FEBS Letters

PERSPECTIVE

Mechanisms and biomedical implications of –1 programmed ribosome frameshifting on viral and bacterial mRNAs

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(Received 9 April 2019, revised 14 May 2019, accepted 26 May 2019, available online 20 June 2019)

doi:10.1002/1873-3468.13478

Edited by Michael Ibba

Some proteins are expressed as a result of a ribosome frameshifting event that is facilitated by a slippery site and downstream secondary structure elements in the mRNA. This review summarizes recent progress in understanding mechanisms of –1 frameshifting in several viral genes, including IBV *1a/1b*, HIV-1 *gag-pol*, and SFV *6K*, and in *Escherichia coli dnaX*. The exact frameshifting route depends on the availability of aminoacyl-tRNAs: the ribosome normally slips into the –1-frame during tRNA translocation, but can also frameshift during decoding at condition when aminoacyl-tRNA is in limited supply. Different frameshifting routes and additional slippery sites allow viruses to maintain a constant production of their key proteins. The emerging idea that tRNA pools are important for frameshifting provides new direction for developing antiviral therapies.

Keywords: frameshifting; protein synthesis; recoding; regulation; ribosome; RNA; translation; tRNA

Translation is a tightly regulated step of gene expression which ensures the synthesis of proteins according to the sequence of an mRNA produced during transcription. The ribosome, a macromolecular machine that synthesizes proteins in all cells, controls accurate decoding of mRNA triplets by the respective aminoacyl-tRNAs (aa-tRNAs) [1–6]. Stringent tRNA selection mechanisms ensure a very low overall error rate of mRNA decoding in the range of 10^{-7} to 10^{-5} [7]. Each time an amino acid is incorporated into the growing peptide chain, the ribosome moves by one codon along the mRNA to read the next codon. Keeping the codon-wise step of translation is even more essential than preventing missense errors, because slippage by one or two nucleotides results in a completely altered sequence of the synthesized peptide. In fact, spontaneous frameshifting errors are rather infrequent, $<10^{-5}$ per codon [8]. However, in some cases, this rigorous reading frame control is abrogated allowing the ribosome to recode genetic information in response to specific stimulatory signals embedded in the mRNA sequence or structure [9,10]. Programmed ribosome frameshifting (PRF) is a recoding event that allows to produce multiple proteins from the same mRNA by shifting the reading frame in the forward (+PRF) or backward (–PRF) direction [9–12]. Slippage occurs typically by 1 nucleotide, although 2-, 4-, 5-, and 6-nucleotide shifts were also reported [13–17]. –1PRF is found in all kingdoms of life including higher eukaryotes, but is particularly prevalent in viruses and mobile genetic elements [9,10,18]. The biological significance of PRF is to increase the genome-coding capacity, to control the stoichiometric ratio between proteins and to regulate gene expression by influencing mRNA

Abbreviation
IBV, infectious bronchitis virus; LSU, large ribosomal subunit; PRF, programmed ribosome frameshifting; SFV, Semliki Forest virus; smFRET, single-molecule FRET; SSU, small ribosomal subunit; TRIT, tRNA Inhibition Therapy.

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stability [9,10]. Many human pathogenic viruses require frameshifting for their viability, because it ensures production of certain viral enzymes that are not encoded in the 0-frame and modulates the ratio between viral structural proteins necessary for virion assembly [18–21]. Among the viral proteins produced upon –1PRF, many contribute to viral infectivity either directly by evading the host antiviral response [22] or indirectly by hindering viral particle formation and release, thereby decreasing viral titer [20,23].

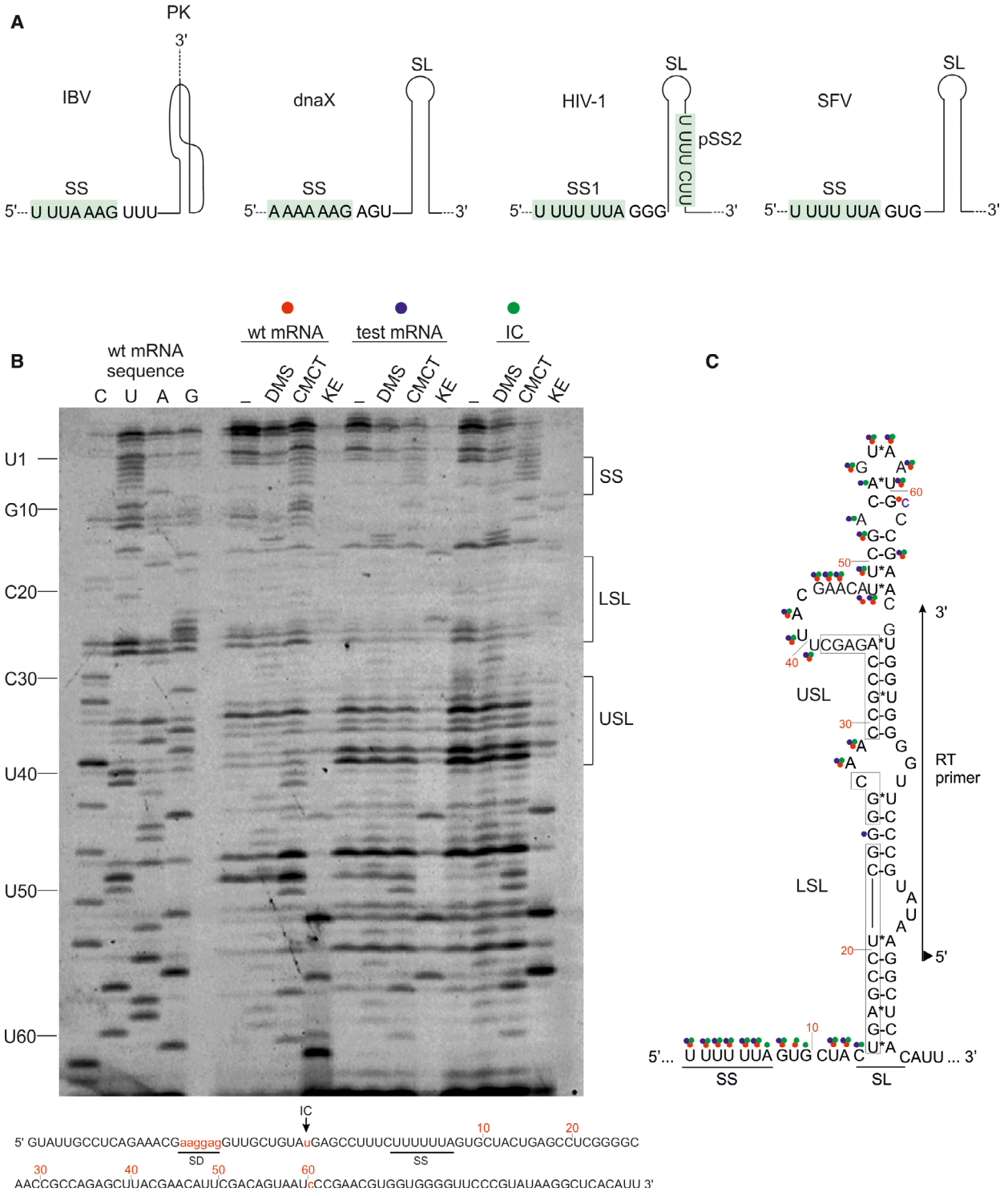
In this review, we summarize the recent progress in understanding the mechanisms of –1PRF, including the different routes of –1PRF and the mechanisms leading to alternative reading frames, for example, +1 and –2 frameshifting, which we call alternative slippages. In addition to two well-studied cases of –1PRF in avian infectious bronchitis virus (IBV) and bacterial *dnaX*, we focus on examples of –1PRF from two phylogenetically distant human viruses, human immunodeficiency virus type 1 (HIV-1), and Semliki Forest virus (SFV). We show how tRNA abundance can modulate the routes and efficiency of –PRF and discuss how different routes and additional slippery sites result in robust –1PRF which is needed for virus life cycle.

