Global Incorporation of Synthetic ATP Analogs Reveals Poly(A)-Dependent Translation Differences in mRNA

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Abstract

Chemical modifications of nucleosides are essential for enhancing the efficacy of therapeutic mRNAs. While uridine analogs like N1-methylpseudouridine (m1Ψ) are well studied, adenine modifications remain underexplored, despite adenine's abundance and exclusive role in the poly(A) tail of eukaryotic mRNA. Inspired by the translational benefits of N4-acetylcytidine (ac4C), we designed and synthesized a series of novel N6-acylated ATP analogs. we systematically evaluated these analogs against established modifications, including N6-methyl (m6A), 2-amino (am2A) and 7-deaza (c7A) derivatives, by incorporating them into mRNAs via global substitution. Our investigation highlights the efficacy of the newly developed N6-acetyl (ac6A) modification. In non-polyadenylated mRNAs, ac6A substitution significantly enhanced translation, achieving a threefold increase over unmodified mRNA. Importantly, in polyadenylated mRNAs, ac6A mRNA maintained translation efficiency comparable to natural mRNA, demonstrating its high biocompatibility. Surprisingly, am2A modification displayed striking poly(A)-dependent translational behavior. While am2A modified mRNA showed a threefold translation increase in the absence of a poly(A) tail, its efficiency dropped to 6% of natural levels upon poly(A) addition. Structural simulations revealed that am2A group introduces steric clashed and electrostatic repulsion with poly(A) binding protein (PABP), hindering closed-loop formation and reducing translation. Overall, this work expands the chemical space of adenine modifications with effective N6-acylated analogs and highlights that region-specific modifications can be strategically exploited to optimize translation efficiency for mRNA therapeutic design.

Keywords: mRNA, Adenosine analogs, N6-acylation, Poly(A) tail, Translation efficiency

INTRODUCTION

Messenger RNA (mRNA) has emerged as a transformative platform in modern therapeutics, and chemical modification has become a core principle in mRNA design, as it profoundly influences mRNA stability, immune recognition, and translation efficiency [1]. Synthetic chemical modifications targeting the base, ribose, and phosphate backbone have been developed to fine-tune mRNA properties [2-6]. The clinical success of Pfizer-BioNTech and Moderna COVID-19 mRNA vaccines, which utilize N1-methylpseudouridine (m1Ψ) to suppress innate immune activation and maximize protein production, highlights the critical role of synthetic chemical modification [7-9].

In contrast to the extensive exploration of uridine modifications, the therapeutic potential of adenosine modification strategies remains largely unexplored, despite its unique functional significance. Adenosine is not only ubiquitous within coding and untranslated regions but also constitutes the entirety of the 3' poly(A) tail, a critical structural domain that binds the poly(A)-binding protein (PABP) [10-12]. The PABP-poly(A) complex interacts with the 5' cap-binding factor eIF4G to form a closed-loop structure, which synergistically enhances translation initiation by increasing eIF4E's functional affinity for the 7-methylguanosine (m7G) cap, and protect the mRNA from degradation [5, 13, 14]. This synergy, recapitulated in nuclease-treated rabbit reticulocyte lysates (RRL), is abrogated when PABP binding to poly(A) is disrupted, highlighting the poly(A) tail's indispensable role in optimal mRNA function [15-17]. Thus, chemical modification of adenosine could exert profound effects on both local RNA structure and the global formation of this critical regulatory complex.

The functional versatility of adenosine is exemplified by N6-methyladenosine (m6A), the most abundant internal modification in eukaryotic mRNA. m6A orchestrates nearly all aspects of mRNA metabolism, including splicing, nuclear export, translation, and decay, through the recruitment of reader proteins [18-20]. This naturally occurring modification highlights the profound regulatory potential inherent to the adenosine N6-position. However, while m6A serves as a dynamic epitranscriptomic mark, its direct application as a synthetic substitute for unmodified adenosine in therapeutic mRNAs has not yielded consistent translational benefits -suggesting that the chemical space for optimizing adenosine function extends beyond methylation [21].

Recent chemo-enzymatic advances have enabled the synthesis of diverse modified ATP analogs for incorporation into RNA[22, 23]. For example, substitution with 2-

