Review

N1-Methyl-Pseudouridine: The Evolution, Impact, and Future of a Key mRNA Vaccine Modification

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Review

N1-Methyl-Pseudouridine: The Evolution, Impact, and Future of a Key mRNA Vaccine Modification

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Abstract

Nucleoside modifications to the template of mRNA vaccines were important in achieving the desired level of antigen expression and immune response for the vaccine platform to function successfully during the COVID-19 pandemic. The innovation solved a crucial issue and resulted in the awarding of the 2023 Nobel Prize in Medicine to Katalin Karikó and Drew Weissman. Despite the spotlight on nucleoside modifications, several important aspects of its behavior in living systems were only discovered recently, particularly context specific translational accuracy, and the stimulation of +1 ribosomal frameshifting at specific "slippery sequences". The latter has been observed in living subjects (mouse and human) to produce both off-target antigens and an off-target immune response. While adverse effects cannot currently be attributed to poor translational fidelity, this may spell issues for future iterations of mRNA therapeutics if not addressed. Fortunately, in most cases, the transcript sequence can be modified to allay these concerns and produce faithful protein products, by avoiding sequence contexts resulting in either incorrect amino acid incorporation or +1 frameshifting. The modularity of the mRNA platform makes these fixes easy to incorporate, as long as this is accounted for in future mRNA therapeutics design.

Keywords: n1-methyl-pseudouridine; mRNA vaccines; Pfizer/BioNTech BNT162b2; Moderna mRNA-1273

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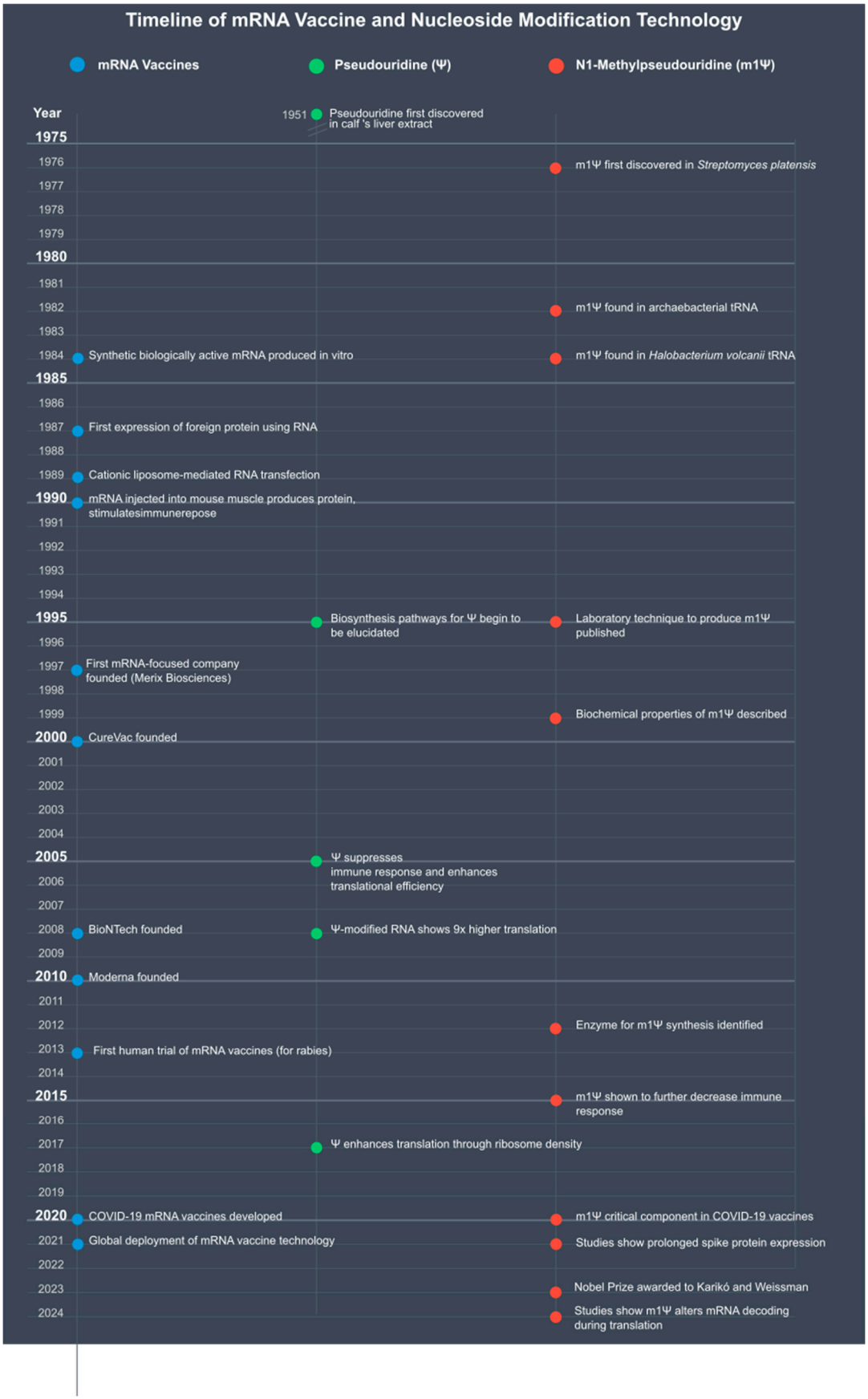