Frameshifting elements in the mRNA

Typically, –1PRF is governed by two cis-acting elements—a slippery site (SS) and a downstream mRNA secondary structure (Fig. 1). The SS is a repetitive heptanucleotide sequence of the type X_1 XXY_4 YYZ_7, which allows the two tRNAs that read the 0-frame codons XXY and YYZ to re-pair with their XXX and YYY codons after the slippage into the –1-frame [24]. The mRNA secondary structure—a stem-loop (SL) or a pseudoknot (PK) or a kissing loop [25–28]—acts as a roadblock to hinder translocation and thereby promote frameshifting [29,30]. In addition, Shine–Dalgarno-like (SD-like) sequences in bacteria [31], trans-acting proteins in viruses [32–35], G-quadruplexes [36,37] and miRNAs in mammalian cells [32] can modulate the –1PRF efficiency.

In most cases, the structure of the downstream mRNA secondary structure is known from bioinformatics, chemical probing, mutagenesis, or structural studies. For example, the PK at the frameshifting site of IBV was discovered based on mutational analysis and its structure predicted by bioinformatics (Fig. 1A), whereas the atomic structure of this PK is still lacking [28,38]. The structure of the SL in bacterial *dnaX* was initially suggested based on mutational analysis and structural probing [39]; recent cryo-EM studies determined the structure of this SL bound to the bacterial ribosome (Fig. 1A) [40]. The SL structure of HIV-1 is one of the best-studied examples of mRNA secondary structures that modulate –1PRF. Its structure was solved using mutagenesis and enzymatic probing [41], thermodynamic and NMR analysis [28,42,43], as well as toeprinting and chemical probing in the presence of the bacterial ribosome (Fig. 1A) [44].

The secondary structure element of SFV was predicted to be an extended SL by bioinformatics and mutational analysis [27]. We validated the structure by chemical probing, which can distinguish single- and double-stranded RNA regions by their accessibility to chemical modification (Fig. 1B). The chemicals were chosen such as to modify the Watson–Crick positions of the nucleotide base; double-stranded regions are protected from chemical modifications due to base pairing to the complementary strand. Modification causes a stop in the progression of the reverse transcriptase (RT) resulting in the production of short cDNA fragments, which can be then visualized by sequencing (Fig. 1B). In agreement with the bioinformatics predictions [27], the SL element in SFV contains a long lower stem encompassing nucleotides 15–26 after the SS (counting from nucleotide 1 of the SS), as seen from the lack of chemical modifications of this region (Fig. 1B,C). Nucleotides 28–29 form a small unstructured loop between the lower and upper stems, consistent with their accessibility to modifications. According to the bioinformatics analysis, C27 also belongs to this loop; however, its modification status is unclear. The upper stem was predicted to span nucleotides 30–35; however, our results suggest that also the adjacent nucleotides 36–39 are protected from modifications and thus might belong to the upper stem, although the accessibility of the complementary strand nucleotides (nucleotides 64–68) is unclear (Fig. 1C). The upper stem is closed by a large bulge spanning nucleotides 40–48, as predicted by the bioinformatics analysis and supported by the chemical probing data. Nucleotides 49–52 are predicted to form a small stem, but appear to be in a single-stranded region according to chemical probing. According to the bioinformatics analysis, C54 is base paired to G61, suggesting that both should be inaccessible to chemical modification. This is, however, not the case: C54 is indeed inaccessible, but G61 is modified. In addition, when G61 is mutated to C61, it becomes protected, suggesting that the interaction pattern is more complex than predicted. Finally, we confirm the presence of the predicted AGUAAU loop closing the upper stem (Fig. 1C). Hence, chemical probing largely supports the structure of the SFV SL predicted by the bioinformatics analysis [27]. The major differences concern the length of the



upper stem, which appears to include nucleotides 36–39 based on chemical probing, and the absence of the predicted small stem spanning nucleotides 49–52. As expected, the SS sequence is single-stranded. Nucleotides between the SS and the SL (nucleotides 8–14) show a very high degree of modification indicating that they do not belong to the SL but represent a single-stranded spacer between the two

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Fig. 1. Sequence and structure of mRNA frameshifting motifs. (A) From left to right, schematics of frameshifting motifs of IBV *1a/1b*, *dnaX* of *E. coli*, *gag-pol* of HIV-1, and *6K* of SFV. Slippery sites (SS) are indicated and highlighted in green. The regulatory downstream mRNA element is a pseudoknot (PK) or a stem-loop (SL), as indicated. pSS2 in HIV-1 stands for the second putative SS. (B) Chemical probing of the mRNA secondary structure element in the SFV 6K mRNA. *In vitro* transcribed mRNA was treated with dimethyl sulfate (DMS; A- and C-specific), 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMCT; U-specific and low reactivity toward G) and β-ethoxy-α-ketobutyraldehyde (kethoxal KE; G-specific, and analyzed base modifications by primer extensions [109]. (–) indicates untreated mRNA. Positions of reverse transcription (RT) stops due to modification were visualized on a sequencing gel using fluorescence primer complementary to positions 109–129 nucleotides of the mRNA (60–80 nucleotides downstream the SS). wt mRNA has the native sequence; test mRNA has been optimized for translation in *E. coli* (see lower panel; SD is Shine–Dalgarno sequence, AUG is the start codon, G61 is mutated to C to remove a potential initiation codon); IC is the initiation complex of test mRNA with 70S ribosomes. C, U, A, G are sequencing lanes. Numbered nucleotides to the left refer to the nucleotides in the SFV mRNA starting from the slippery site as indicated in c. LSL is lower stem-loop, USL is upper stem-loop. (C) Secondary structure of SFV 6K mRNA based on bioinformatics prediction [27] and probing results. Modified nucleotides are marked with circles: red for the wt mRNA, blue for the test mRNA and green for the test mRNA in the IC. Sequences in boxes indicate nucleotides forming lower (LSL) and upper (USL) stems. Primer-binding site for RT is marked with an arrow; triangle on the 5′ of the primer indicates its fluorescence label Atto647*N*.