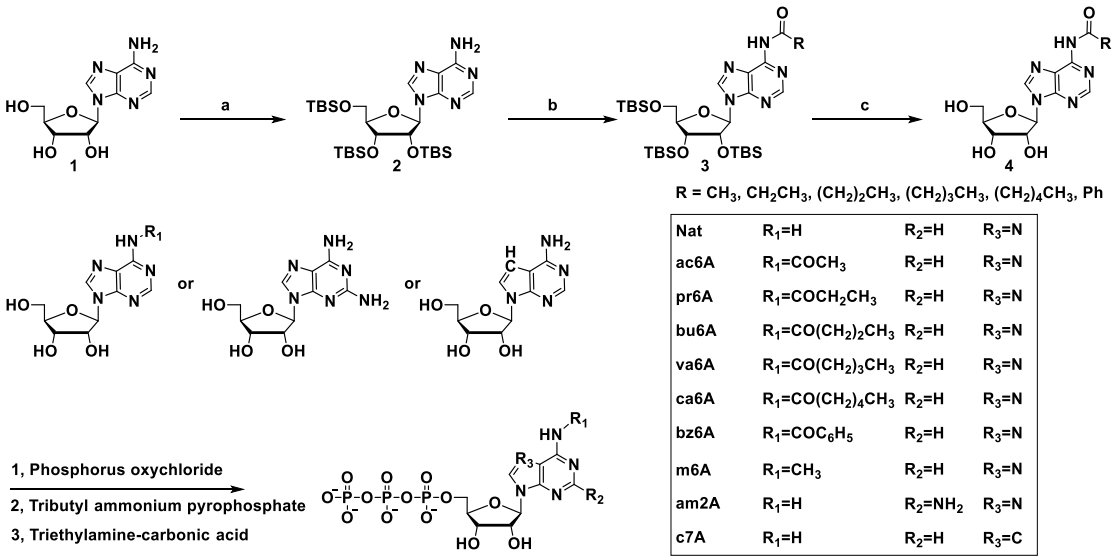
aminoadenine (diaminopurine) in full-length mRNAs was shown to enhance translational capacity [24], while backbone modifications such as phosphorothioate linkages can be strategically incorporated into the poly(A) tail to prevent deadenylation without impairing protein expression [25]. Further expanding the toolbox, modified ATP analogs bearing bioorthogonal groups enable intracellular mRNA tracking [26-28], and complex architectures such as "multi-tailed" mRNAs have been shown to extend protein expression [29, 30]. These studies demonstrate the power of adenosine modifications in optimizing mRNA function. However, the limited diversity of ATP analogs with translational benefits restricts our ability to fully exploit this chemical space. Notably, N4-acetylcytidine (ac4C) has recently been identified as a potent enhancer of translation [31], but analogous acylated strategies for adenosine remain unexplored.

To bridge this gap, we drew inspiration from the regulatory significance of the N6-site, which is exemplified by m6A, and the translation-promoting chemistry of acylation, which is exemplified by ac4C. Here, we designed and synthesized a series of novel N6-acylated ATP analogs (**Fig. 1A**) and evaluated them alongside them against established modifications, including N6-methyl (m6A), 2-amino (am2A), and 7-deaza (c7A) variants. By globally incorporating these analogs into firefly luciferase mRNAs, we systematically profiled their impact on translation, stability, and immunogenicity. Our investigation not only identifies N6-acetyl (ac6A) as a highly effective modification. It enhances translation threefold in tail-less mRNAs while maintaining native levels in polyadenylated mRNAs. Our work also uncovers a striking context-dependent effect: the translational outcome of adenine modifications is profoundly dictated by the presence of a poly(A) tail. This phenomenon was most dramatically exemplified by am2A, which boosted translation in tail-less mRNAs yet suppressed it in polyadenylated mRNAs to ~6% of native levels. Structural simulations revealed that the am2A group likely disrupts PABP binding, which hinders closed-loop formation. Overall, this work expands the chemical space of adenine modifications through the development of functional N6-acylated analogs and demonstrates that the functional impact of base modifications is highly context-dependent. This behavior is directly dictated by the presence of a poly(A) tail. These findings highlight the importance of tailoring modification strategies to distinct mRNA regions for the rational design of optimized mRNA therapeutics.

Results and Discussions

Synthesis and Characterization of Base-modified ATP Analogs

The chemical synthesis of base-modified adenosine triphosphates was conducted through a systematic modular approach (**Scheme 1**). Initially, the 2', 3', and 5'-hydroxyl groups of adenosine were protected using *tert*-butyldimethylchlorosilane (TBSCl) and imidazole in *N*,*N*-dimethylformamide to yield the fully protected nucleoside **2** with a 90% yield. To introduce diverse N6-modifications, compound **2** was reacted with various acyl chlorides in anhydrous pyridine, followed by selective desilylation using triethylamine trihydrofluoride to furnish the N6-modified adenosine derivatives (compound **4**). The resulting modified nucleosides were then converted into their corresponding triphosphates using a one-pot, three-step strategy, and purified by C18 reverse-phase HPLC. This procedure afforded a series of ATP analogs, including N6-acylated analogs N6-acetyladenosine (ac6A), N6-propionyladenosine (pr6A), N6-butyladenosine (bu6A), N6-valeryladenosine (va6A), N6-caproyladenosine (ca6A), N6-benzoyladenosine (bz6A) and non-acylated analogs N6-methyladenosine (me6A), 2-aminoadenine (am2A), and 7-deazaadenine (c7A). All final products were rigorously characterized by ^{1}H and ^{31}P NMR spectroscopy and high-resolution mass spectrometry (HRMS) to confirm their structural integrity and purity (Supplementary NMR/HRMS spectra).



Nat	R_1=H	R_2=H	R_3=N
ac6A	R_1=$COCH_3$	R_2=H	R_3=N
pr6A	R_1=$COCH_2CH_3$	R_2=H	R_3=N
bu6A	R_1=$CO(CH_2)_2CH_3$	R_2=H	R_3=N
va6A	R_1=$CO(CH_2)_3CH_3$	R_2=H	R_3=N
ca6A	R_1=$CO(CH_2)_4CH_3$	R_2=H	R_3=N
bz6A	R_1=COC_6H_5	R_2=H	R_3=N
m6A	R_1=CH_3	R_2=H	R_3=N
am2A	R_1=H	R_2=NH_2	R_3=N
c7A	R_1=H	R_2=H	R_3=C

Scheme. 1 The synthetic scheme for the base-modified ATP analogs. Reagents and conditions: a: t-butyl dimethyl chlorosilane, imidazole, DMF; b: corresponding acyl chloride, anhydrous pyridine; c: triethylamine trihydrofluoride, THF. For convenience, the nucleoside analogs were abbreviated: N6-acetyladenosine (ac6A), N6-propionyladenosine (pr6A), N6-butyladenosine (bu6A), N6-valeryladenosine (va6A),

N6-caproyladenosine (ca6A), N6-benzoyladenosine (bz6A), N6-methyladenosine (m6A), 2-aminoadenine (am2A), and 7-deazaadenine (c7A).