Introduction

Messenger RNA (mRNA) vaccines were well poised to respond to the challenge of the COVID-19 pandemic. While SARS-CoV-2 was spreading rapidly, the teams at Pfizer/BioNTech and Moderna produced prototype vaccines merely from the sequence released on January 10, 2020[1]. While

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previous generations of vaccines required laborious culturing from infectious material, Pfizer/BioNTech and Moderna were able to leverage the digital revolution in biology, and develop the vaccine sequence based on the publicly available genome of SARS-CoV-2 on GenBank [1].

At the core of the vaccine is the genetic code. All of the major COVID-19 vaccines, mRNA and otherwise, express the spike (S) protein from SARS-CoV-2, with some modifications. Importantly, for the vaccines to function, the genetic code needs to remain intact long enough to enter a cell, to find a ribosome, and be translated by the ribosome to produce the antigen, in this case the modified SARS-CoV-2 S protein.

Early mRNA vaccines used naked mRNA, composed of the same bases as mRNA in all organisms, cytosine (C), guanine (G), adenine (A) and uridine (U), which takes the place of the DNA base thymine (T) [2]. The development of mRNA vaccines demonstrated promising results in generating targeted immune responses and validating the platform's core concept [3]. However, initial formulations faced significant challenges due to rapid degradation of unprotected mRNA strands by host ribonucleases and unintended activation of innate immune pathways [4]. These biological defense mechanisms, which normally break down cellular transcripts or detect viral/bacterial RNA, often intercepted therapeutic mRNA before it could trigger the desired adaptive immunity [3]. Researchers subsequently addressed these issues through innovations in nucleotide modification and lipid nanoparticle delivery systems [5].In 2005, Karikó and Weissman demonstrated that by modifying the uridines to pseudouridines (Ψ), the immune response was lessened, and the modified mRNA was able to remain intact and express the target antigen in greater amounts than with naked mRNA [6](Figure 1). A further upgrade came in 2015, when experiments demonstrated that n1-methyl-pseudouridine (m1Ψ) modifications increased the translational efficiency further still [7]. However, although this was not an active concern at the time, Ψ-modified RNA was later found to exhibit reduced translational fidelity compared to naked RNA, which may result in the production of unintended proteins [8].. In 2019, m1Ψ-RNA demonstrated high translational fidelity compared to Ψ-RNA, at a similar level to naked mRNA, the standard for translational fidelity [9]. Other developments critical for the development of mRNA vaccines are shown in Figure 1, adapted from the events in [5]. These are broken down by events related to the development of mRNA vaccines [5,10–15], events related to pseudouridine [6,16–18], and events related to n1-methylpseudouridine [8,19–27].

