

# Stem–loop structures can effectively substitute for an RNA pseudoknot in –1 ribosomal frameshifting

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## ABSTRACT

–1 Programmed ribosomal frameshifting (PRF) in synthesizing the gag-pro precursor polyprotein of *Simian retrovirus type-1* (SRV-1) is stimulated by a classical H-type pseudoknot which forms an extended triple helix involving base–base and base–sugar interactions between loop and stem nucleotides. Recently, we showed that mutation of bases involved in triple helix formation affected frameshifting, again emphasizing the role of the triple helix in –1 PRF. Here, we investigated the efficiency of hairpins of similar base pair composition as the SRV-1 gag-pro pseudoknot. Although not capable of triple helix formation they proved worthy stimulators of frameshifting. Subsequent investigation of ~30 different hairpin constructs revealed that next to thermodynamic stability, loop size and composition and stem irregularities can influence frameshifting. Interestingly, hairpins carrying the stable GAAA tetraloop were significantly less shifty than other hairpins, including those with a UUCG motif. The data are discussed in relation to natural shifty hairpins.

## INTRODUCTION

Ribosomal frameshifting is a translational recoding event in which a certain percentage of ribosomes are forced to shift to another reading frame in order to synthesize an alternative protein. This switch occurs at a specific position on the mRNA, called the slip site or slippery sequence, and can be either forwards (+1) or backwards (–1). The nature and efficiency of frameshifting depends on several factors, including tRNA availability and modifications, and mRNA primary and secondary structure (1,2).

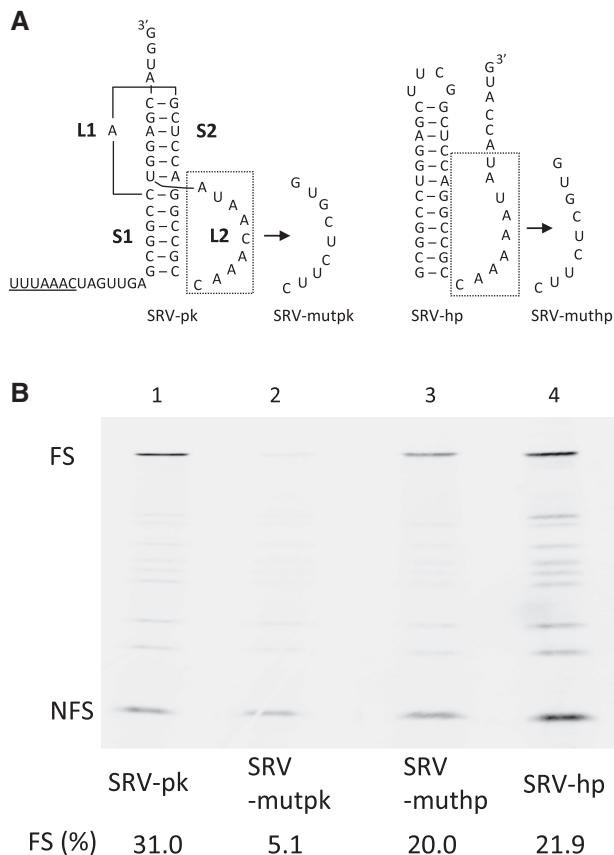
The signals that are responsible for –1 frameshifting comprise two elements: a slippery sequence where the actual reading shift takes place, and a downstream located structural element which greatly stimulates the efficiency

of frameshifting. Although the mechanism is still elusive, the present view is that the downstream structure forms a physical barrier that blocks EF-2 function and causes ribosomes to stall in their translocation step. This ‘road-block’ puts tension on the mRNA–tRNA interaction. The tension can be relieved by the realigning of A-site and P-site tRNAs in the 5'-direction, whereafter EF-2 can do its work and the ribosome resumes translation in the –1 reading frame (3).