In vitro transcription of mRNA with Modified ATPs

To assess the transcriptional compatibility of the modified ATP analogs, we designed three firefly luciferase-encoding mRNA templates in parallel: one containing a poly(A) signal but no encoded tail ("signal Tail", **Fig. 1B**) which will introduce a native polyA tail in living cells, one lacking both the signal and tail ("Tail^{-}", **Fig. 1C**), and one bearing an encoded 110-nt poly(A) tail ("Tail^{+}", **Fig. 1D**). All transcripts were synthesized using T7 RNA polymerase in the presence of the respective ATP analogs and co-transcriptionally capped using anti-reverse cap analogs (ARCA) [32]. Transcription yields were quantified relative to a native mRNA control set at 100%.

In the "signal Tail" configuration (**Fig. 1B**), transcription yields with N6-modified analogs displayed a clear dependence on substituent size. Relatively small acyl groups (ac6A, pr6A, bu6A, va6A) were well tolerated, yielding 76%-83% as that of the control, whereas bulkier bz6A led to markedly reduced yields. Notably, m6A resulted in the lowest incorporation efficiency (~14%). am2A and c7A also impaired transcription, yielding ~44% and ~56%, respectively. A similar trend was observed for the "Tail^{-}" templates (**Fig. 1C**). Strikingly, the presence of an encoded 110-nt poly(A) tail ("Tail^{+}", **Fig. 1D**) substantially improved transcription output across most analogs. ac6A and pr6A reached ~89% and ~109% of the native ATP level, respectively, and even the poorly incorporated am2A and c7A analogs in the first two templates showed significant recovery.

Collectively, these results indicated that transcriptional efficiency is governed by the chemical nature and steric hindrance of adenine modification. Small N6 substituents (e.g., ac6A, pr6A) are well-tolerated by T7 polymerase, while bulkier groups or m6A significantly impair incorporation [33]. A defined 110-nt poly(A) tail provides a clear termination signal, which may facilitate efficient polymerase release and reduce non-specific extension, thereby ensuring the production of full-length, homogeneous mRNA [34-36].