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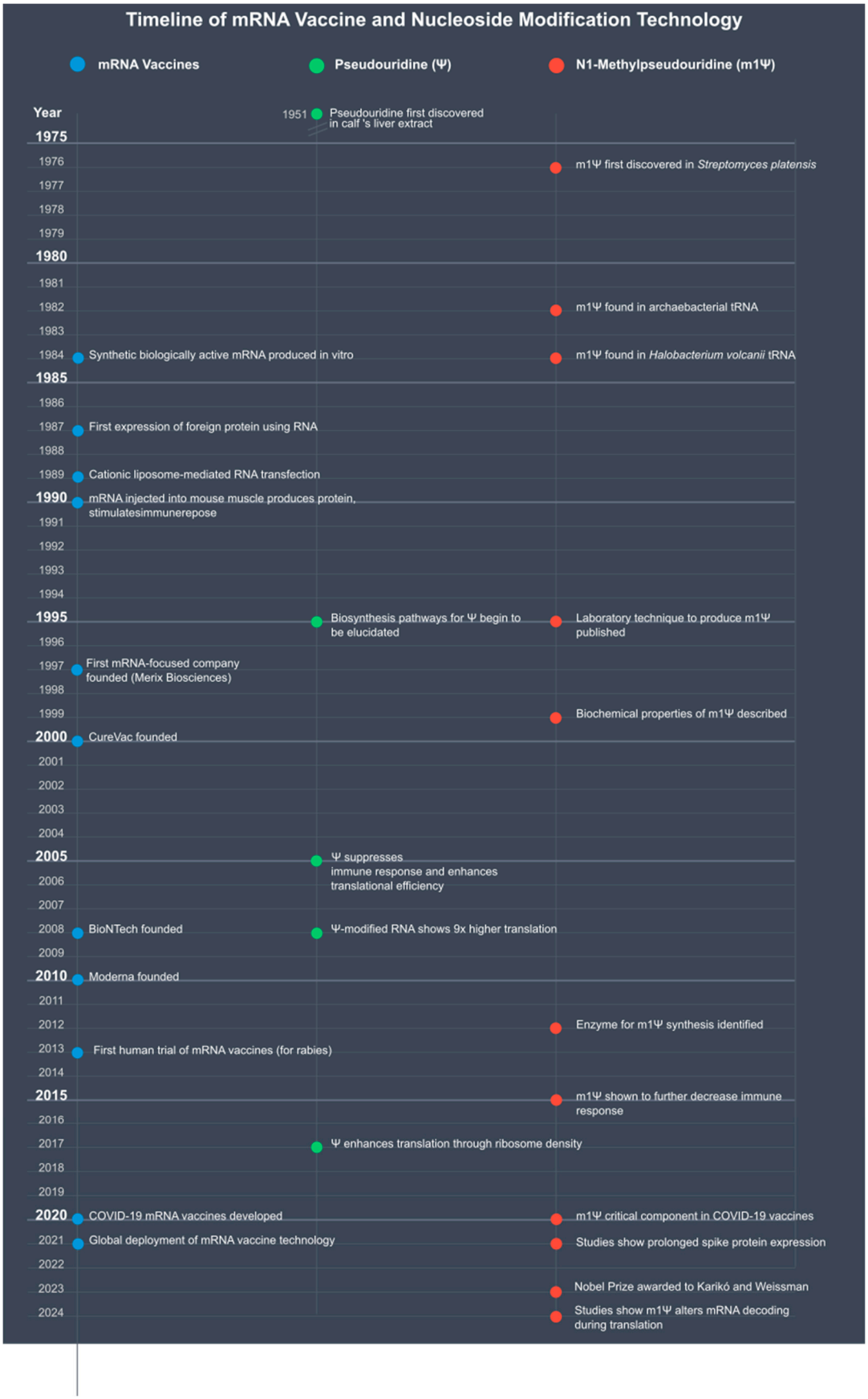

Figure 1. Timeline of events in the use of modified nucleosides in mRNA vaccines. Adapted from [5]. Timeline sources: mRNA vaccines events [5,10–15], pseudouridine [6,16–18], n1-methylpseudouridine [8,19–27].

