

# Messenger RNA change drives protein slip-up

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Modified components of messenger RNA can cause the protein-production machinery to stall during the process of translation. This might change the protein being made, a finding with implications for vaccines or therapies. **See p.189**

Advances made in messenger RNA technology led to two mRNA vaccines that target SARS-CoV-2 having an important role in the response to the COVID-19 pandemic. Rather than making the mRNA using cells, these vaccines use mRNA that is made *in vitro* in the laboratory and is known as *in vitro* transcribed (IVT) mRNA. Understanding the details of how IVT mRNA encodes protein during the process of translation (protein production mediated by the machinery of ribosomes and transfer RNAs) might contribute to future applications of this type of technology. On page 189, Mulrone *et al.*<sup>1</sup> reveal that the ribosome can stall at 'slippery' sequences along modified versions of mRNA (ones that include some nucleoside components that have been chemically modified); this might result in aberrant protein products. In the context of such nucleoside-modified mRNA vaccines and other non-vaccine therapeutic RNAs, this might lead to unintended consequences.

Cells contain numerous protein receptors for defence that sense and respond to viral RNAs. Extensive work has demonstrated that IVT mRNA can cause immune-system overstimulation<sup>2</sup> owing to these innate-sensing mechanisms, which can interfere with the utility of this mRNA for vaccines or therapies. A key finding relating to the production of IVT mRNA is that, for the nucleoside part of nucleotides, replacing the nucleosides uridine and cytosine with modified versions – respectively, N1-methyl pseudouridine (abbreviated as m1Ψ) and 5-methyl cytosine – can counteract non-specific reactivity of the immune system<sup>3</sup>. However, whether such modifications might affect the translation fidelity of mRNA has not been previously reported.

Mulrone and colleagues investigated the effect of m1Ψ modifications on mRNA translation fidelity by designing an IVT mRNA reporter system that enables the identification of a specific translational error known as +1 frameshifting. This process causes the ribosome to 'slip' as it processes the mRNA, missing out the first of three nucleotides

(termed a codon) that encode an amino acid (Fig. 1). Instead, the second nucleotide in the sequence becomes the first nucleotide of the codon, which now contains a third nucleotide that would normally be part of the next codon. Each codon thereafter becomes offset. This can lead to protein products that differ from the expected 'in-frame' sequence of amino acids, because translation is shifted one nucleotide 'out-of-frame' of the normal coding sequence.

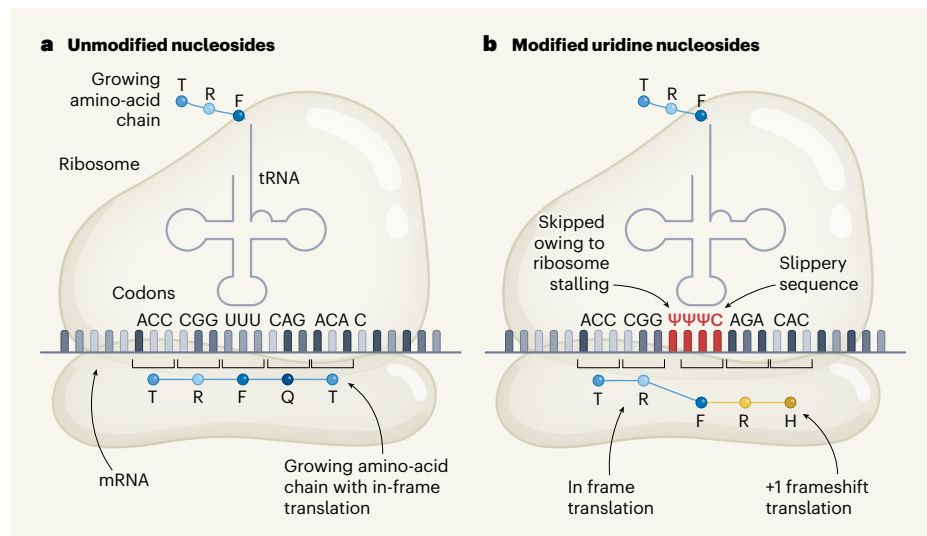
The authors' data indicate that m1Ψ modifications result in +1 frameshifting. This reached a level of approximately 8% of the corresponding amount of in-frame protein. Frameshifts can

result in misfolded, truncated or otherwise aberrant proteins. This observation was not observed with unmodified mRNA or with other modified nucleosides, such as 5-methylcytosine.