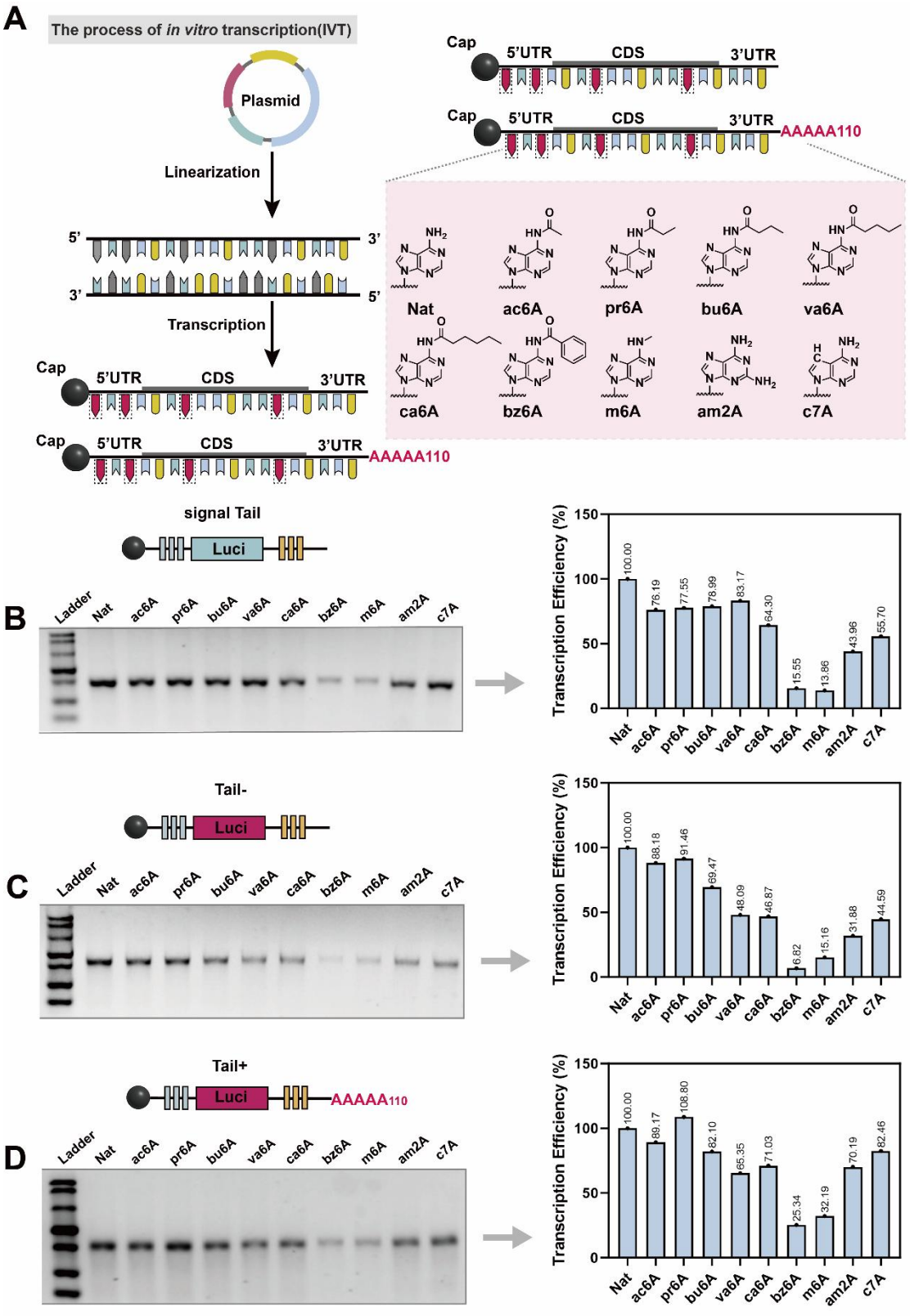


Fig.1 Transcriptional Incorporation Efficiency of Base-Modified ATPs. (**A**) Schematic workflow of IVT using canonical ATP or base-modified ATP analogs, with global substitution of adenine nucleotides. (**B-D**) Representative agarose gels and quantification of luciferase mRNAs synthesized from three templates: capped without

poly(A) tail (**B**, **C**) and capped with 110-nt poly(A) tail (**D**). All analogs supported full-length mRNA synthesis, though transcription efficiencies were reduced compared with canonical ATP. bz6A and m6A exhibited the lowest yields, whereas other modifications were only slightly impaired relative to the unmodified control.

In vitro translation of of Modified mRNAs in Cell-Free and Cellular Systems

We next evaluated the translational efficiency of the base-modified mRNAs using RRL, a well-established eukaryotic cell-free system that enables precise assessment of intrinsic translational capacity independent of cellular stability or transport variables [15, 17], along with validation in mammalian cells (**Fig. 2A**).

In the RRL system, capped mRNAs lacking a poly(A) tail ("signal Tail" and $Tail^{-}$) exhibited pronounced, modification-dependent variability in protein output (**Fig. 2B, C**). Strikingly, mRNAs bearing ac6A or am2A substitutions produced the strongest luminescence signals, reaching approximately threefold higher levels than the native ATP control, which is consistent with previous reports that cytidine acetylation enhances translation efficiency [31]. Moderate enhancement was observed for pr6A, bu6A, and va6A derivatives, whereas bz6A, m6A, and c7A analogs severely impaired translation.

The introduction of a 110-nt poly(A) tail ($Tail^{+}$) dramatically reshaped this landscape (**Fig. 2D**). While most analogs exhibited largely homogenized translational output, two distinct behaviors emerged. First, modifications that were incompatible in the tail-less context, namely bz6A, m6A, and c7A, remained repressed regardless of tail presence. This finding suggests a fundamental incompatibility with the translation machinery that is independent of poly(A) regulation. More strikingly, the am2A analog exhibited a complete functional reversal. It shifted from the highest expression in non-tailed mRNAs to nearly undetectable levels upon poly(A) addition. This represents a reduction of approximately 17-fold, to just ~6% of native mRNA levels. By contrast, ac6A demonstrated remarkable robustness. It maintained translation efficiency comparable to native mRNA in the presence of the poly(A) tail while retaining its enhancement capacity in tail-less contexts.

These trends were recapitulated in mammalian cells, where only capped and polyadenylated mRNAs were sufficiently stable for reliable measurement (**Fig. 2E**). Consistent with RRL findings, ac6A supported translation at levels comparable to native mRNA. Meanwhile, am2A remained severely repressed. This confirms that the

poly(A)-dependent suppression is not an artifact of the cell-free system.

Mechanistically, the observed effects align with distinct molecular origins. For m6A, the severe reduction across all contexts is consistent with reports that global incorporation interferes with canonical initiation [37]. It also impairs ATP-dependent scanning, reduces start codon recognition, and increases ribosome pausing during elongation. Additionally, m6A recruits YTHDF proteins. These proteins accelerate mRNA decay and promote sequestration into stress granules, which collectively diminish translation output [20, 38-41]. For am2A, however, the context-dependent reversal points to a distinct mechanism. This mechanism specifically involves poly(A) tail function. Previous studies have suggested that am2A substitution within the poly(A) tail may diminish PABP affinity [24] [42]. This raises the possibility that global incorporation disrupts PABP engagement.

To directly test whether altered PABP-poly(A) interactions underlie this reversal, we performed molecular dynamics simulations based on the PABP-poly(A)-eIF4G ternary complex. This is discussed in the next section. Collectively, these findings underscore that translation efficiency is determined not solely by nucleotide identity, but by the concerted interplay between adenine modification and poly(A)-mediated regulation.