The importance of these modifications to the effectiveness of mRNA vaccines is illustrated by a counterexample. CureVac, founded in 2000 to deliver RNA therapeutics, uses unmodified mRNA

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with codon-optimized sequences selected to minimize the use of uridine [28], as GU sequences can stimulate toll like receptors 7 and 8 to provoke an immune response [29,30]. However, CureVac's prospective vaccine performed comparatively poorly compared to the modified nucleoside mRNA vaccines, with the CureVac® vaccine achieving a mere 48% efficacy [31], significantly lower than the modified nucleoside mRNA vaccines [14,15]. CureVac has changed to using modified nucleosides in its recent vaccine candidates [32].

In short, nucleoside modifications are important for mRNA vaccines, as they allow the genetic code to enter the cytoplasm of the cell and be expressed [33]. Chemical modification of RNA allows them to escape host immune response [6,34,35] and also independently increase host ribosomal density [36]. Both of these factors increase the number of copies of the antigen produced. Karikó and Weissman's discovery enabled mRNA vaccines to express enough of the antigen for recognition by the host immune system [37]. Compared to unmodified RNA, pseudouridinylated RNA is translated roughly 9x more [18].

The discovery solved the crucial challenge of mRNA vaccines, allowing the lipid nanoparticle-encapsulated mRNA to produce amounts of the target antigen sufficient to stimulate a robust immune response. In 2020, their decades of work came to fruition when clinical trials of the Pfizer and Moderna messenger RNA vaccines, both using n1-methyl-pseudouridine in place of uridine, demonstrated efficacies of 95% (95% Confidence interval (CI), 90.3,97.6%) [14] and 94.1% (95% CI, 89.3 to 96.8%) [15], respectively. Katalin Karikó and Drew Weissman were awarded the 2023 Nobel Prize in Medicine for their breakthrough development of the chemical modifications of mRNA [38], which solved a crucial challenge in mRNA vaccines, and are likely responsible for millions of lives saved from COVID-19 [39].

The biology of Ψ and m1Ψ

The nucleobases of RNA were elucidated during the first half of the 20th century [40]. Pseudouridine, the first RNA modification to be discovered, and the most ubiquitous, was first discovered in 1951 in ribonucleic acid extracted from calf liver [16]. Following its discovery, over 150 additional nucleic acid modifications have been discovered [41], with wide ranging effects on gene expression [42]. Pseudouridine is ubiquitous throughout diverse organisms [43], including humans, where 0.2 to 0.6% of human RNA uridines are converted to pseudouridines by endogenous enzymes [44].

N1-methyl-pseudouridine was first discovered in 1976 in *Streptomyces platensis*, a bacterium [19]. Later discoveries of m1Ψ in 1982 in an archaea (*Halococcus* morrhuae) transfer RNA [21] and again in 1984 in *Halobacterium volcanii* [22]. Additional archaea species containing m1 Ψ have since been discovered, shown in Figure 2, including *Methanococcoides burtonii* [45], *Methanosarcina barkeri* [46], *Archaeoglobus fulgidus* [47], *Methanococcus voltae* [48], *Methanococcus jannaschii* [49], *Methanomicrobium mobile* [50], *Halococcus morrhuae* [51], Haloferax volcanii [52], *Halobacterium salinarum* [53], *Pyrobaculum islandicum* [54], *Thermoproteus neutrophilus* [55], *Methanococcus igneus* [56], *Methanothermus fervidus* [57] and *Methanococcus vannielii* [58].

