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Reprogramming ribosomes during viral protein synthesis

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It is estimated that ~10^{31} viruses exist on planet earth, and all are entirely dependent on host cell ribosomes for viral mRNA translation. Ribosomes, the engines of protein synthesis, can be regarded as the 'compilers' of life—responsible for interpreting the genetic instruction set in a consistent way and creating protein molecules with executive functions, each of which contributes towards the phenotype of the cell. Even subtle changes in how the ribosome normally operates can have dramatic consequences, as illustrated by the lethality of many antibiotics that target the protein synthesis machinery. For viruses, millions of years of evolutionary pressure have led to every possible avenue being explored to optimise the encoding of information within a (typically) small genome. This has led to the emergence of mechanisms to reprogram translation in a variety of ways, collectively termed 'recoding'. For example, elongating ribosomes can be induced to shift reading frame and access overlapping coding sequences or to continue elongating through a stop codon. Here, we review the discovery of these phenomena, explore the role of RNA structures in these processes, and outline mechanistic questions that remain unanswered.

New tricks for an old dog(ma)

Soon after the publication of the Maxam & Gilbert DNA sequencing methodology in 1977, the first sequences of retroviruses began to appear, and with them, new challenges in our understanding of protein synthesis. One of the earliest complete sequences was that of Rous sarcoma virus (RSV), well known for its ability to cause tumours in chickens. The genetic material (the genome) of RSV has only four major coding regions *gag, pol, env* and *src*, encoding, respectively, proteins involved in making the virus particle, viral enzymes, the cell attachment protein and the oncogene responsible for immortalisation of cells (Figure 1).

The RSV genome sequence was largely consistent with earlier biochemical analyses of RSV-infected cells, which had detected Gag, Env and pp60-vSrc proteins, and a Gag-Pol fusion protein of lower abundance. Surprisingly, the sequence revealed that the *gag* and *pol* coding sequences overlapped by seven nucleotides at the 3' end of *gag*, with the *pol* coding sequence encoded in a different reading frame (so-called –1). Initially, it was thought that Gag-Pol would be translated from a spliced viral mRNA in which the two reading frames would be aligned in-frame, but this turned out not to be the case and instead; Gag-Pol was found to be expressed by programmed –1 ribosomal frameshifting (–1 PRF). Remarkably, about 5% of the ribosomes synthesising Gag change reading frame just before the *gag* stop codon and carry on in the –1 reading frame, synthesising a Gag-Pol fusion protein. This had never been seen before – accurate maintenance of the reading frame is normally essential for the production of functional proteins – so what was going on, and why?

In answering these questions, some progress has been made in terms of understanding the *biological* relevance of –1 PRF. In infected cells, the Gag protein self-assembles into virus particles (virions), and the Gag-Pol protein synthesised by frameshifting is also incorporated through Gag self-interactions. Thus, –1 PRF can be used as a mechanism to package the Pol protein into virus particles. This is an essential step for retroviruses, as they need to bring their own enzymes into a newly-infected cell for reverse transcription and integration of their genetic material into the host chromosome. The efficiency of the frameshift event also sets the precise ratio of Gag:Gag-Pol in virions (in RSV it is 20:1, consistent with a 5% frameshift efficiency), which is optimised for the virus in question. Indeed, drugs or mutations that inhibit –1 PRF are severely detrimental to virus replication. Frameshifting is known to occur in a huge number of viruses, particularly those with RNA genomes. These include human pathogens like HIV-1, SARS-CoV-2 and others, and also many viruses of veterinary and agricultural importance. So, we are keen to understand the *mechanism* of frameshifting to give us insights into the best way to target the process for antiviral intervention.

Twists and turns: the critical role of RNA structure (and proteins!)

Soon after its first description in 1985, seminal works from the group of Harold Varmus at the University of

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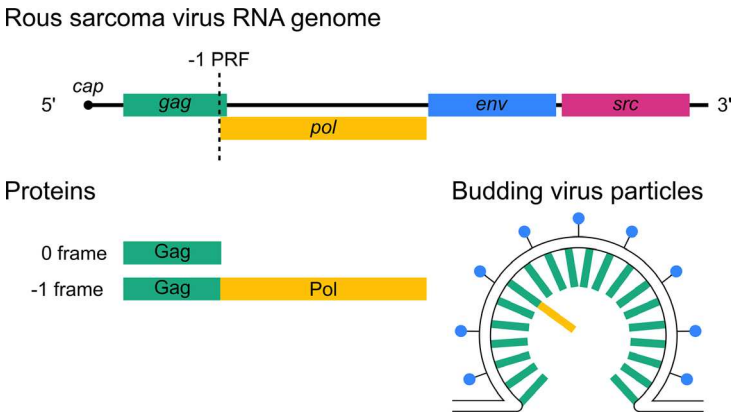