In general, a pseudoknot is more efficient in stimulating frameshifting than a hairpin of the same sequence composition. This difference is likely related to a higher thermodynamic stability of the pseudoknot. Indeed, from thermodynamic analysis it appears that pseudoknots are more stable than their hairpin counterparts (4–6). Recent studies employing mechanical ‘pulling’ of frameshifter pseudoknots have shown a correlation between the mechanical strength of a pseudoknot and its frameshifting capacity (7,8), and the influence of major groove and minor groove triplex structures (9). The higher strength of a pseudoknot can be primarily attributed to the formation of base triples between the lower stem S1 and loop L2 (Figure 1A), making it more resistant against unwinding by an elongating ribosome (8,10). Base triples in several pseudoknots, such as *Beet western yellows virus* (BWYV) p1–p2 (11), *Pea enation mosaic virus type-1* (PEMV-1) p1–p2 (6), *Sugarcane yellow leaf virus* (ScYLV) p1–p2 (12) and *Simian retrovirus type-1* gag-pro (SRV-1) (13,14) have been shown to play an essential role in frameshifting. For pseudoknots with a longer stem S1 of 10–11 bp, like that of *Infectious Bronchitis Virus* (IBV), base triples do not appear to contribute to frameshifting (15).

Although a hairpin is considered to be a less efficient frameshift-inducing secondary structure than a pseudoknot, some viruses like *Human immunodeficiency virus* (HIV) (16), *Human T-lymphotropic virus type-2* (HTLV-2) (17) and *Cocksfoot mottle virus* (CfMV) (18) make use of a simple hairpin to stimulate substantial levels of frameshifting. In addition, frameshifting in the prokaryotic *dnaX* gene requires, next to an upstream

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**Figure 1.** Hairpin derivative of the Simian retrovirus type-1 (SRV-1) *gag-pro* frameshift pseudoknot is an efficient frameshift stimulator. (A) Schematic representation of the SRV-1 pseudoknot (SRV-pk) and its hairpin derivative (SRV-hp). Mutations in SRV-pk loop L2 (SRV-mutpk) and SRV-hp (SRV-muthp) are indicated. The slippery sequence is underlined. (B) SDS-PAGE analysis of <sup>35</sup>S-methionine-labeled translation products in rabbit reticulocyte lysate (RRL). -1 Ribosomal frameshifting is monitored by appearance of the 65-kD product (FS). The non-shifted zero-frame product is indicated by NFS. Quantitative analysis of frameshifting efficiency [FS (%)] is described in 'Materials and Methods' section.

enhancer, the presence of a hairpin as well (19). A few studies have investigated a correlation between hairpin stability and frameshift efficiency of natural shifty hairpins (19,20). Nonetheless, certain studies have shown that a hairpin composed of the same base pairs as a frameshifter pseudoknot is not very efficient in inducing frameshifting in mammalian cells and lysates (21–23) but is in other systems (24).

Here, we have carried out a systematic analysis of the frameshift-inducing efficiency of hairpins derived from the SRV-1 *gag-pro* frameshifter pseudoknot. Investigation of about 30 different hairpin constructs revealed that next to thermodynamic stability, also loop size and composition, and stem irregularities can significantly influence frameshifting. Our data showed that there exists no base specific contacts between the hairpin and the ribosome during frameshifting and suggests that the hairpin primarily serves as a barrier to allow repositioning of tRNAs at the slippery site.

## MATERIALS AND METHODS

Mutations in the SRV-1 *gag-pro* frameshifting signal were made in an abridged version of plasmid SF2 (25) which is derivative of pSFCASS5 (26), a frameshift reporter construct. In this version, the entire BglII–NcoI fragment of pSF2 was replaced by a synthetic dsDNA fragment (5'-G ATCTTAATACGACTCACTATAGGGCTCATTTAA ACTAGTTGAGGGGCCATATTTTCGC-3', a SpeI restriction site is underlined). This yielded plasmid pSF208 in which the original GGGAAAC slippery sequence has been replaced by the more slippery UUUAAAC sequence (26). pSF208 was digested with SpeI and NcoI, and sets of complementary oligonucleotides corresponding to the various mutants were inserted. A list of oligonucleotides is available upon request. All constructs were verified by automated dideoxy sequencing using chain terminator dyes (LGTC, Leiden).