frameshifting elements, as predicted [27]. Binding of the ribosome to the start codon (initiation complex, IC) does not affect the mRNA structure, most likely because the ribosome binds to the mRNA region distal from the SL (Fig. 1B,C). Nucleotides C14, A53, A55 show different reactivity in the IC than in the free wt mRNA (Fig. 1B,C), however, this effect is most likely caused by the mutations in the test mRNA sequence introduced to ensure translation initiation in *E. coli*, rather than by the presence of the ribosome.

Mechanisms of –1PRF on IBV *1a/1b* and *E. coli dnaX* mRNAs

–1PRF takes place during the elongation phase of translation, but due to a lack of kinetic data, the exact timing of –1PRF was until recently unknown. This led to the proposal of a number of different models that differ in the timing of the –1PRF during the elongation cycle, for example, some models predicted that –1PRF occurs during aa-tRNA accommodation in the A site of the ribosome (integrated & 9 Å models), before peptidyl transfer (simultaneous slippage model), during hybrid state formation in translocation (dynamic model), in the post-translocation state of the ribosome (mechanical model) or during the next round of elongation requiring three-tRNA slippage (three-tRNA model) (summarized in [12]). The lack of a unifying model has prompted several groups to investigate –1PRF by ensemble kinetics or single molecule methods [14,15,29,30,45,46]. Compared to results obtained previously with cellular lysates (e.g., rabbit reticulocyte lysate), the *in vitro* assays used in these experiments have the advantage of providing a fully controlled environment where every elemental step of translation elongation can be dissected. Results for two unrelated frameshifting examples, gene *1a*/*1b* of IBV [29] and *dnaX* of *E. coli* [14,15,30] suggested that –1PRF occurred predominantly during the tRNA translocation step and provided no support for any of the other models listed above. In the following we will discuss the current kinetic models of frameshifting.

The frameshifting motif of IBV gene *1a*/*1b* consists of a SS motif U_1 UUA_4 AAG_7 encoding Leu (UUA) and Lys (AAG) in 0-frame, and a PK positioned 6 nucleotides downstream of the SS (Fig. 1A) [38]. Comparison of the ensemble kinetics [29] and single molecule Fluorescence Resonance Energy Transfer (smFRET) [47,48] results with the structural intermediates of translocation [49–51] suggests that frameshifting occurs when the 3′ ends of the P- and A-site tRNAs have moved to the E and P site, respectively, on the large ribosomal subunit (LSU), but the tRNA anticodon domains are not yet fully translocated on the small ribosomal subunit (SSU) [29,30,46,52], that is, frameshifting occurs when two tRNAs are bound to the ribosome. Because the ribosome adopts a conformation in which the SSU head domain swivels relatively to the body domain, the tRNA anticodons are placed in an intermediate position with respect to the SSU head and body domains [49,52,53]. In this so-called chimeric (with respect to SSU) hybrid (to LSU) state, the codon–anticodon interaction is destabilized, which favors frameshifting [54]. The PK structure in the mRNA downstream of the SS impedes the closing movement of the 30S SSU head domain, which, in turn, hinders the release of the deacylated tRNA from the E site and the completion of translocation [29,45]. The presence of the mRNA secondary structure element leads to translational pausing and opens a kinetic window in which tRNAs can slip into a different reading frame. Notably, –1PRF appears favorable for translation because the ribosomes that shifted into the –1-frame complete translocation and release EF-G faster than those remaining in 0-frame [29]. Hence,

–1PRF could be considered as a rescue mechanism to resolve a persistent translational block caused by a secondary structure and resume translation at its normal rate.

Another well-studied example of –1PRF is on the *dnaX* mRNA of *E. coli*. The slippery site contains the SS motif A_1 AAA_4 AAG_7 encoding two Lys (AAA and AAG) in 0-frame, the downstream SL and an SD-like sequence upstream of the SS (Fig. 1A) [39,55,56]. Here –1PRF proceeds via two alternative routes, one of which is identical to that described for IBV *1a*/*1b*, whereas the other is activated by aa-tRNA limitation [15]. –1PRF was studied on an *dnaX* model mRNA in detail using smFRET, mass spectrometry and rapid ensemble kinetics [14,15,30,46]. smFRET and ensemble kinetics show that the presence of the downstream SL hinders translocation, whereas the rates of A-site tRNA delivery and peptidyl-transfer are unchanged [15,30,45]. The presence of the downstream SL in the *dnaX* mRNA also hinders the E-site tRNA release [30,45], as was shown with the PK of IBV [29]. While pausing at the SL, ribosomes undergo multiple conformational transitions between classical and hybrid states in the presence of EF-G [30,46]. EF-G may take multiple attempts to complete translocation while the ribosome tries to resolve the secondary structure to continue canonical decoding in 0-frame [14,46,57]. This prevalent translocation-dependent route, which corresponds to the two-tRNA slippage mechanism suggested earlier [57–62], is similar in IBV 1a/1b and *E. coli dnaX*.