SARS-CoV-2 mRNA vaccines that encode the viral spike protein, such as BNT162b2 (Pfizer/BioNTech) and mRNA-1273 (Moderna), contain m1Ψ-modified mRNA. The authors addressed whether +1 frameshifting might affect mRNA-vaccine-induced immunity by examining the SARS-CoV-2 spike-specific response by immune cells called T cells in animals and humans.

Two key immune signatures associated with antiviral protection are the induction of antibody responses and T-cell responses. During a defence process known as antigen presentation, immune cells termed dendritic cells prime T cells with peptide fragments that span the length of the antigen (the part of the protein that drives an immune response). The production of +1 frameshifted proteins could lead to unintended peptide presentation and the priming of off-target T-cell responses.

The authors compared the T-cell response in mice vaccinated with BNT162b2 against the response in ones that were given a non-mRNA vaccine, ChAdOx1 nCoV-19. Both vaccines elicited T-cell responses to peptides produced from in-frame SARS-CoV-2 spike protein. However, BNT162b2-immunized mice also elicited a T-cell response to +1 frameshifted spike peptides, whereas ChAdOx1-immunized mice did not.



**Figure 1 | How messenger RNA modifications can alter the encoded protein.** Protein is produced when mRNA undergoes a process termed translation; this is mediated by machinery that includes the ribosome and transfer RNAs that process along the mRNA. The tRNAs match a sequence of three nucleotides (termed a codon) and thereby add a particular amino acid (names of amino acids indicated by single-letter codes beside the circular residues) to the growing chain of amino acids that form an encoded protein. The usual nucleotides in mRNA are adenine (A), cytosine (C), guanine (G) and uracil (U). U forms from the nucleoside uridine. When mRNA is made *in vitro*, a modified version of uridine (Ψ, also abbreviated as m1Ψ) is sometimes used instead of uridine to form U. Mulrone *et al.*<sup>1</sup> report that certain sequences that include modified uridine can lead to a phenomenon called +1 frameshifting. When this happens, ribosomal stalling can result in 'slippage' and the tRNA can skip a nucleotide and match instead to an offset group of three nucleotides (the same tRNA can match either UUU, UUC, ΨΨΨ or ΨΨC). This offsetting alters the subsequent sequence of amino acids.

These findings were extended by studying human samples from individuals immunized with either BNT162b2 or ChAdOx1. Both vaccines generated T-cell responses to in-frame peptides. However, BNT162b2 elicited T-cell responses to +1 frameshifted peptides. This illustrates that a lack of fidelity in translation can result in unintended consequences arising from a non-uniform protein output from the vaccine, leading to the induction of off-target immune responses and probably affecting product potency. No evidence has been reported linking this phenomenon to safety issues for COVID-19 mRNA vaccines through the many systems that monitor vaccine safety after licensing – including the Vaccine Adverse Event Reporting System, the Vaccine Safety Datalink and the Clinical Immunization Safety Assessment project.

Numerous natural mechanisms can lead to frameshifting<sup>4,5</sup>. The authors examined whether m1Ψ-modified mRNA affects ribosome stalling, a process that can lead to +1 frameshifting. The rate of translation of mRNA is not constant along the length of an mRNA transcript. This is because of differences in the abundance of transfer RNAs that bind to matching mRNA codons and provide specific amino acids during protein production. The ribosome might stall on a ‘slippery’ mRNA sequence for which few corresponding tRNAs are available, and this could lead to a +1 frameshift to accommodate a more abundant tRNA instead<sup>6</sup>.