### *In vitro* transcription

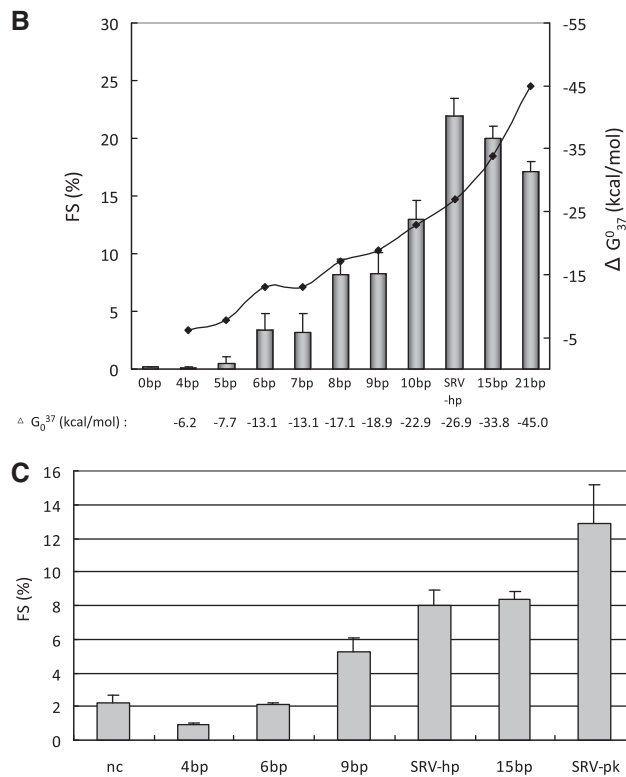
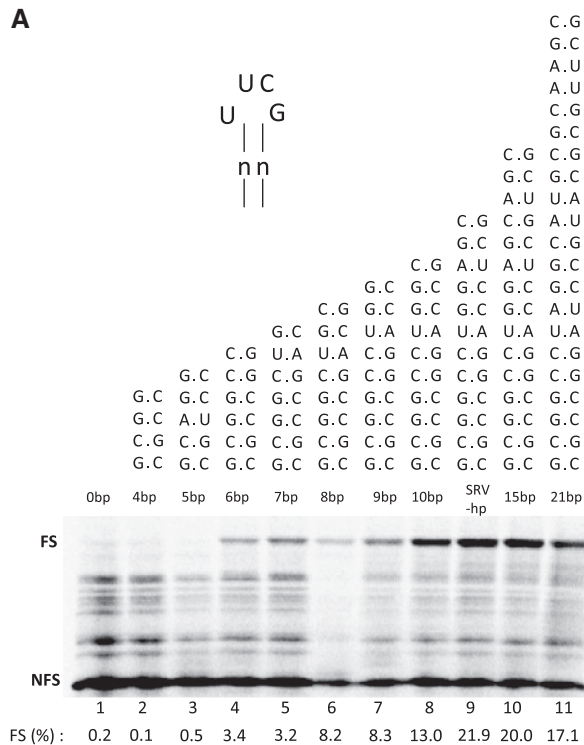
DNA templates were linearized by BamHI digestion and purified by successive phenol/chloroform extraction and column filtration (Qiagen, Benelux). SP6 polymerase directed transcriptions were carried out in 50 μl reactions containing ~2 μg linearized DNA, 10 mM NTPs, 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 10 mM DTT, 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 6 U of RNase inhibitor (RNasin, Promega, Benelux) and 15 U of SP6 polymerase (Promega, Benelux). After an incubation period of 2 h at 37°C, samples were taken and run on agarose gels to determine the quality and quantity of the transcripts. Appropriate dilutions of the reaction mix in water were directly used for *in vitro* translations. Alternatively, transcripts were purified by phenol/chloroform extraction and isopropanol precipitation and quantified by UV absorption as described previously (14).