Another pathway for frameshifting on *dnaX* was found to be operational when the A site remains vacant due to the absence of the cognate aa-tRNA. In this case, delayed decoding eventually allows slippage of the single P-site tRNA. Once the ribosome encounters a codon for which an aa-tRNA is available, normal translation resumes. Compared to translocation-dependent –1PRF, this so-called 'hungry' or one-tRNA frameshifting is very slow and does not require the presence of the downstream mRNA secondary structure [14,15]. Also, smFRET experiments suggest the presence of two branch points determining the reading frame: one during translocation, as described [15,30] and one during $tRNA^{Lys}$ sampling in the A site [46]. Mass spectrometry and optical tweezers data suggest that during translocation the ribosome attempts multiple trajectories or excursions along the mRNA and that the slippage into the new frame might occur from different 0-frame codons [14]. Many frameshift attempts fail due to codon–anticodon base pair mismatches in the alternative frame leading to the accumulation of incomplete peptides [14]. This scenario explains many cases of alternative frameshifting events at conditions of *in vitro* translation, for example, −4 and +2 frameshifting and decoding-related frameshifting observed on *dnaX* [14,46] and may explain the heterogeneity of frameshifting products observed in previous *in vivo* studies [63,64]. The 'hungry' frameshifting also relates to a common mechanism for how frameshift suppressor tRNAs can rescue mRNA frameshift mutants by a –1 frameshift [65]. For example, a translation defect caused by an G insertion in the mRNA resulting in a sequence -GGG-GAA-AGA- can be rescued by loss-of-function mutations in $tRNA^{Arg}$ reading the AGA codon. The delay in decoding allows $tRNA^{Glu}$ bound at the P-site GAA codon to slip into the –1-frame, which restores translation in 0-frame [66].

Given that –1PRF in IBV *1a*/*1b* and *E. coli dnaX* proceeds via the same translocation-dependent two-tRNA slippage, the question arises whether the same mechanism is utilized in other cases as well or if any of the earlier proposed mechanisms can be identified. One of the most widely known cases of –1PRF is the *gag-pol* overlap in HIV-1, which prompted us to study the mechanism of –1PRF in this system. In addition, we studied –1PRF in SFV, a virus phylogenetically distant from HIV-1, which has the same slippery site sequence as HIV-1 with a remarkable degree of conservation in different virus subtypes [20]. In the following section we will describe the mechanism of –1PRF on these two mRNAs.

Mechanism of –1PRF on slippery sites of *gag-pol* in HIV and *6K* in SFV

–1PRF in HIV-1 takes place at the *gag-pol* gene overlap and defines the ratio between the structural proteins of the capsid (Gag, 0-frame) and the viral enzymes (Gag-Pol, –1-frame). Changes in the frameshift efficiency impede viral particle formation and are detrimental for viral viability and infectivity [21,67]. Notably, –1PRF on the *gag-pol* mRNA results in two different –1-frame products in addition to the 0-frame peptide. Although numerous models were proposed to explain –1PRF in HIV-1 [21,59,61,62,68], the origin and biological significance of the two frameshifting products remained unknown, which prompted us to dissect the frameshifting event on the *gag-pol* mRNA using our *in vitro* reconstituted translation system with either mammalian or bacterial components [69]. The reported efficiency of *gag-pol* –1PRF is about 10%, as measured with dual-luciferase reporters *in vivo* in human cell culture [70,71]. We note that this frameshifting efficiency has been recapitulated in bacteria, yeast, and mammalian cells