Figure 1. Frameshifting in Rous sarcoma virus (RSV). The RSV genome contains a –1 programmed ribosomal frameshift (–1 PRF) site between the *gag* and *pol* genes. Frameshifting generates the correct ratio of Gag to Gag-Pol proteins (20:1) for optimal virion assembly and budding, which takes place at the plasma membrane of the cell.

California defined three main components of a –1 PRF signal; a homopolymeric slippery sequence, where the ribosome changes reading frame, separated by a short 'spacer' of single-stranded mRNA from a 3' stimulatory mRNA element (Figure 2).

These early experiments suggested a model, still relevant in many aspects, where interaction with the stimulatory element pauses the translating ribosome while the ribosome-bound tRNAs are decoding the slippery sequence codons in the peptidyl- (P) and aminoacyl- (A) sites. At this point, movement of the tRNAs into the –1 frame occurs, with the homopolymeric nature of the slippery sequence allowing sufficiently stable post-slippage base-pairing between mRNA and tRNAs that ribosomes can continue elongation in the –1 reading frame. Even though 40 years have passed since –1 PRF was first described, we are still some distance from a complete understanding of the mechanism of frameshifting. What we do know is that frameshift stimulatory elements are highly diverse.

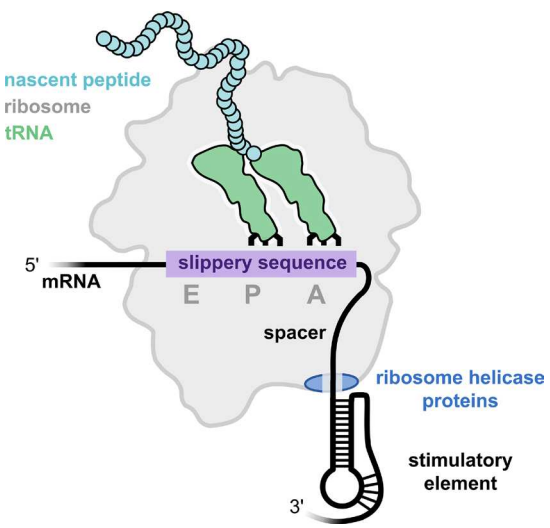

Figure 2. Overview of –1 PRF. Three elements are typically present at sites of –1 PRF, a slippery sequence, a short spacer and a structured RNA element – here an RNA pseudoknot – that stalls the elongating ribosome. Transfer RNAs in the peptidyl- (P) and aminoacyl- (A) sites of the ribosome slip into the -1 frame during a frameshift.

The most common stimulatory signal is an RNA secondary structure – usually a stem-loop or RNA pseudoknot. The latter 'motif' is an elaboration of the stem-loop, where some of the loop nucleotides base-pair to a run of bases in the mRNA downstream generating a two-stem structure connected by single-stranded loops (Figures 2 and 3).

It is termed a pseudoknot, because the downstream RNA does not thread through the loop of the stem-loop (as it would in a true knot), rather just base-pairs to it. Several decades of X-ray crystallography, NMR spectroscopy and more recently, electron cryo-microscopy (cryo-EM) on core stimulatory RNA elements have revealed that in addition to *secondary* structures like base-paired stems, *tertiary* structural features (for example A-minor interactions, 2′-OH interactions) can play a major role. For example, the 28-nucleotide pseudoknot from beet western yellows virus has more hydrogen bonds stabilising tertiary interactions than the number involved in Watson–Crick base pairs (Figure 3). Variations on this theme have been described in different viruses, including stem bulges, kinks and triple- or quadruple base-pairing interactions near the stem junctions. In the last ten years or so, examples have also emerged of the involvement of protein factors. A frameshift signal identified in the genome of porcine reproductive and respiratory syndrome virus (an important pig pathogen) was found to lack a structured stimulatory RNA but had a functionally important stretch of cytosine bases downstream of the slippery sequence. Further work established that this C-rich stretch was the