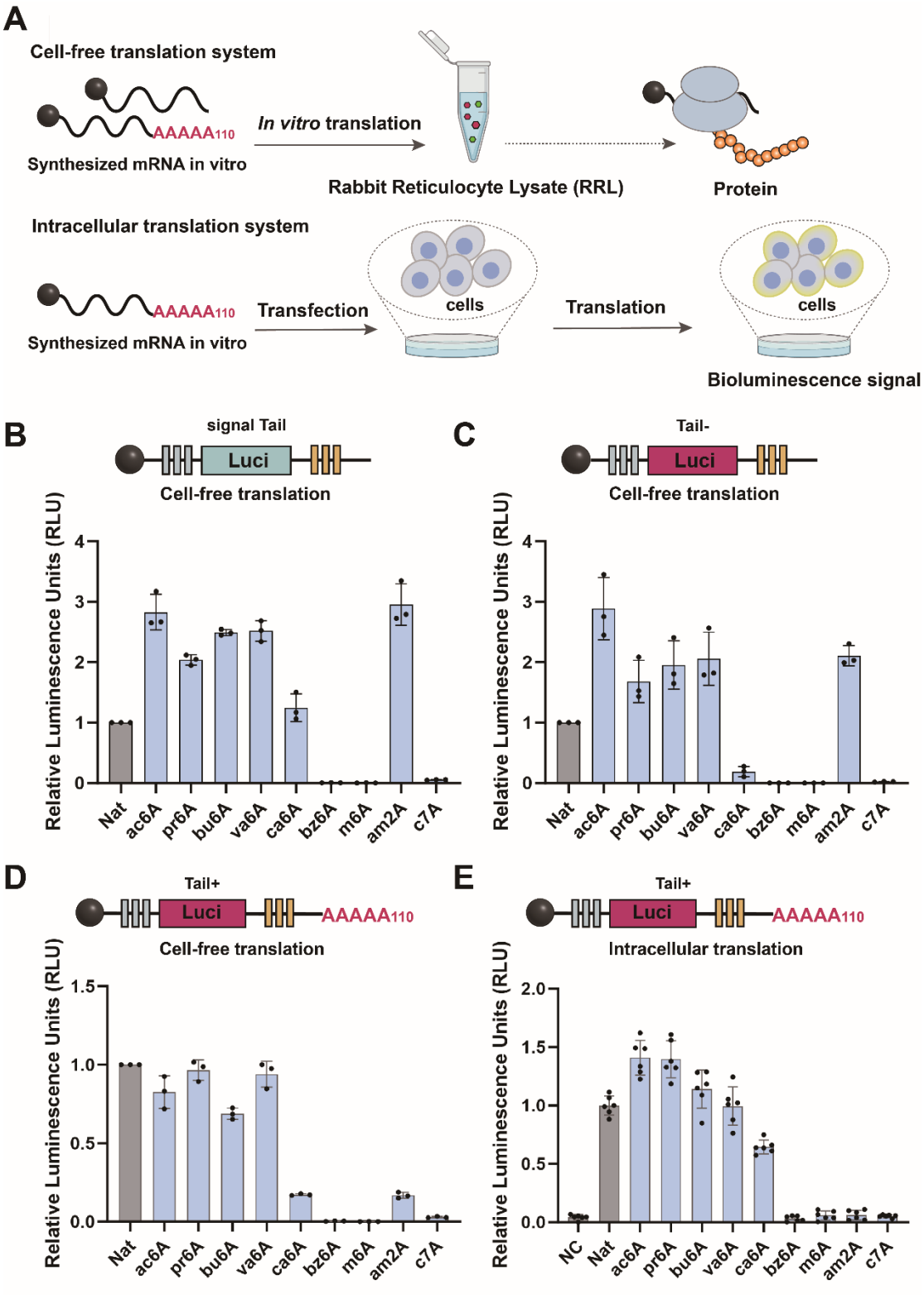


Fig.2 Translation Efficiency of Base-Modified mRNAs in Cell-Free and Cellular Systems. (**A**) Experimental workflow for assessing translation in rabbit reticulocyte lysate (RRL) and mammalian cells. (**B-D**) Translation efficiencies of capped luciferase mRNAs with or without 110-nt poly(A) tails, fully substituted with indicated ATP analogs. For non-polyadenylated transcripts, N6 modifications and the am2A analog

enhanced translation relative to canonical ATP, with ac6A reaching ~3-fold higher protein levels. In contrast, polyadenylated transcripts exhibited attenuated or suppressed translation, particularly for the am2A analog, which showed ~15-fold lower efficiency compared to the unmodified control. (**E**) Comparison of translation efficiency in Hela cells, showing consistent trends with in vitro assays.

Computational modelling of modified poly(A) tails with PABP

The striking context-dependent reversal of the am2A modification points to a tail-specific regulatory mechanism. This modification enhances translation in tail-less mRNAs but suppresses it upon poly(A) addition. Given that global incorporation modifies every adenosine within the poly(A) tail, we hypothesized that am2A substitution disrupts PABP recognition. This disruption thereby destabilizes the closed-loop complex essential for cap-dependent initiation [13]. To test this, we performed molecular dynamics (MD) simulations of the PABP-poly(A)-eIF4G ternary complex (PDB: 4F02). We compared natural poly(A) with poly(am2A) and poly(m6A) in these simulations.

Simulations revealed that natural poly(A) rigidified the PABP structure (RMSD <2.0 Å). In contrast, poly(am2A) failed to stabilize the protein and exhibited high structural lability that was comparable to RNA-free PABP (RMSD >5.0 Å) (**Fig. 3A, S1**). Atomic-level analysis identified the mechanistic basis. The exocyclic am2A group created a direct steric clash and strong electrostatic repulsion with the positively charged guanidinium group of PABP residue Arg83 (R83) (**Fig. 3F**). This conflict disrupted canonical π-π stacking between A4 and A5. It also forced A5 out of the binding pocket and broke the cooperative inter-domain lock required for stable PABP function (**Fig. 3D**). By contrast, poly(m6A) destabilized PABP through a distinct mechanism. This mechanism involved a hydrophobic clash that induced backbone bending. It ultimately converged on the same outcome: impaired PABP-poly(A) engagement (**Fig. S2, 4E**).

These simulations demonstrate that the am2A modification directly compromises PABP-poly(A) interaction through atomic-level clashes. This observation provides a mechanistic explanation for the poly(A)-dependent translational suppression of this modification. Together with the robust performance of ac6A across contexts, these findings reinforce the concept of "base-tail interplay" as a critical determinant in mRNA therapeutic design.