Using labelled nucleotides to track translation, the authors observed that the translation rate was slower for m1Ψ-modified mRNA than for unmodified mRNA. If m1Ψ-modified codons lead to ribosome stalling, enlarging the pool of available tRNAs should prevent stalling. Using the drug paromomycin, which enables the binding of non-matching tRNAs, the authors report that the translation rate of m1Ψ-modified mRNA improved with this treatment, which supports the hypothesis that ribosomal stalling is responsible for this phenomenon.

To investigate a possible remedy for ribosomal stalling, the authors used their *in vitro* system. They identified slippery sites in their reporter system and altered the corresponding mRNA sequence so that it had some synonymous substitutions that changed the mRNA sequence but not the encoded amino acid. The goal was to retain the correct sequence of amino acids in frame and limit the effect of the slippery sequence. Substitutions in the sequence reduced +1 frameshifted products *in vitro*, illustrating a method to ameliorate this phenomenon.

This study has implications for the development of modified mRNA products. Further evaluation of the T-cell and antibody response to +1 frameshifted protein products made from vaccine antigens encoded from m1Ψ-modified

mRNA vaccines would be informative. Off-target T-cell or antibody responses have the potential to be misdirected against non-relevant target proteins, compromising product performance and resulting in unintended *in vivo* product complexity. Mulrone and colleagues’ study highlights a key aspect for future study that might aid our understanding of the design of modified mRNA sequences, thereby enabling further-improved outcomes. Studies to confirm and extend the implications of *in vivo* frameshifting resulting from mRNA modifications, as well as to investigate other methods of amelioration, are warranted.

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## Genomics

# Gene expression of single cells mapped in tissues

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A tool that tags individual cells in a tissue with a unique barcode means that the gene-expression profile of each cell can be plotted in its original location. This allows spatial information to be captured at single-cell resolution. **See p.101**

Over the past decade, there have been major advances in techniques that can inform scientists about the properties of single cells, such as their gene-expression profiles<sup>1</sup>. Similar progress has been made with methods that provide spatial information, such as identifying where genes are being expressed in a tissue<sup>2</sup>. On page 101, Russell *et al.*<sup>3</sup> present a tool that they name Slide-tags, which could integrate the best aspects of both of these approaches.

Information about gene expression in a given biological sample can be acquired by sequencing the RNA transcripts present (known as transcriptomics), or by identifying regions of the genome that are accessible to the molecular machinery that controls gene transcription (one example of an epigenomic approach). More broadly referred to as genomics, such techniques can be used to obtain the genetic profiles of single cells, or to spatially map gene expression in whole tissues. Although the two methods have found widespread application in biomedical research, many challenges remain.

The main problem for single-cell approaches is that it is difficult to maintain the natural state of cells while attempting to accurately characterize them during analysis. For example, information about the location of a cell in a tissue is lost when cells are dissociated from one another and mixed together

during sample preparation. Retaining spatial information is important because it can reveal how cell types are organized in tissues, which is valuable when trying to understand developmental processes or how certain diseases (such as cancer) progress.

Isolating cells from tissues can also be disruptive to the native architecture of the cell itself. Just how disruptive often depends on the composition of the tissue in question. Some cells, such as those in the blood, can be isolated relatively easily, because they are free-floating. By contrast, cells in frozen or fragile tissues are difficult to isolate, because they are prone to breaking apart during tissue dissociation. Thus, when working with tissues of this nature, it is usually only the nuclei of cells that can be isolated<sup>4</sup>.

For spatial genomics, resolution is a key concern. When mapping a piece of tissue onto a physical surface, the surface can be divided into a matrix in which each element (referred to as a ‘pixel’) represents a particular spatial location. The size of the pixels determines the resolution. Although pixel sizes that provide single-cell resolution are technically achievable, spatial pixels usually contain information from a mixture of cells, and it has so far been hard to work out exactly which data belong to which cells in a tissue<sup>2,5</sup>.

The possibility of combining single-cell and