### *In vitro* translation

Experiments were carried out in duplicate using serially—diluted mRNAs with final concentrations of 5 nM. Reactions contained 4 μl of an RNA solution, 4.5 μl of rabbit reticulocyte lysate (RRL, Promega), 0.25–1 μl of <sup>35</sup>S methionine (Amersham, *in vitro* translation grade), 0.5 μl of 1 mM amino acids lacking methionine and were incubated for 60 min at 28°C. Samples were boiled for 3 min in 2× Laemmli buffer and loaded onto 12% SDS polyacrylamide gels. Gels were dried and exposed to phosphorimager screens. Band intensity of 0-frame and -1 frameshift products was measured using a Molecular Imager FX and Quantity One software (Biorad). Frameshift percentages were calculated as the amount of -1 frameshift product divided by the sum of 0 and -1 frame products, corrected for the number of methionines (10 in the 0-frame product and 28 in the fusion product), multiplied by 100.

### Frameshift assays in mammalian cells

Candidates of interest were constructed in a dual luciferase vector, pDUAL-HIV(0), essentially as described previously (14,27). In short, pDUAL-HIV(0) was digested



**Figure 2.** Influence of stem length of UUCG-capped hairpins on  $-1$  ribosomal frameshifting efficiency *in vitro* and *in vivo*. (A) SDS-PAGE analysis of  $^{35}\text{S}$ -methionine-labeled translation products in RRL using mRNAs with hairpins of various stem lengths. See legend to Figure 1B for more details. The base composition of the various stems is shown. The 0bp is the control without a hairpin. (B) Graph showing the relation between frameshifting efficiency (indicated by bars) on the left y-axis and predicted thermodynamic stability by MFOLD [indicated by a solid diamond (filled diamond) on the right y-axis].

by KpnI and BamHI, followed by insertion of complementary oligonucleotides to clone the SRV-1 *gag-pro* pseudoknot, various hairpins as shown in Figures 2C and 5, and a negative control (NC) which formed no apparent secondary structure downstream of the slippery sequence. An in-frame control was constructed by inserting an A-residue upstream of the cytosine in the UUUAA AC slippery sequence of a 12 bp hairpin frameshift construct. HeLa cells were cultured in DMEM/high glucose/stable glutamine (PAA Laboratories GmbH, Germany) and supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. Cells were kept in a humidified atmosphere containing 5%  $\text{CO}_2$  at 37°C. Assay protocols were described previously (14). Briefly, cells were transfected with 300 ng of plasmid using 1  $\mu\text{l}$  of lipofectamine-2000 (Invitrogen) in a 24-well plate. Cells were lysed 24 h after transfection and luciferase activities were quantified by Glomax-multidetector (Promega, Benelux) according to manufacturer's protocol. Frameshifting efficiency was calculated by dividing the ratio of Renilla luciferase (RL) over Firefly luciferase (FL) activity of the mutant by the RL/FL ratio of the in-frame control, multiplied by 100.

## RESULTS

### Hairpin derived from the SRV-1 *gag-pro* pseudoknot is an efficient frameshift stimulator

In contrast to earlier reports involving the IBV frameshifting pseudoknot (21,22), we found that in the case of the SRV-1 *gag-pro* frameshift inducing pseudoknot a hairpin of similar composition as the pseudoknot did stimulate frameshifting *in vitro* (Figure 1A and B). The 12 bp hairpin derivative of the SRV-1 pseudoknot (SRV-hp) showed 22% frameshifting efficiency, whereas the SRV-1 pseudoknot (SRV-pk) in this context yielded 31%. The pseudoknot in these experiments is a modified version of the wild-type SRV-1 pseudoknot previously used for NMR and functional analysis (14). We note that the UUUAAAC slippery sequence was used to enhance the sensitivity of the *in vitro* frameshifting assay. This sequence is  $\sim 1.5$ -fold more slippery than the wild-type GGGAAAC slippery sequence (28). In the latter context, the hairpin was indeed less efficient (data not shown) while a non-slippery variant, GGGAAAGC, was not effective at all ( $<0.2\%$ , data not shown). Two other known efficient slip sites, AAAAAAC and UUUUUUA, caused 23 and 27%, respectively, of ribosomes to switch frame in the presence of the 12 bp hairpin (data not shown). These data showed that the 12 bp hairpin is a genuine stimulator of frameshifting.