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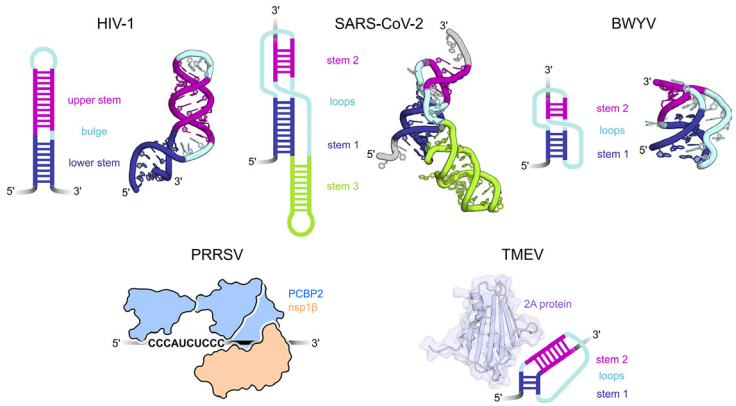

Figure 3. Structural diversity of frameshifting stimulatory elements. Examples are shown of stem-loop (HIV-1, PDB 1Z2J) and pseudoknot (SARS-CoV-2, beet west yellow virus BWYV, PDB 7O7Y and 437D) stimulatory RNAs. Theiler's murine encephalitis virus (TMEV) requires the virally encoded 2A protein (PDB 7NBV) to bind to the RNA to stimulate frameshifting. The PRRSV stimulator is a complex of a cellular and viral protein that binds to the mRNA at a C-rich region.

target of a protein complex composed of cellular poly(C)-binding protein and a viral protein, nsp1β. These findings represented a seismic shift in the field, demonstrating for the first time that protein factors can be involved in PRF and opening the possibility that frameshifting can be regulated during virus infection. This was classically demonstrated in studies of another positive-strand RNA virus, Theiler's murine encephalitis virus (TMEV), a mouse pathogen. The TMEV signal is incredibly efficient (>85% of ribosomes frameshift) yet the spacer between the slippery sequence and the stimulatory RNA is longer than normal, apparently too far away to stop the ribosome on the slippery sequence. It turns out that TMEV frameshifting requires a virus protein called 2A, which in infected cells can bind to the stimulatory RNA and together, the RNA-protein complex positions the ribosome in the correct place. Early in infection, 2A is present at low levels, there is no frameshifting and the virus synthesises its replication proteins. Later in the infection, 2A accumulation turns on the frameshift signal and ribosomes frameshift efficiently to make only the structural proteins needed to build virus particles.

A new pathway in translocation?

As the ribosome moves along the mRNA, it moves in three-nucleotide steps, one codon at a time, and at each step, several things happen. First, the growing peptide chain is transferred from the P-site tRNA to the new aminoacyl-tRNA in the A-site. Then, the two tRNAs located in the P- and A-sites of the ribosome each move by three nucleotides across the ribosome, a process called translocation, with the P-site tRNA moving into the exit (E-) site and the A-site tRNA into the P-site. This movement is mediated by the action of elongation factor 2 (eEF2) and internal rotations within the small ribosomal subunit (40S). These rotations lead to a ratcheting effect, where tRNA movement is coupled to movement of the mRNA and, importantly (from our perspective), the unwinding of any RNA structures coming into the ribosome. Inspiring work in the laboratory of Harry Noller has revealed that, as translocation takes place, three proteins which line the narrow entrance to the ribosome (the mRNA tunnel) can interact with the mRNA, and as the internal 40S subunit rotations take place, they can act as a helicase to peel apart base-paired regions close to the entry channel (Figure 2). This is essential, as mRNA must be single-stranded when it enters the decoding site. We hypothesise that the frameshift-stimulatory elements resist unwinding at the entry channel and the process of accurate translocation becomes compromised, leading to movement by two nucleotides rather than the full codon step, and thus a –1 frameshift. This could be mediated by tension in the mRNA, where the pulling force of translocation is resisted by the 'jammed' helicase, and a way to relieve the tension is to detach the tRNA anticodons, which re-pair in the –1 frame. However, there remain many unanswered questions and alternative hypotheses. How can this unwinding model accommodate the variety of stimulatory signals in nature? Can stem-loops, pseudoknots and protein–RNA complexes conceivably 'tweak' the helicase by the same route? How are the intricate movements of translocation reset to allow elongation to continue in the new frame? How long is ribosome movement delayed at the stimulatory RNA? It is known that extended pausing of ribosomes can activate cellular mechanisms that remove 'blocked' ribosomes and degrade the mRNA, not an ideal situation for an RNA virus. Based on kinetic studies, it is speculated that frameshifting is a default pathway that permits ribosomes to escape from a stall induced

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by a stimulatory element. Indeed, the encounter of the stimulatory signal might send the ribosome into a slow mode of translocation that is more prone to frameshift.