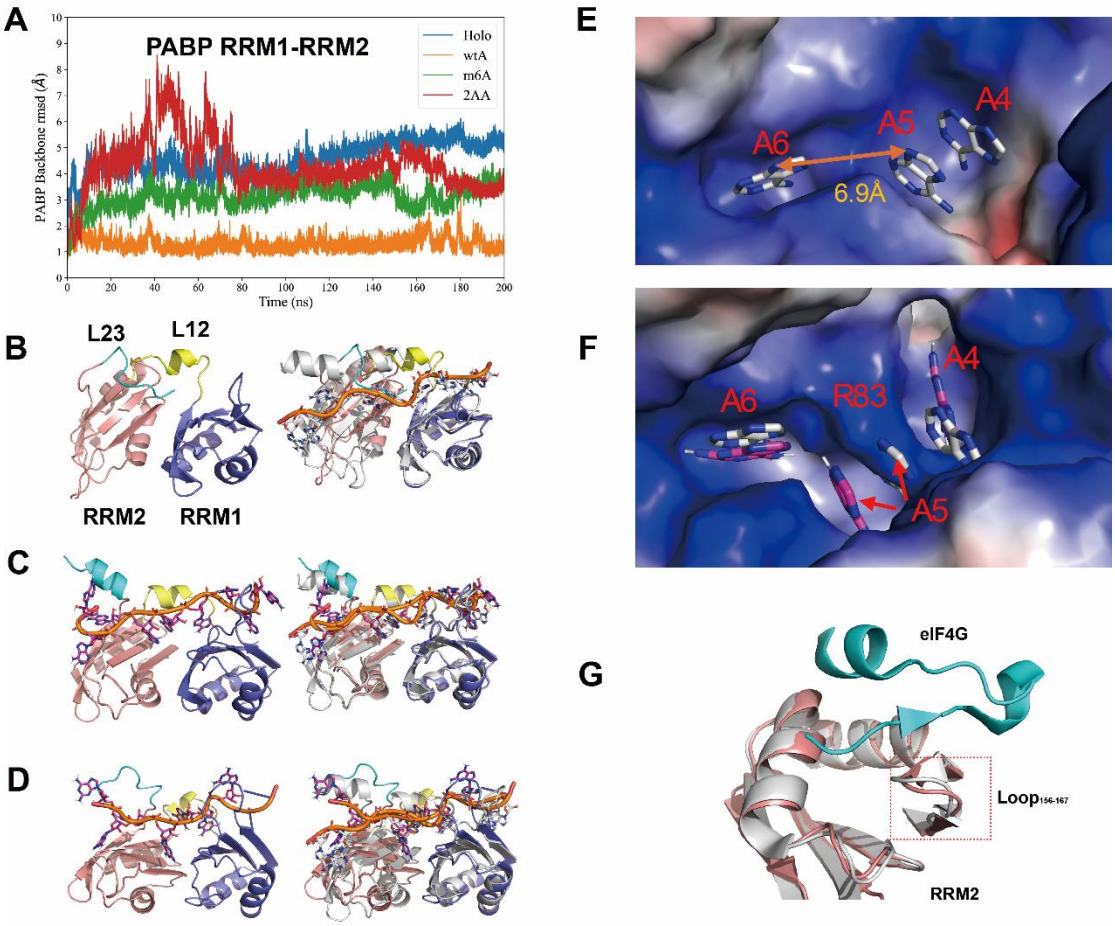


Fig.3. Structural basis of poly(A)-dependent destabilization of PABP induced by am2A adenine. (**A**) Backbone RMSD trajectories of PABP RRM1-RRM2 domains during 200 ns MD simulations in complex with natural poly(A), poly(m6A), poly(am2A) (2AA), and RNA-free PABP (holo). Natural poly(A) stabilizes the protein conformation, whereas modified poly(A) variants exhibit increased structural fluctuations comparable to the holo form. (**B**) Domain organization of PABP highlighting RRM1, RRM2, and linker regions (L12, L23). (**C-D**) Representative conformations from the final 10 ns of simulations showing altered positioning of the poly(A) tract and inter-domain rearrangement in modified complexes compared to the crystal structure. The left column is the protein/complex from MD simulations, while the right column shows the structural compassion to the crystal structure (PDB ID 4F02, colored in light gray) by superimposing the RRM1 domains. The protein is depicted as cartoon models with RRM1, L12 linker, RRM2 and L23 linker colored in tv-blue, yellow, pink and cyan, respectively. The backbone of polynucleotides is colored in orange with carbons of bases colored in purple. (**E**) Binding geometry of the central nucleotide triplet (A4–A6) in the wt poly(A)–PABP complex, maintaining canonical stacking interactions. (**F**) In the poly(am2A) complex, the exocyclic am2A group

introduces steric and electrostatic conflict with Arg83 (R83), disrupting base stacking and displacing A5 from the binding pocket. (**G**) Superposition of RRM2 domains reveals structural deviation in the eIF4G-interacting loop (residues 156-167), indicating allosteric perturbation of the closed-loop assembly interface.

Intracellular stability of modified mRNAs

The MD simulations suggested that am2A modification destabilizes the PABP-poly(A) interaction, which is known to protect mRNA from deadenylation and decay. To investigate whether these structural disruption leads to accelerated the modified mRNA degradation in cells, HeLa cells were transfected with polyadenylated ($Tail^+$) luciferase mRNAs substituted with various ATP analogs (**Fig. 4A-C**). At 6 h post-transfection (**Fig. 4B**), bz6A and m6A transcripts were nearly undetectable. This minimal signal likely reflects rapid clearance and impaired PCR amplification due to steric hindrance from bulky modifications [43-45]. For m6A, the accelerated decay is consistent with the recruitment of YTHDF proteins and subsequent sequestration into stress granules[20]. Conversely, the instability of bz6A may stem from altered base-stacking and disrupted secondary structures that increase nuclease susceptibility [46].