### Figure 2. Continued

The average FS (%) and error bars were from at least three independent experiments. (C) Selected hairpins with different stem lengths were assayed for their ability to induce  $-1$  ribosomal frameshifting in HeLa cells. The frameshifting efficiency was obtained by measuring dual-luciferase activity of a frameshift reporter construct (see 'Materials and Methods' section). The *in vivo* experiments were done at least three times in triplicate.

Since the hairpin construct also contained sequences resembling those of L2 of the pseudoknot construct, it was theoretically possible that these nucleotides could take part in the same base triples. To investigate this possibility, we replaced the downstream sequence in the hairpin construct (SRV-muthp). This did not affect the frameshift efficiency of the hairpin construct. In contrast, the same mutations in the pseudoknot context (SRV-mutpk) reduced its activity about 6-fold (Figure 1B). Thus, it is unlikely that triple helix formation or other tertiary interactions contribute to hairpin-dependent frameshifting; the hairpin as such seems to be sufficient.

### Effect of hairpin stem size on frameshifting efficiency

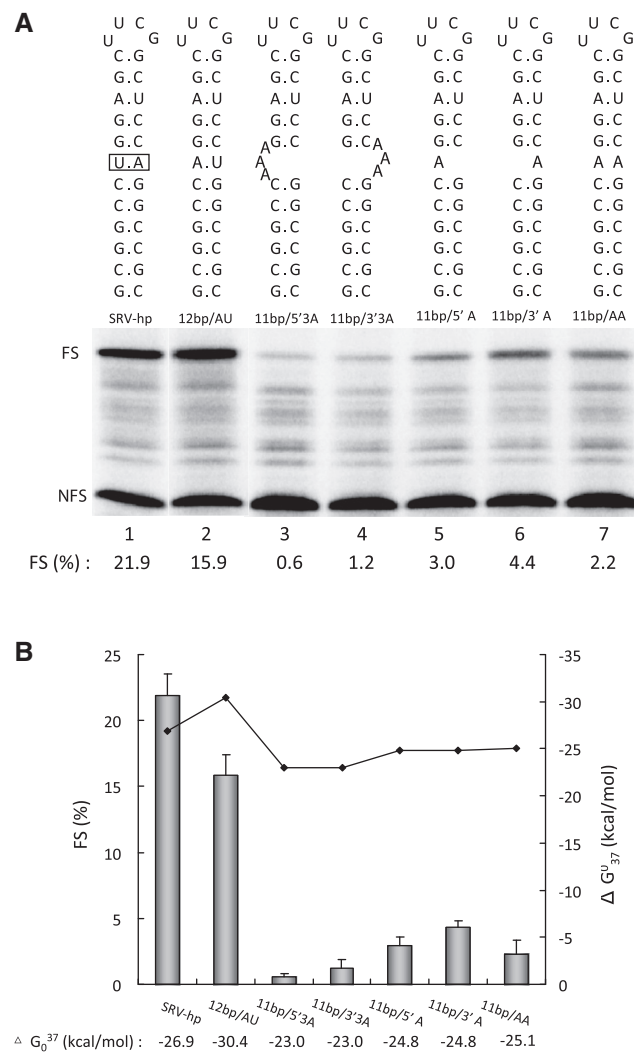
Next, we investigated the role of stem length on frameshifting efficiency. Increasing stem size from 12 to 15 or 21 bp did not significantly alter frameshifting (Figure 2A). On the other hand, decreasing stem size led to a steady decrease in frameshifting efficiency which seemed to vanish around a stem size of 5 bp or  $\Delta G^{37}$  of  $-7.7$  kcal/mol (Figure 2B). Thermodynamic stabilities were calculated at the MFOLD website using version 2.3 parameters (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3>), as these were previously shown to better fit *in vivo* hairpin stabilities (29). These data support the notion that downstream structures serve as barriers to stall translating ribosomes to stimulate frameshifting, and demonstrate that there is a correlation between the thermodynamic stability of a hairpin and its frameshift inducing capacity.