The qUAGmire deepens: stop codon readthrough

A closely-related phenomenon with interesting parallels is stop codon readthrough (SCR). Unlike sense codons, stop codons are decoded by release factors (RFs); in eukaryotes, a complex of eRF1 and eRF3 enters the decoding site, recognises the stop codon and then releases the completed polypeptide. Returning to the early days of nucleic acid sequencing, publication of the genome of murine leukaemia virus (MuLV) in 1981 also threw up a conundrum. In MuLV, a retrovirus of rodents, the Gag and Pol coding sequences are in the same reading frame but separated by an in-frame stop codon. Here, expression of Gag-Pol, at a frequency of 5% of that of Gag alone, requires 'suppression' of the *gag* UAG stop codon, which is instead decoded by a glutamine tRNA (which normally recognises CAG). Inspection of flanking sequences revealed an RNA pseudoknot located just downstream of the stop codon (Figure 4).

Like –1 PRF, SCR signals are found in many viruses, with diverse stimulatory RNA elements. As yet, we have no high-resolution images of ribosomes on SCR signals, but presumably, the stimulatory signal interferes with RF action and perhaps facilitates erroneous decoding by a tRNA, possibly by suppressing ribosome activities that detect errors.

Looking forwards

Sites of –1 PRF seem to be extremely rare in cellular genes, a potential advantage if recoding events could be targeted for antiviral intervention. It might be possible to develop drugs that modulate frameshifting to the detriment of virus replication, without host toxicity. Another possible avenue is the generation of frameshift-defective, attenuated viruses for vaccine purposes. It will be fascinating to witness future advances in the recoding field, and what they will tell us about protein synthesis, RNA structure–function relationships and virus gene expression. ■

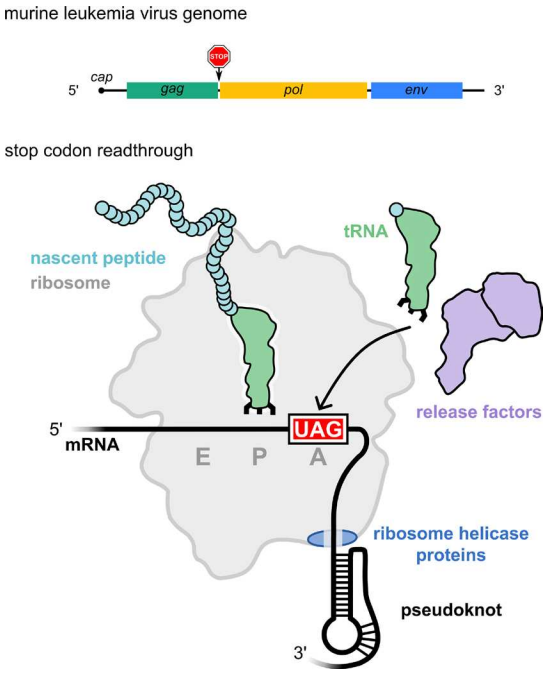

Figure 4. Stop codon readthrough in murine leukaemia virus (MuLV). The *gag* and *pol* genes are in the same reading frame but separated by a stop codon (UAG). The RNA pseudoknot 'suppresses' stop codon recognition by the release factor complex eRF1/ eRF3, resulting in the production of a Gag-Pol fusion protein.

Further reading

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- Hill CH, Brierley I. (2023). Structural and functional insights into viral programmed ribosomal frameshifting. *Annual Rev. Virol.* 10(1):217-242. PMID: 37339768.
- The ViralZone site (at the Swiss Bioinformatics Resource Portal) has information on both frameshifting (https://viralzone.expasy.org/860) and stop codon readthrough (https://viralzone.expasy.org/859).
- An expert video is 'Targeting programmed -1 ribosomal frameshifting in COVID-19 with Jonathan Dinman of UMD'. https://www.youtube.com/watch?v=8OccCntltX4
- Recoding is the subject of a book edited by experts Professor John Atkins and Professor Raymond Gesteland entitled 'Recoding: Expansion of Decoding Rules Enriches Gene Expression' (Springer).

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