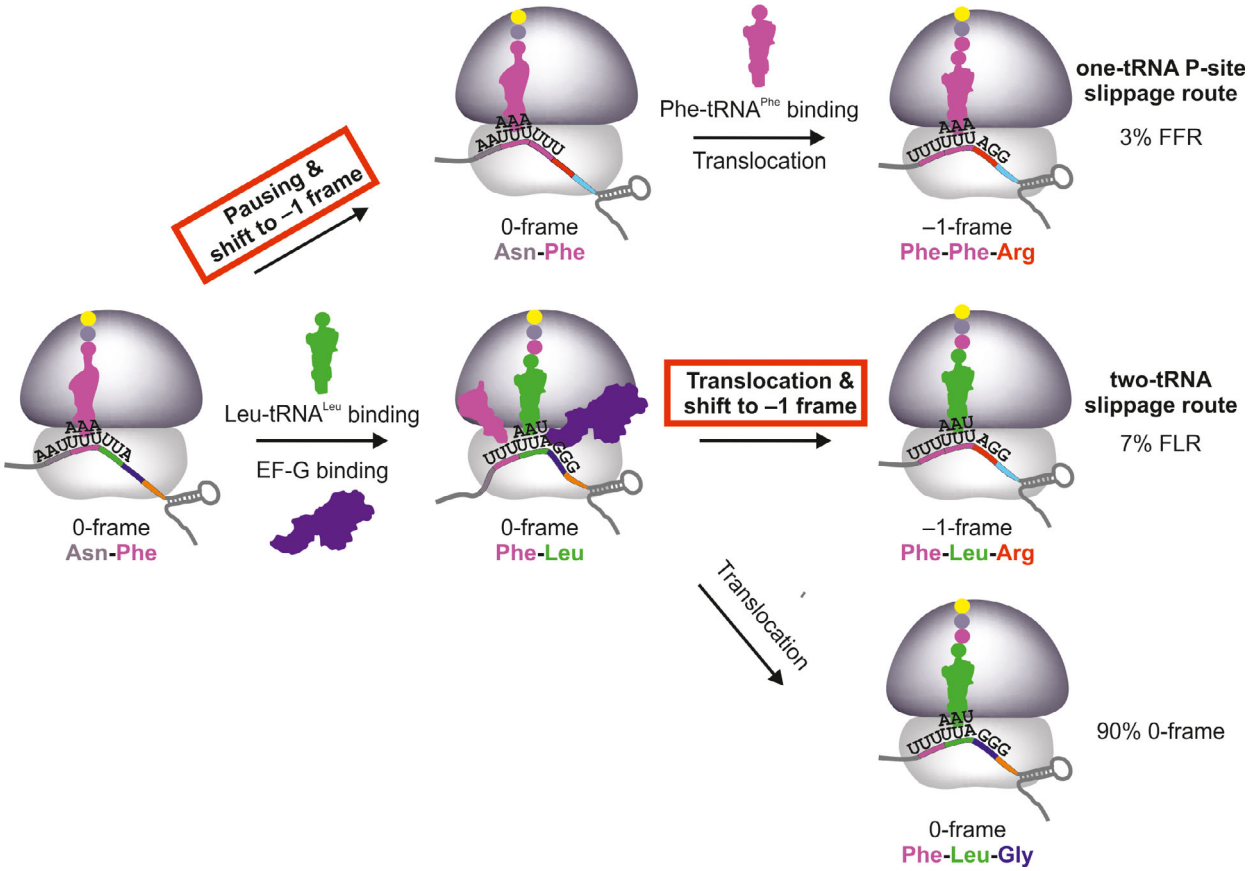

Fig. 2. Kinetic mechanisms of FFR (upper) and FLR (lower) –1PRF pathways on the *gag-pol* mRNA of HIV-1. FFR results from one-tRNA slippage with peptidyl-tRNA^{Phe} in the P site (in magenta) when the A site is vacant due to low availability of Leu-tRNA$^{Leu(UAA)}$. FLR arises upon frameshifting during translocation of tRNA^{Phe} and peptidyl-Phe-Leu-tRNA$^{Leu(UAA)}$ (in green) and is prevalent at excess of Leu-tRNA$^{Leu(UAA)}$. After reading the slippery site, translation can continue in the –1-frame by incorporating Arg at the AGG codon (red) or in 0-frame by decoding Gly at the GGG codon (blue). The –1-frame commitment steps on the FFR and FLR routes is marked in red. –1PRF on SFV *6K*, IBV *1a/1b*, and *E. coli dnaX* can, in principle, follow the same two routes. The existence of the two-tRNA route is well-documented [15,29,30,45,46,57]. The prevalence of the one-tRNA route for SFV 6K depends on the concentration of tRNA$^{Leu(UAA)}$ in the infected neuronal cells, which is not known (see text below). The one-tRNA slippage on IBV *1a/1b* could occur before decoding of the first slippery seqence codon UUA by the respective rare tRNA$^{Leu(UAA)}$, however, the existence of the respective –1-frame peptide product containing Phe-Lys, rather than Leu-Lys, has not been tested and the abundance of tRNA$^{Leu(UAA)}$ in avian host cells is unknown. For *dnaX*, tRNA^{Lys} that reads the slippery site codons is abundant and the one-tRNA frameshifting pathway is only elicited by starvation.

in vivo and in the respective *in vitro* translation systems, suggesting that –1PRF relies on the highly conserved components of the translational apparatus [21,68,72]. The frameshifting motif of HIV-1 consists of a main SS, SS1, with a sequence of U_1 UUU_4 UUA_7 encoding Phe (UUU) and Leu (UUA) in 0-frame with a downstream SL element (Figs 1A and 2) [21,43]. Of the two distinct frameshifting products, one contains the 0-frame peptide Phe-Leu followed by the –1-frame amino acid sequence (FLR) and the other one a Phe incorporated instead of Leu, that is, Phe-Phe followed by the –1-frame sequence (FFR) (Fig. 2). The ratio of the two –1PRF products is about 70% to 30% [21,61,62,64].

Kinetic analysis shows that –1PRF in HIV-1 proceeds via the same two routes as previously described for IBV *1a*|*1b* and *E. coli dnaX* mRNAs (Fig. 2) [69]. Intriguingly, we found that the two routes depend on the availability of Leu-tRNA$^{Leu(UAA)}$ to read the UUA codon of the SS1 (Fig. 2) [69]. At limited supply of Leu-tRNA$^{Leu(UAA)}$ decoding is slow. While the ribosome waits for the tRNA delivery, the P-site peptidyl-tRNA^{Phe} bound to the UUU codon slips backwards and re-pairs with another UUU codon in –1 frame, resulting in the change of the identity of the A-site codon from UUA to UUU and the incorporation of the second Phe into the peptide, which gives rise to the FFR –1-frame product (consistent with earlier

suggestions [59,61,64,68]) (Fig. 2). At low Leu-tRNA-Leu(UAA) concentrations, the rate of Leu incorporation is lower than that of P-site $tRNA^{Phe}$ slippage; hence, the one-tRNA slippage and the respective FFR route prevail. With increasing Leu-$tRNA^{Leu(UAA)}$ concentration, its incorporation rate increases, which abolishes the FFR route and favors the two-tRNA slippage resulting in FLR pathway of frameshifting (Fig. 2). Along with kinetic data [69], one of the main arguments supporting this finding is the lack of competition between $tRNA^{Gly}$ (0-frame) and $tRNA^{Arg}$ (–1-frame) reading the codon GGG (or AGG in –1-frame) following the SS1 [69]. This observation indicates that the commitment to –1-frame happens upon Leu incorporation but prior to the next codon decoding.

Another example of viral frameshifting is found in gene *6K* of the alphavirus SFV. Here –1PRF defines the ratio between two structural proteins, 6K (0-frame) and TransFrame (TF, –1-frame), which play a role in the envelope protein processing, membrane permeabilization, virion assembly, virus budding, and contribute to infectivity [20]. The efficiency of –1PRF in SFV measured with dual-luciferase reporters in human cells is about 15% [27]. Having probed the mRNA secondary structure element on the 6K mRNA (Fig. 1B, C), we studied the mechanism of –1PRF as described for IBV, *dnaX*, and HIV-1. Because SFV and HIV-1 have the same SS sequence U_1 UUU_4 UUA_7, we hypothesized that –1PRF in SFV also results in two peptides, in this case FFS and FLS, depending on the presence of the Leu-$tRNA^{Leu(UAA)}$ isoacceptor (Fig. 3A). In the absence of Leu-$tRNA^{Leu(UAA)}$, the FFS product is formed and its yield depends on the concentration of Phe-$tRNA^{Phe}$ (Fig. 3B), suggesting that the slippage occurs prior to and independent of $tRNA^{Leu(UAA)}$ incorporation, similarly to 'hungry' slippage in HIV-1. With the increase in Leu-$tRNA^{Leu(UAA)}$ concentration, the –1PRF efficiency decreases dramatically from about 70% in the absence of $tRNA^{Leu(UAA)}$ to 18% at $tRNA^{Leu(UAA)}$ saturation (Fig. 3C). Thus, the FFS route in SFV is operational when $tRNA^{Leu(UAA)}$ is absent or in limited supply, whereas under saturating translation conditions the FLS route becomes prevalent. To better understand the FLS regime, we tested the competition between 0-frame Val-$tRNA^{Val}$ and –1-frame Ser-$tRNA^{Ser}$ for binding at the codon following the SS, GUG. Titration of these tRNAs in the presence of equimolar amounts of $tRNA^{Leu(UAA)}$ (1:1 molar ratio to 70S) does not change the frameshifting efficiency appreciably (Fig. 3D), which suggests that, similarly to HIV-1, FLS products result from the dual slippage of the SS tRNAs $tRNA^{Phe}$ and $tRNA^{Leu(UAA)}$ in the late stage of translocation before the GUG codon is presented in the A site.