A selection of above hairpins was cloned into a dual-luciferase reporter plasmid and their frameshifting efficiency assayed in mammalian cells (Figure 2C). Although the absolute level of frameshifting was lower than *in vitro*, the trend was similar and showed maximal frameshifting of  $\sim 8\%$  around 12–15 bp. The pseudoknot in these assays was 1.6 times more efficient than the 12 and 15 bp hairpins, close to the *in vitro* ratio of 1.4 (see above). Thus, the hairpin derivative can effectively substitute for the SRV-1 pseudoknot in  $-1$  ribosomal frameshifting.

### Bulges and mismatches decrease frameshifting efficiency

Bulges and mismatches are known to change twisting and bending of a regular stem and are thus expected to influence the way in which a ribosome encounters a hairpin structure (11,30). To investigate a possible effect of helical twisting and bending on frameshifting, we introduced mismatches and bulges in the 12 bp stem at a position corresponding to the junction in the SRV pseudoknot (Figure 3A). Introduction of an A•A mismatch halfway through the stem (11 bp/AA) decreased frameshifting about 10 fold, although its predicted thermodynamic stability of  $-25.1$  kcal/mol is comparable to that of a regular hairpin of 10 bp, yielding 13% frameshifting (Figure 2B). The frameshift inducing ability was recovered when the base pair was restored to A–U (12 bp/AU).

We also introduced a single or triple adenosine bulge at either side of the stem, to investigate potential bending effects on frameshifting. Figure 3A and B show that the single adenosine bulge mutant decreased frameshifting,

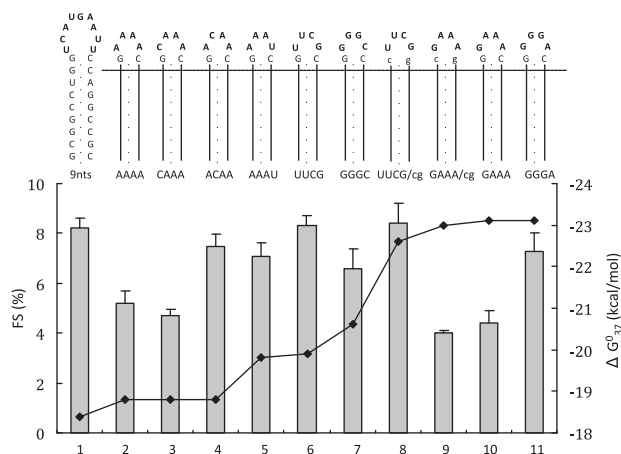


**Figure 3.** Influence of bulges and mismatches in the middle part of the hairpin on frameshifting efficiency. (A) SDS-PAGE analysis of  $^{35}$ S-methionine-labeled translation products in RRL using mRNAs containing the indicated hairpins. See legend to Figure 1b for more details. (B) Graph showing the relation between the predicted thermodynamic stability and frameshift efficiency. See legend to Figure 2B for more details.

depending on the location of the bulge, five to seven fold compared to the 12 bp hairpin construct. When the bulge was enlarged to three adenosines the frameshifting was almost abolished. Interestingly, the effect of bulges at the 3' side of the stem was less dramatic than those at the 5' side.

### Loop composition affects frameshifting efficiency

The loop composition plays a major role in hairpin stability, RNA/RNA and RNA/protein interaction. These factors may directly influence hairpin-induced ribosomal frameshifting efficiency. To explore the correlation between loop composition and frameshifting efficiency, a number of loop mutations were introduced in the context of a 9 bp stem (Figure 4). We note that the UUCG tetraloop with a CG closing base pair (cbp) has higher