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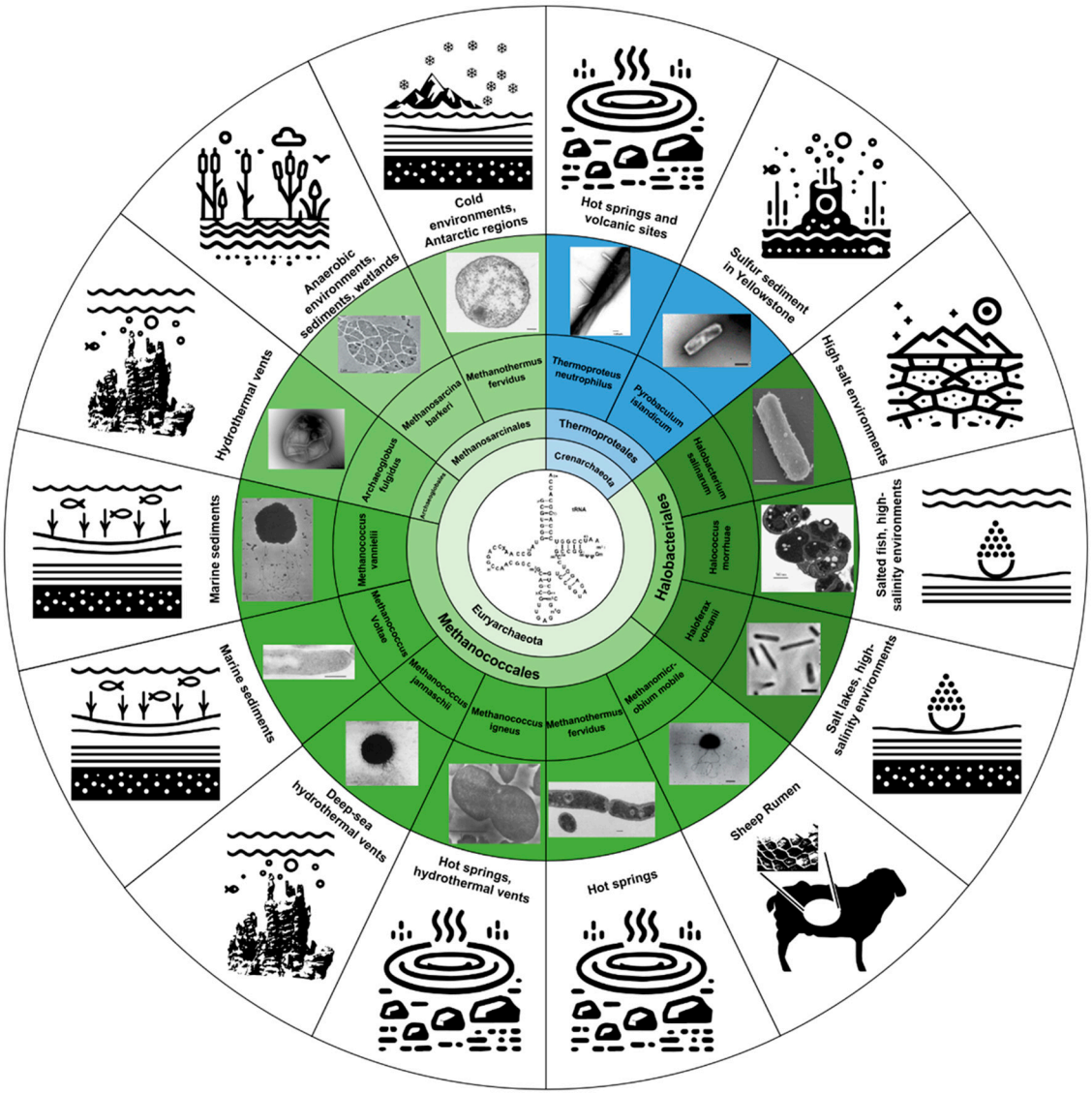