Effect of the tRNA pool on frameshifting and the implications for potential antiviral therapies

As described above, the Leu-$tRNA^{Leu}$ isoacceptor reading the UUA codon of the SS in HIV-1 and SFV acts as the main modulator of the –1PRF efficiency and defines the frameshifting route in these two viruses. Interestingly, the UUA codon along with other A-ending codons is rare in humans, but accounts for more than 45% of all Leu-coding codons in late-expressing genes of HIV-1 including *gag* and *pol* [73,74]. Leu-$tRNA^{Leu(UAA)}$ is dramatically underrepresented in $CD4^+$ T-lymphocytes, the primary target cells for HIV-1 infection in humans, that is, it is 20-fold less abundant than the major Leu isoacceptor Leu-$tRNA^{Leu(CAG)}$ [69]. The high demand for Leu-$tRNA^{Leu(UAA)}$ to achieve efficient *gag* and *gag-pol* translation may additionally deplete the pool of free Leu-$tRNA^{Leu(UAA)}$ in the cell, thus further decreasing its availability for decoding at the *gag-pol* frameshifting site. Furthermore, tRNA levels are known to fluctuate in response to interferon activation and to changes in gene expression triggered by viral infection [75,76]. In light of these observations, FFR and FLR –1-frame products resulting from two different routes in HIV-1 may represent an adaptation strategy of the virus to the changing availability of the crucial Leu-$tRNA^{Leu(UAA)}$. When the concentration of $tRNA^{Leu(UAA)}$ decreases, the ribosome switches to the FFR route leading to robust –1PRF, thereby maintaining a stationary frameshifting level independent of small $tRNA^{Leu(UAA)}$ fluctuations that are realistic *in vivo*. We also note that unlike in *dnaX*, where the 'hungry' frameshifting pathway is activated only under starvation conditions, HIV-1 can use both 'hungry' (FFR) and translocation-dependent (FLR) pathways constitutively to achieve a constant –1PRF efficiency. Unlike HIV-1, SFV primarily infects neuronal cells (neurons and oligodendrocytes) [77], in which the level of $tRNA^{Leu(UAA)}$ is not known; thus the physiological relevance of the two potential –1PRF pathways in SFV remains to be elucidated.

Given the low level of $tRNA^{Leu(UAA)}$ in human T-lymphocytes, the question remains how HIV-1 can satisfy its high demand for this tRNA to achieve an efficient translation of its late-expressing genes. HIV-1 can package some cellular tRNAs, among them $tRNA^{Lys}$, $tRNA^{Ile}$ and to a lesser extent $tRNA^{Leu(UAA)}$, during

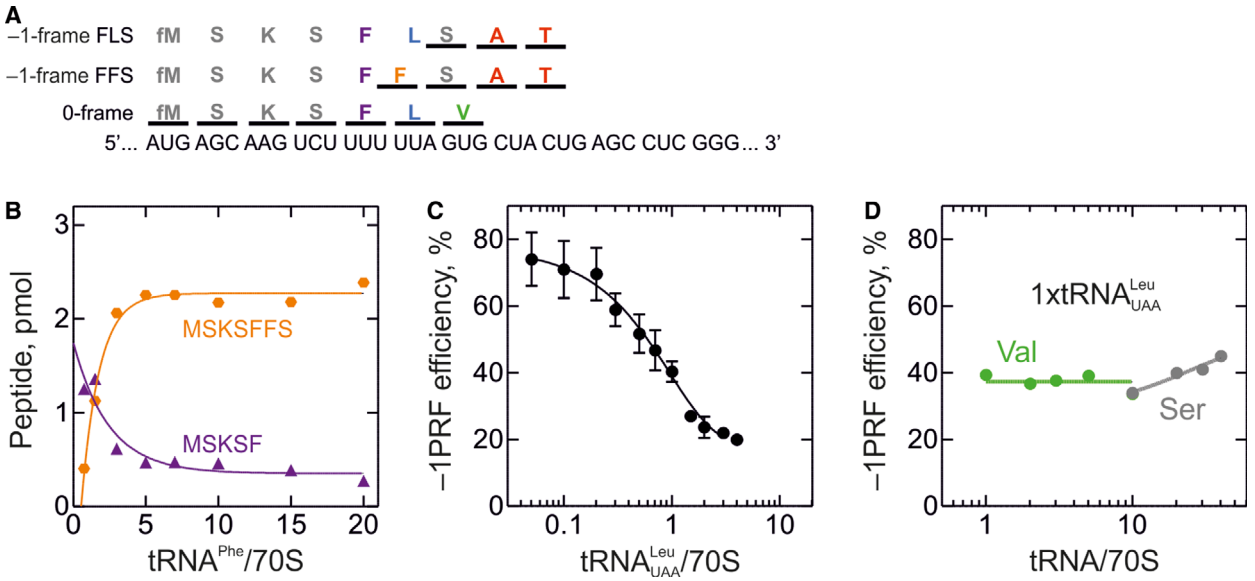

Fig. 3. Mechanism of –1PRF on the SFV 6K mRNA. Translation was carried out in HiFi buffer at 37 °C as described in [69] for the *gag-pol* mRNA; concentrations were Ser-tRNA^{Ser} and Phe-tRNA^{Phe} (with 0.8 μM each) and Lys-tRNA^{Lys}, Val-tRNA^{Val}, Ala-tRNA^{Ala} and Thr-tRNA^{Thr} (0.25 μM each) and IC (0.08 μM) programmed with the 6K mRNA. Translation products were separated by reversed phase high-performance liquid chromatography [69]. 0-frame products were identified based on the incorporation of [^{14}C]Val, –1-frame peptides using [^{14}C]Ala and [^{14}C]Thr. The –1PRF efficiency was calculated as a ratio between –1-frame peptides and the sum of –1-frame and all 0-frame products, multiplied by 100%. (A) Schematic of the frameshifting site. The model SFV mRNA containing native SS and SL is optimized for translation in *E. coli* by introducing a SD sequence and a start codon AUG followed by AAG (Lys) to improve translation efficiency. (B) Effect of Phe-tRNA^{Phe} on FFS peptide formation in the absence of Leu-tRNA$^{Leu(UAA)}$. Translation was carried out using tRNAs aminoacylated with M, S, K, F. (C) Dependence of –1PRF on Leu-tRNA$^{Leu(UAA)}$ concentration. Translation was carried out with M, S, K, F, L, V, A, and T aa-tRNAs. (D) Effect of Val-tRNA^{Val} (green circles) and Ser-tRNA^{Ser} (gray circles) concentrations on –1PRF efficiency. Translation was carried out using an equimolar concentrations of Leu-tRNA$^{Leu(UAA)}$ and aa-tRNAs as in C. The large excess of Ser-tRNA is required to ensure efficient translation of the SFV mRNA, which contains three Ser codons read by different tRNA^{Ser} isoacceptors in the total tRNA^{Ser} used in these experiments.

virion assembly [78]. Because tRNA packaging happens passively governed by the concentration gradient, these tRNAs must be present in the cell at significant concentrations. There are multiple indirect indications that HIV-1 itself can affect the tRNA pools by yet unknown mechanisms [74] and that HIV infection can change the cellular localization of individual aa-tRNA synthetases from the multi-aa-tRNA synthetase complex [79], which may affect their aminoacylation activity. Other viruses whose genomes have a codon usage different from their host can alter the free tRNA pools by changing polysome-associated tRNA levels (vaccinia and influenza A) or by tRNA misacylation (influenza A and adenovirus) [76,80], but the mechanism of how HIV-1 could modulate tRNA concentrations remains unknown.