In contrast, pr6A and bu6A analogs-maintained mRNA levels 2.5- to 3.0-fold higher than the native control, supporting their sustained translation. Notably, am2A and c7A modifications exhibited a striking discrepancy between mRNA abundance and protein production. At 6 h, while am2A mRNA levels were comparable to the native control, its protein production was negligible (6% of control), indicating that the suppressed translation results from translational inactivation rather than transcript instability. However, by 24 h (**Fig. 4C**), am2A and c7A transcripts underwent a drastic decline, whereas N6-acylated analogs remained stable. This accelerated decay may reflect the well-established coupling between translation efficiency and mRNA stability, whereby mRNAs that fail to efficiently engage the translational machinery become increasingly susceptible to cellular mRNA decay pathways [47, 48].

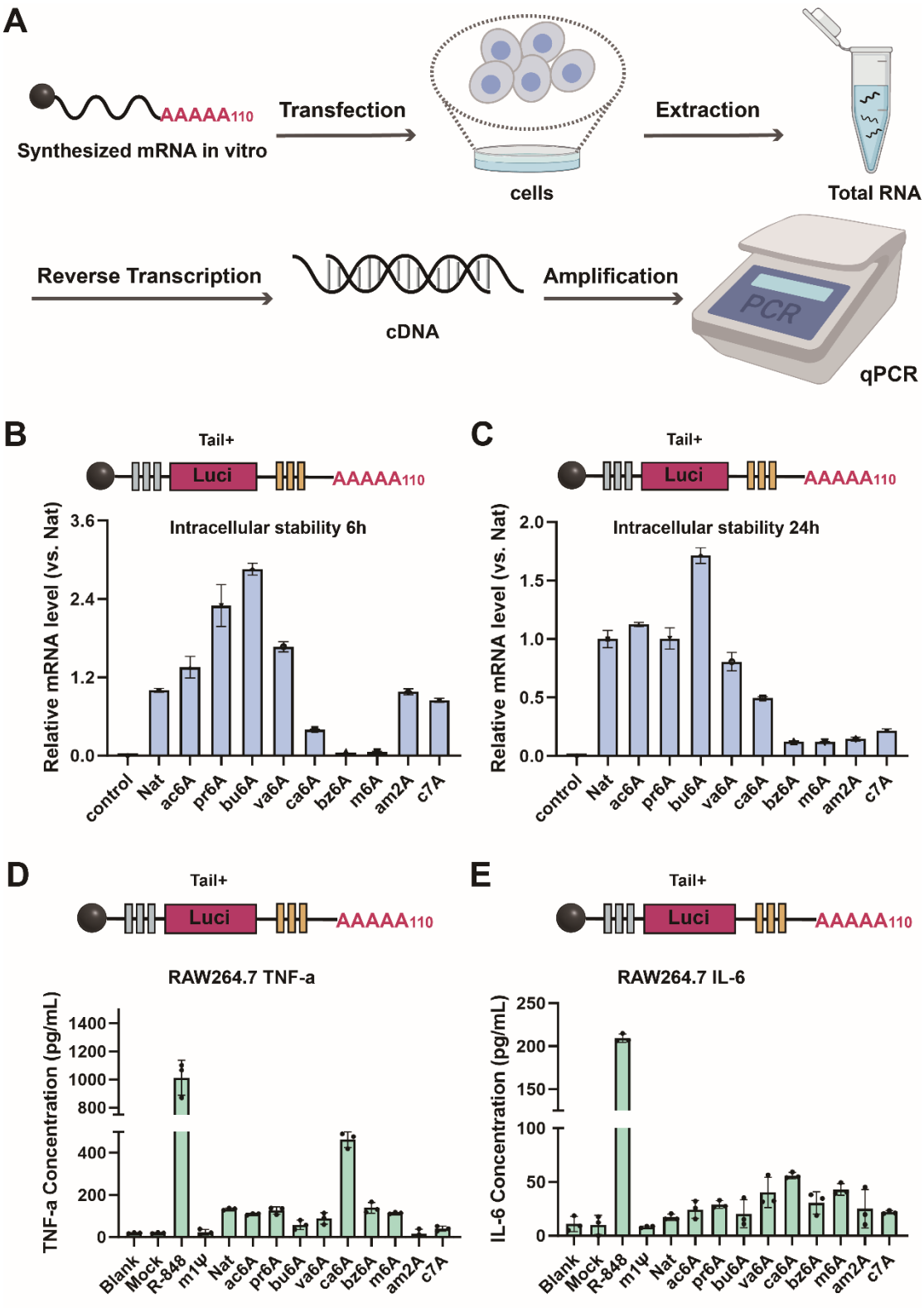


Fig. 4. Intracellular Stability and Immunogenicity of Base-Modified mRNAs. (**A**) Schematic of the assay to measure intracellular stability of capped and polyadenylated luciferase mRNAs fully substituted with ATP analogs in HeLa cells after transfection; mRNA abundance was quantified by RT-qPCR at 6 h and 24 h. (**B**-**C**) Quantification of intracellular mRNA levels at 6 h (**B**) and 24 h (**C**) post-transfection. N6 modifications

increased intracellular RNA abundance at 6 h, whereas am2A and c7A analogs exhibited reduced stability by 24 h. (**D-E**) Immunogenicity analysis in RAW264.7 macrophages compared with R848 (positive control), lipoMAX (mock control), and pseudouridine-containing mRNA. ca6A elicited the strongest immune response, whereas bu6A, am2A, and c7A analogs reduced immune stimulation relative to canonical ATP.