Figure 2. Archaeal species with naturally occurring m1Ψ, and their habitats. Adapted from [59]. Images for Methanosarcina barkeri [46], *Archaeoglobus fulgidus* [47], *Methanococcus voltae* [48], *Methanococcus jannaschii* [49], *Methanomicrobium mobile* [50], *Halococcus morrhuae* [51], Haloferax volcanii [52], *Halobacterium salinarum* [53], *Pyrobaculum islandicum* [54] are reproduced under Creative Commons licenses, from their respective references. The image for *Thermoproteus neutrophilus* [55] is reproduced with permission from Prof. Lennon. Image for *Methanococcoides burtonii* is reprinted from A Methanogenic Archaeon from Ace Lake, Antarctica: *Methanococcoides burtonii sp. nov.*," P.D. Franzmann, N. Springer, W. Ludwig, E. Conway De Macario, M. Rohde, *Systematic and Applied Microbiology*, Vol. 15, Issue 4, pp. 573–581, 1992. [45], with permission from Elsevier. Image for *Methanococcus igneus* is reprinted from *Systematic and Applied Microbiology*, Vol. 13, Siegfried Burggraf, Hans Fricke, Annemarie Neuner, Jakob Kristjansson, Pierre Rouvier, Linda Mandelco, Carl R. Woese, Karl O. Stetter, *Methanococcus igneus sp. nov., a Novel Hyperthermophilic Methanogen from a Shallow Submarine Hydrothermal System*, pp. 263-269. Reference [56], Copyright (1990), with permission from Elsevier. The images of *Methanothermus fervidus* [57] and *Methanococcus vannielii* [58] are reproduced from their associated references, with permission from Elsevier.

In archaea, m1Ψ has only been found at position 54 of tRNA (Figure 3A). In *Saccharomyces cerevisiae*, the enzyme Nep1 catalyzes the conversion of the uridine at position 1191 of 18S ribosomal RNA (rRNA) into m1Ψ (Figure 3B). While, to our knowledge, m1Ψ has not been directly observed

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in other eukaryotic cells, it is plausible that m1Ψ may be present in the 18S rRNA of other eukaryotic species sharing Nep1 or an ortholog [59]. m1Ψ is thus rarely observed in nature, and its ubiquitous use as artificially incorporated in mRNA COVID-19 vaccines is without precedent.