Along with HIV-1 and SFV, –1PRF in the extended CAG repeats in the human huntingtin mRNA is also modulated by the availability of the aa-tRNA [81]. Expansion of CAG-encoded poly-glutamine (polyQ) stretches beyond a certain threshold leads to the development of progressive neurodegenerative diseases including Huntington's disease and spinocerebellar ataxia [82]. Translation of the extended CAG repeats results in the depletion of tRNA$^{Gln(CUG)}$ which recognizes the CAG codon. The depletion of the pool of free tRNA, in turn, causes –1PRF, the efficiency of which depends on the length of polyQ stretches [81]. Here –1PRF likely proceeds via the one- tRNA (or 'hungry') slippage mechanism, as discussed above for *dnaX*, HIV-1 *gag-pol* (FFR) and SFV *6K* (FFS). Interestingly, tRNA$^{Gln(CUG)}$ shows tissue-specific expression levels in humans, and the lowest concentration of this tRNA was detected in the brain region called striatum, which is the primary site for Huntington's disease [81]. In summary, the low level of the target tRNA is linked to the increased frameshifting levels and accumulation of the mutated huntingtin protein, thereby contributing to the disease severity in Huntington's patients.

The strong inhibition of –1PRF in HIV-1 by excess amounts of tRNA$^{Leu(UAA)}$ offers a potential new approach in antiviral therapy. Based on codon usage differences between retroviruses and the human host, multiple tRNA species were predicted that are critical for retroviral protein synthesis but dispensable for

human translation, laying the foundation for the hypothetical tRNA Inhibition Therapy (TRIT) [83]. Inactivation of these tRNAs should drastically reduce the elongation rate of viral protein synthesis leaving the host translation unaffected. One of the best targets of TRIT, which could be exploited in HIV-1 and other retroviruses (HIV-2, HTLV-1 and 2), is $tRNA^{Leu(UAG)}$ reading the CUA Leu codon [83]. Furthermore, interferon-induced protein Schlafen 11 (SLFN11) was shown to selectively inhibit the expression of late HIV-1 proteins in a codon-dependent manner [84]. SLFN11 induces a selective cleavage of tRNAs with a long variable loop, such as $tRNA^{Ser}$ and $tRNA^{Leu}$ [85]. Because HIV-1 *gag-pol* mRNA harbors multiple UUA codons, its translation is susceptible to the action of SLFN11 overexpression due to the $tRNA^{Leu(UAA)}$ depletion [85]. Recently, another interferon-induced protein, Shiftless, was shown to dysregulate –1PRF and inhibit replication of HIV-1 [35]. However, its potential use in antiviral therapy remains unclear because Shiftless binds to the ribosome and frameshifting sequences of many viral and even human cellular mRNAs [35].

Alternative slippages: examples and mechanisms

Frameshifting events are not limited to −1- and +1-slippages: ribosomes can slip by −2, +2, +4, +5, or +6 nucleotides [13–17]. For example, the SS of *dnaX* in *E. coli* supports not only –1PRF, but also −2, +2, and even +4 frameshifting [14,15]. In dsDNA tailed phages, the tail assembly is typically regulated by a highly conserved –1PRF event; however, in Mu phage a −2 slippage is required instead [86]. The synthesis of the full-length human antizyme requires +1PRF, which can proceed via +1PRF (predominant) and –2PRF in fission yeast, but results from –2PRF in budding yeast; here –2PRF occurs via a one-tRNA slippage in the presence of the empty A site, as described for 'hungry' –1PRF [17]. Porcine reproductive and respiratory syndrome virus utilizes both –1 and –2 slippage on the same SS G_1 GUU_4 UUU_7 to produce different replicase polyproteins [87]. This is the only described example of –2PRF where the –2 slippage requires the presence of a special mRNA stimulatory sequence downstream of the SS, similar to enhancers known to regulate +1PRF [87,88]. We note that a small amount of +1 and −2 products in HIV-1 may also result from the transcriptional slippage of RNA polymerase on the *gag-pol* SS1 [89].

The SS1 of HIV-1 was also reported to support –2PRF [16]. Because in those experiments, the frameshifting sequence was placed into an unnatural context followed by an antisense oligonucleotide-binding site, we were prompted to study the extent of alternative slippages on the native *gag-pol* mRNA frameshifting site [69]. HIV-1 *gag-pol* SS1 indeed allows –2 and even +1 slippage, but their contribution to the overall frameshifting efficiency becomes significant only when certain aa-tRNAs are omitted. Thus, alternative slippages on SS1 must follow the 'hungry' route of frameshifting.

In HIV-1, ribosomes that continue translation in the +1- or –2-frame, soon encounter one of the multiple downstream stop codons, which leads to premature termination. Premature termination is often used upon slippage on nonprogrammed tetra- and heptanucleotide slippery sites to abort production of nonfunctional peptides, especially under conditions of aa-tRNA limitation [90]. Premature termination upon frameshifting can also result in the production of functional proteins. One example is *E. coli* gene *copA* encoding a copper ion transporter [91]. Here –1PRF causes premature termination and formation of a truncated peptide CopA(Z), which turned out to be a copper chaperone protecting cells from excessive copper concentrations in the environment [91]. On the other hand, premature termination upon –1PRF in human CCR5 mRNA leads to mRNA degradation by the nonsense-mediated decay pathway, thus, regulating mRNA stability and gene expression [32].