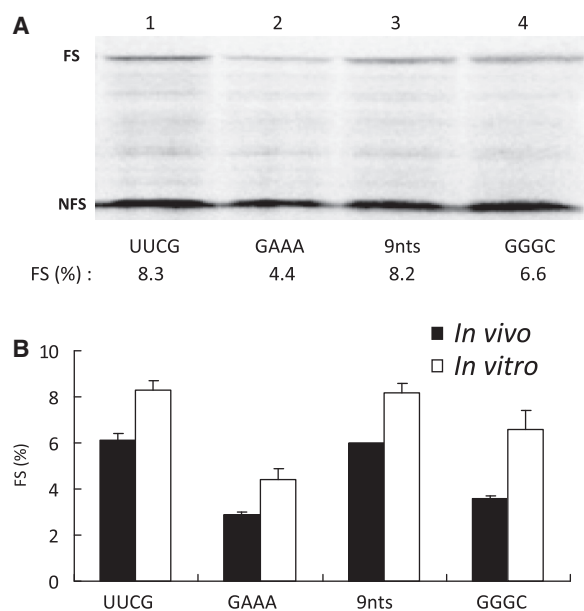
**Figure 4.** Influence of loop sequence and closing base pair (cbp) on  $-1$  ribosomal frameshifting efficiency. The composition of various loops capping a 9 bp stem is shown in bold, and CG-cbps are shown in lower case. The constructs are named after their loop sequence followed by the ‘/cg’ extension when the cbp was changed from G–C to C–G. Slippery sequence and spacer are the same as in the construct shown in Figure 1A. Graph is similar to that of Figure 2B except that on the right y-axis  $\Delta G$  starts from  $-18$  kcal/mol.

stability ( $\sim 2$  kcal/mol) than that with a GC cbp (31). Therefore, we first tested if this different cbp affected frameshifting efficiency. Our results showed that there is no difference in frameshifting efficiency between UUCG and UUCG/cg constructs (Figure 4, bars 6 and 8). Replacing the UUCG tetraloop by GGGC which, due to its high content of purines, is among the most disfavored tetraloops (32) had only a marginal effect on frameshifting (Figure 4, compare bars 6 and 7 and Figure 5A, lanes 1 and 4). Interestingly, increasing the loop size to 9 nt, which is predicted to lower the stability of stem did not affect frameshifting (Figure 4, bar 1; Figure 5A, lane 3).

Substituting UUCG by another stable tetraloop sequence (GAAA) resulted in a 2-fold decrease in frameshifting (Figure 5A, lanes 1 and 2) either with GC (Figure 4, bar 10) or CG cbp (Figure 4, bar 9). We designed another five loop mutants to try to explain the low efficiency of the GAAA tetraloop constructs. Constructs AAAA and CAAA induced 5.2% and 4.7% frameshifting, respectively (Figure 4, bars 2 and 3), which is close to that of the GAAA constructs. The efficiency of two other A-rich loop mutants, ACAA and AAUU, was 7.5% and 7.1%, respectively (Figure 4, bars 4 and 5), thereby closely matching that of the UUCG constructs. Finally, the GGGA tetraloop construct, belonging to the stable GNRA tetraloop family, induced 1.7-times more frameshifting than its GAAA sibling (Figure 4, bar 11). These data suggest that the presence of 3 or 4 adenines at the 3' side of a tetraloop is unfavorable for frameshifting.

#### Loop composition affects frameshifting efficiency *in vivo*

To further examine the role of the loop identity or size in ribosomal frameshifting, we cloned some of the above loop mutants into a dual-luciferase reporter plasmid and



**Figure 5.** Comparison of *in vitro* and *in vivo* frameshifting efficiencies induced by four selected 9 bp hairpins with different loops. (A) SDS-PAGE analysis of  $^{35}\text{S}$ -methionine-labeled translation products in RRL of mRNAs containing the 9 bp hairpin with the indicated loop sequence. See legend to Figure 1B for more details. (B) Comparison of  $-1$  ribosomal frameshifting *in vitro* and *in vivo*. The *in vitro* efficiency (white bar) was obtained by quantifying autoradiograms and averaging of at least three independent experiments. *In vivo* frameshifting efficiency (black bar) was obtained by measuring dual-luciferase activity of a frameshift reporter construct in HeLa cells (see ‘Materials and Methods’ section). The *in vivo* experiments were done at least three times in triplicate.

assayed their frameshifting efficiency in mammalian cells (Figure 5B). Our data show that the effects of loop nucleotides are comparable *in vitro* and *in vivo*. The stable GAAA tetraloop construct again had the lowest frameshifting efficiency (Figure 5B, 2.9%), which was half that of the UUCG construct (Figure 5B, 6.1%).