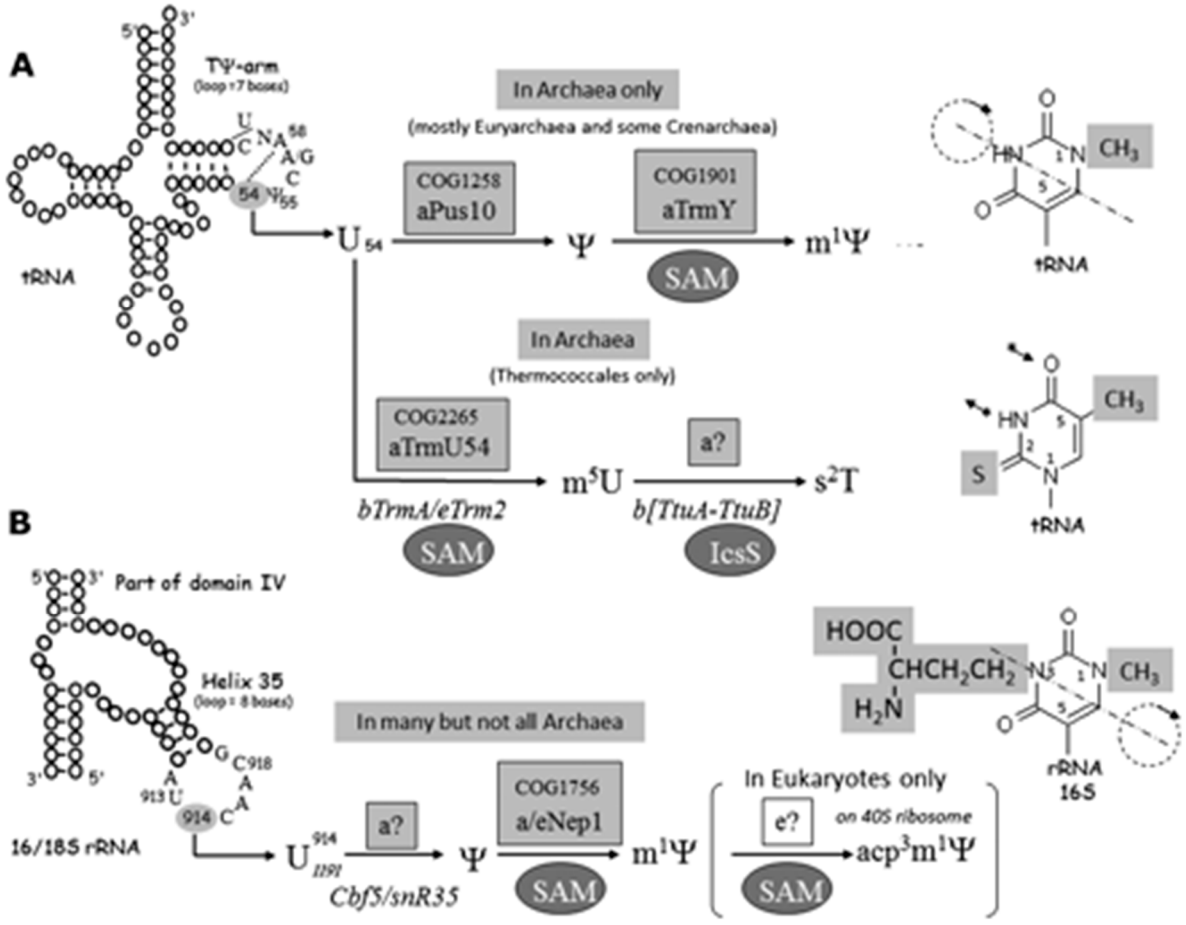

Figure 3. Two biological pathways where n1-methyl-pseudouridine is present. Panel A. tRNA modifications at position 54 in the conserved TΨ-loop of tRNA. The modification pathway varies by organism type, in most archaea, U54 is first converted to pseudouridine (Ψ54) by the enzyme aPus10, then can be further methylated to m1Ψ54 by the methyltransferase TrmY1. In Thermococcales, Bacteria, and Eukarya, U54 is directly methylated to m5U54 by different methyltransferases (aTrmU54, bTrmA, and eTrm2p respectively). Panel B. Modifications in the 16S rRNA helix 35 region. In Archaea U914 is converted to pseudouridine by an unidentified archaeal enzyme system. In Eukarya the corresponding U1191 in 18S rRNA is modified by a snoRNP complex containing the eCbf5 pseudouridine synthase and snR35 guide RNA. The pseudouridine can be methylated to m1Ψ by the Nep1 methyltransferase, and in some eukaryotes, further modified to acp3m1Ψ. The right side of the figure displays the chemical structures of these various uridine derivatives, with dashed lines indicating the axis of rotation during isomerization and asterisks marking atoms involved in reverse Hoogsteen base pairing.Reproduced from [59] under a Creative Commons License **(Attribution-NonCommercial 4.0 International License)**.

Important differences from uridine

Little is known about the role of m1 Ψ in nature, though recent experiments have elucidated how it behaves in biological systems. As m1Ψ has a different chemical structure than both U and Ψ, there are subtle differences in base pairing energies [8,60]. This may result in context-specific translational fidelity, which has been observed for m1Ψ [8]. Earlier work on the translational fidelity of m1Ψ had characterized tRNA misincorporation levels for a subset of possible codons, finding accuracy similar to uridine-containing mRNA codons [9]. The signal of altered translational fidelity may have been missed in these initial experiments. The error rate for m1Ψ-incorporated transcripts,

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while lesser than that for Ψ-incorporated transcripts, was 25% higher (p=0.006) than for U-containing transcripts (Figure 4A) [61].

