues likely have distinct effects on translation efficiency. Elucidating the underlying mechanisms is a key area for future investigation.

These non-A residues are regular ribonucleotides, which can be easily designed directly into the mRNA synthesis template, ensuring their complete and reproducible incorporation into IVT mRNA product. Moreover, as this new strategy is different from all existing approaches in improving the translation of mRNA drugs, it can be readily incorporated into current mRNA development pipelines together with other optimizations, such as modified nucleosides that reduce immunogenicity and increase stability. In summary, incorporating non-A residues into poly(A) tails provides a straightforward, scalable means to enhance IVT mRNA translation and holds considerable translational potential for advancing mRNA therapeutics and vaccines.

## Compliance and ethics

Yusheng Liu and Falong Lu are named inventors on a patent application filed by Institute of Genetics and Developmental Biology covering the discovery in this manuscript.

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## Supporting information

The supporting information is available online at https://doi.org/10.1007/s11427-025-3078-0. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

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