Immunogenicity of modified mRNAs in macrophages

We further evaluated the innate immunogenicity of the modified mRNAs by monitoring TLR7/8 activation in RAW264.7 macrophages, measuring the secretion of TNF-α and IL-6 as classical pro-inflammatory cytokines induced downstream of TLR7/8 signaling (**Fig. 4D, E**). R848 and lipofectamine-only (mock) were used as positive and negative controls, respectively. m1Ψ-modified mRNA, a well-established modification for reducing immunogenicity [49, 50], significantly suppressed cytokine release compared to the native control. Among the adenosine analogs, most N6-acylated variants elicited immune activation comparable to that of native mRNA. However, ca6A, which possesses the longest aliphatic chain, induced the most robust immune response, with both IL-6 and TNF-α levels elevated by approximately three-fold relative to the native control. The enhanced immunogenicity of ca6A may arise from the increased hydrophobicity introduced by the long aliphatic chain, which could perturb local RNA structure and promote recognition by innate immune sensors.

In contrast, both am2A and c7A modifications consistently reduced immunogenicity. Relative to the native control, IL-6 levels remained comparable, whereas TNF-α induction was reduced by approximately eight-fold and three-fold for am2A and c7A, respectively. Notably, TNF-α levels induced by am2A were even lower than those observed for m1Ψ-modified mRNA. The reduced immune profile of am2A is consistent with recent reports demonstrating that its incorporation yields mRNA with minimal immunogenicity while maintaining high translational capacity [24]. Together, these findings demonstrate that adenine base modifications distinctly modulate innate immune recognition, in addition to their effects on stability and translation. The contrasting immunogenic profiles of ac6A and am2A, c7A analogs underscore the potential for structure-guided engineering to decouple translation efficiency from immune activation, providing key insights for the design of therapeutic mRNAs with optimized safety and functionality.

Polyadenylation of base-modified mRNAs by E. coli poly(A) polymerase

To evaluate whether chemical modifications attenuate the compatibility of these ATP analogs with poly(A) polymerase, we first examined their enzymatic incorporation during poly(A) tail extension. Two RNA templates were used as substrates, and polyadenylation was performed using *E. coli* poly(A) polymerase (PAP) (**Fig. S3 and S4**). With the exception of the 7-deaza-modified ATP, all tested chemically modified ATP analogs were efficiently recognized and incorporated by PAP, indicating that chemical modifications at the adenine base did not impair their capacity as PAP substrates.

We subsequently evaluated these enzymatically polyadenylated mRNAs in rabbit reticulocyte lysate (RRL) translation assays and in cellular transfection experiments. However, the results were not sufficiently conclusive to support clear functional interpretations and are therefore not presented in the main text. One likely reason is that *E. coli* PAP typically generates poly(A) tails with heterogeneous lengths [51]. Because poly(A) tail length critically influences mRNA stability and translation efficiency, this variability complicates the interpretation of downstream functional outcomes. In addition, the modified ATP analogs exhibited strong substrate activity for *E. coli* PAP, frequently producing excessively long poly(A) extensions that could exceed one thousand nucleotides. Such unusually long tails may alter mRNA stability and turnover, further introducing uncertainty into functional analyses [51, 52]. As a result, the tail-length heterogeneity and excessive tail extension made it difficult to unambiguously associate any observed biological effects to the nucleotide modifications themselves.

Conclusion

In this work, we designed and synthesized a series of N6-acylated ATP analogs. This new modification class was inspired by the translation enhancing cytidine modification ac4C. We benchmarked these analogs against established controls including m6A, am2A, and c7A. Among all tested analogs, N6-acyl derivatives, particularly ac6A, exhibited the most balanced performance. They showed efficient transcription, robust translation, sustained stability, attenuated immunogenicity and full compatibility with poly(A)-dependent initiation. These results establish N6-acylation as a promising and chemically tunable strategy for adenosine engineering.

The control modifications behaved largely as expected. m6A impaired transcription and translation, while c7A nearly abolished protein expression. In striking

contrast, am2A adenine revealed an unexpected poly(A)-dependent bifurcation. It strongly enhanced translation in non-polyadenylated mRNAs but became one of the strongest inhibitors upon poly(A) tail addition. Structural modeling attributed this reversal to steric and electrostatic conflict between the exocyclic am2A group and PABP residue Arg83. This conflict disrupts closed-loop formation and abolishes poly(A)-mediated translational enhancement.

Together, these findings highlight how adenine chemistry interfaces uniquely with poly(A) tail function. N6-acyl analogs emerge as a broadly compatible modification class, while the am2A control uncovers a specific vulnerability in PABP-poly(A) recognition. These insights support a shift from global substitution toward region-specific modification strategies. This approach involves designing the coding region and poly(A) tail with distinct chemistries to optimize next-generation mRNA therapeutics in the future.

Acknowledge

We thank members of the Mei laboratory for helpful suggestions and comments on the manuscript. This work was supported by the following funding: Strategic Priority Research Program of the Chinese Academy of Sciences (XDB1410000), Guangdong Provincial Key Laboratory of Synthetic Genomics (2023B1212060054); Shenzhen Key Laboratory of Synthetic Genomics (SYSPG20241211173920047); Shenzhen Institute of Synthetic Biology Scientific Research Program (JCHZ20210002). We are grateful to the Shenzhen Infrastructure for Synthetic Biology for instrument support and technical assistance.

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