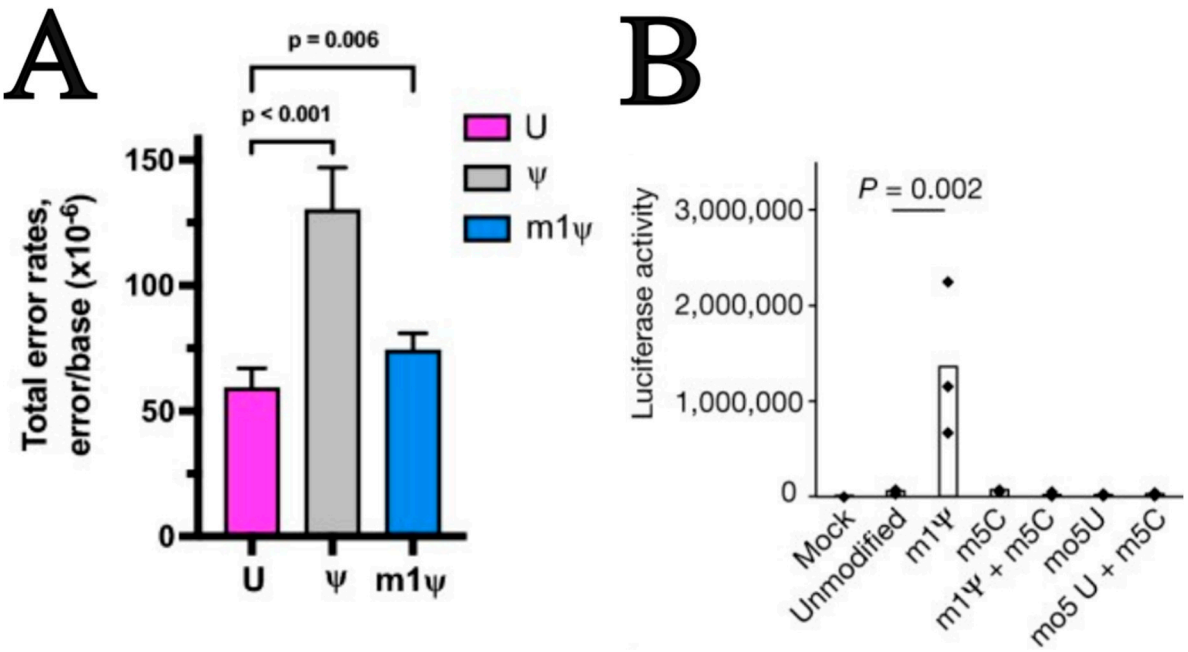

Figure 4. Translational challenges with modified nucleosides. Panel A. Translational error rates (misuse of tRNAs) of modified uridine-containing transcripts. Reproduced from [61] under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). Panel B. Out of frame expression of a firefly luciferase reporter by nucleoside type. m1Ψ, N1-methyl-pseudouridine; m5C, 5-methylcytidine; m1Ψ + m5C, combination of N1-methyl-pseudouridine and 5-methylcytidine; mo5U, 5-methoxyuridine; mo5U + m5C, combination of 5-methoxyuridine and 5-methylcytidine. Reproduced from (23) under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

Another challenge to translational fidelity is m1Ψ-rich sequences inducing +1 translational frameshifting and producing off-target proteins [27] (Figure 4B). This appears to impact the immune response, as antibodies for the frameshifted proteins were observed in both mice and humans injected with BNT162b2 (Pfizer/BioNTech vaccine) [27]. Fortunately, there have not been adverse events attributed to these off-target proteins. However, given the panoply of possible mRNA templates in future products, a space which expands considerably when personalized mRNA transcripts are used for cancer vaccines or gene therapy, it may be difficult to characterize the biological effects of all possible off-target proteins.

Resolution: Moving forward with mRNA

Fortunately, introducing synonymous mutations to the mRNA sequence to avoid "slippery sequences", the specific short sequences which induce +1 ribosomal frameshifting, can limit this issue [27,62]. Fortunately, the slippery sequences appear to be quite specific, and may be avoided without changing the desirable properties of m1Ψ, namely, its decreased immunogenecity, and increased translational efficiency, compared to both unmodified RNA and Ψ-RNA [24]. In the Pfizer/BioNTech BNT162b2 sequence, for example, there are six predicted +1 ribosomal frameshift sites [27]; introduction of synonymous mutations at these six locations may be sufficient to reduce the production of off-target proteins to the same level as in unmodified RNA [27,62].

With dozens of mRNA therapeutic products in development, approval and marketing pipelines [63,64], it is timely that these design challenges were spotted. The strength of mRNA vaccines is their ability to go from sequence to prototype quickly [65], which makes the changes to ensure faithful translation easy to apply to future iterations of the technology. Further studies should be undertaken

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to elucidate additional potential biological impacts and dose effects of m1Ψ in mRNA therapeutics, considering their extreme rare presentation in nature, particularly in eukaryotic cells.

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