## DISCUSSION

Most RNA viruses that make use of ribosomal frameshifting employ pseudoknot structures instead of simple hairpins for this job. The reason for this may be the presence of a triple helix interaction between S1 and L2 in most frameshifter pseudoknots, which has been suggested to be a poor substrate for the ribosomal helicase (13,33) and hence increases ribosomal pausing and the time window for slippage. Although pausing is critical, it is not sufficient for efficient frameshifting (34). Previously, it was shown that a 17 bp hairpin with a calculated stability of  $-31.2$  kcal/mol derived from the minimal IBV pseudoknot induced 5- to 10-fold less frameshifting in RRL (22) than its parent pseudoknot even though both the hairpin and the pseudoknot can pause ribosomes at the same position and to a similar extent (34). In the present study, a 12 bp hairpin derivative of the SRV-1 gag-pro pseudoknot with a calculated stability of  $-26.9$  kcal/mol was capable of inducing 22% of frameshifting, which is only 1.4-fold



frameshift-prone than the standard wheat-germ extracts. Nevertheless, the ratio between pseudoknot and hairpin-induced frameshifting in this system is also 1.5. This number may reflect the additional interactions, like base triples, in a pseudoknot that make it a better frameshift stimulator than a hairpin.

In addition to stem size, loop composition is another determinant of hairpin stability. An important subgroup of hairpin loops is the tetraloop, which is the most common loop size in 16S and 23S ribosomal RNAs (37). The tetraloops with consensus UNCG, GNRA, or CUUG loop sequence form stable loop conformations (38,39). As opposed to the mentioned stable tetraloops, purine-rich (32) and larger loops (40) are considered to be less favorable for hairpin formation. Our results showed that the GGGC loop is indeed less efficient in inducing frameshifting but the larger loop construct (9 bp/9 nt), although having a lower thermodynamic stability, showed comparable frameshifting efficiency to the stable UUCG tetraloop hairpin. This is consistent with previous studies that showed that increasing the size of the loop in a hairpin or pseudoknot can increase frameshift-inducing ability to a certain extent (21,41). Although larger loops seem efficient in inducing frameshifting, in known examples of frameshifter hairpins, there are no loop sizes of more than 5 nt. This could relate to hairpin folding kinetics (40) or to nuclease sensitivity.

Intriguingly, we found that a 9 bp stem capped with a GAAA tetraloop is 2-fold less efficient in inducing frameshifting than its UUCG counterpart *in vitro* and *in vivo*. It has been reported that GAAA tetraloops are frequently involved in RNA tertiary interactions (42). We hypothesize that the GAAA tetraloop may be involved in an unknown RNA tertiary structure with ribosomal RNA, thereby interfering with frameshifting. The fact that in the known natural examples of frameshifter hairpins, the GAAA tetraloop, despite its high stability, is absent can be taken as support for this hypothesis (Olsthoorn, unpublished data). Further investigation of this observation may lead to new insights in ribosomal frameshifting.

In conclusion, our data show that hairpins of various base composition in stem and loop can act as efficient frameshift stimulators. Combined with previous studies on antisense-induced frameshifting (43,44), these data support the notion that downstream structures primarily serve as barriers to stall translating ribosomes to stimulate frameshifting. Although there exists a linear relationship between calculated stability and frameshifting, local destabilizing elements like bulges or mismatches in a hairpin can greatly influence frameshift-inducing activity. Future experiments addressing the mechanical strength of these hairpins (7–9) may help to improve our understanding of the basics of ribosomal frameshifting.

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