Appearance of potential slippery sites in viruses upon antiviral therapy

A hallmark of HIV-1 is its vast genetic diversity and rapid evolution, in particular in response to antiviral therapies. Some of the most potent antiviral drugs target the protease that cleaves the Gag-Pol polyprotein to yield mature functional viral proteins [92,93]. One of the Gag protease cleavage sites, p1/p6, is located in the part of the protein encoded by the UUU-CUU codons in pSS2 downstream of SS1; the cleavage is between Phe and Leu [94,95]. In response to antiviral treatment with protease inhibitors, the protease gene of HIV-1 accumulates mutations that reduce the affinity of the protease for the inhibitor, but simultaneously impair the recognition of its canonical cleavage sites [92,94,95]. This defect is partially rescued by a compensatory mutation of the CUU to the UUU in pSS2, because the resulting Leu to Phe substitution creates a new functional cleavage site by enhancing van der Waals interactions between the substrate and the mutant protease, thereby allowing Gag-Pol processing [96]. The mutation also turns the U_1 UUU_4 CUU_7 sequence into U_1 UUU_4 UUU_7, which can support ribosome slippage (Fig. 1A) [97–101]. The mutated slippery pSS2 supports both –1 and –2 slippage, but

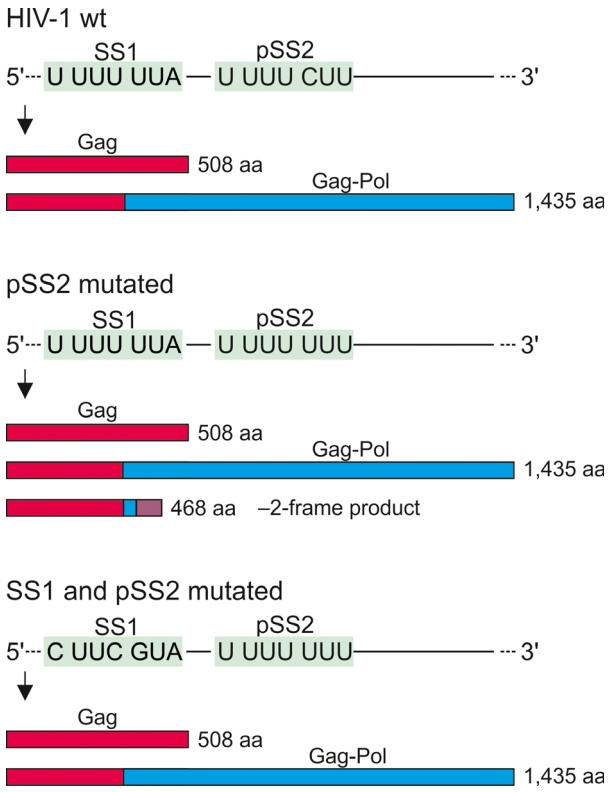

Fig. 4. The role of the pSS2 in maintaining the permissive Gag to Gag-Pol ratio. Top, in the wt gag-pol mRNA, –1PRF on the SS1 accounts for most of the Gag-Pol product and pSS2 is silent. Middle, a compensatory mutation in pSS2 that emerges in response to treatment with inhibitors targeting the viral protease makes pSS2 slippery. The production of Gag and Gag-Pol is unchanged, but a small fraction of ribosomes slips into –2-frame, resulting in synthesis of a truncated protein. Bottom, when SS1 is mutated, –1-frameshifting on pSS2 restores Gag-Pol production to about 70% of that on the wt sequence.

its contribution to the overall frameshifting efficiency depends on the availability of SS1 (Fig. 4) [69]. In case of mutated SS1, the native pSS2 cannot rescue the –1PRF efficiency suggesting that its contribution to frameshifting is negligible. When SS1 is functional and pSS2 harbors the C_5U mutation, the overall –1 frameshifting efficiency remains unchanged but a significant amount of –2 frameshifting is observed. Most importantly, when SS1 is dysfunctional, the C_5U mutation in pSS2 can restore about 70% of the wild-type –1PRF efficiency, which might be enough to sustain the viral lifecycle [69].

Interestingly, HIV-1 is not the only virus which develops an alternative slippery site in response to antiviral treatment. Herpes simplex viruses resistant to acyclovir treatment accumulate mutations in their thymidine kinase (TK) gene turning previously silent repeats of G (G-string) or C (C-cord) nucleotides into functional slippery sites, supporting +1 and –1 slippage, respectively [102,103]. –1PRF in the C-cord is thought to proceed via single P-site tRNA slippage and is stimulated by a nonstop mRNA, presumably, by stalling the ribosome on the polyA-tail [102]. These frameshifting events provide a sufficient level of TK and thus represent a rescue mechanism for the virus to ensure its viability despite the damage caused by the antiviral therapy [102,103].

Conclusions and future perspectives

Frameshifting sites utilize a great variety of the mRNA secondary structure elements. Despite this diversity, –1PRF seems to operate via two major evolutionary conserved kinetic pathways: through two-tRNA slippage during translocation or through one-tRNA P-site slippage at limited supply of the tRNA that decodes the 0-frame A-site codon. The choice of the frameshifting pathway is defined by the availability of the tRNA reading the slippery site codons, which is of crucial importance for pathogenic viruses whose codon usage is different from that of their hosts. Pathogenic viruses have developed different strategies, such as utilizing alternative frameshifting routes, newly emerging slippery sites or alternative slippages, to ensure robust frameshifting efficiencies and thus robust viral cycle progression, regardless of environmental or therapy-induced changes.

Dependence of –1PRF on cis-acting elements—the slippery sites and the mRNA secondary structures—is well understood, whereas the list of trans-acting factors modulating frameshifting is still growing. Recent evidence suggests that multiple proteins of the human immune system can influence the –1PRF efficiency in infectious viruses by acting directly on the frameshifting sites or competing for the translation resources [84,85]. Small interfering RNAs represent another group of important players in modulating frameshifting [32]. Better understanding of trans-acting factors will provide not only novel insights into the mechanism of frameshifting, but will also yield the necessary understanding of the host–pathogen–host interaction and coadaptation, which could lay a foundation for future therapeutic approaches.

Classical antiviral therapies targeting key components of the viral life cycle lead to the emergence of resistant virus isolates due to selection pressure and high mutation rates in viral genomes [104]. Frameshifting sites and especially slippery heptamers represent attractive targets for antiviral drug design because their sequences are highly conserved among virus subtypes [20,105] and frameshifting often determines virus viability. Attempts to develop antiframeshifting therapeutics against HIV-1 using both

natural and synthetic molecules has so far not succeed in clinical trials because of their cytotoxicity and off-target effects [106–108]. This is because –1PRF relies on highly conserved elements of the translation machinery, which makes it likely that both the viral and host components are targeted. Our recent understanding of frameshifting modulation suggests that exploitation of the host cellular resources could become an alternative approach to dysregulate frameshifting in pathogenic viruses. Because of the differences in codon usage between HIV-1 and its human host, a strategy worth exploring would be to change the levels of the tRNAs crucial for the decoding of the viral frameshifting motif but dispensable for the host translation, such as $tRNA^{Leu(UAA)}$; or overexpression of human genes with virus-like codon usage to deplete the available translation resources. Thus, frameshifting—which is a remarkable recoding event—remains an interesting target for future medical research.

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