Functional analysis of the SRV-1 RNA frameshifting pseudoknot

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ABSTRACT

Simian retrovirus type-1 uses programmed ribosomal frameshifting to control expression of the Gag-Pol polyprotein from overlapping gag and pol open-reading frames. The frameshifting signal consists of a heptanucleotide slippery sequence and a downstream-located 12-base pair pseudoknot. The solution structure of this pseudoknot, previously solved by NMR [Michiels,P.J., Versleijen,A.A., Verlaan,P.W., Pleij,C.W., Hilbers,C.W. and Heus,H.A. (2001) Solution structure of the pseudoknot of SRV-1 RNA, involved in ribosomal frameshifting. *J. Mol. Biol.*, 310, 1109–1123] has a classical H-type fold and forms an extended triple helix by interactions between loop 2 and the minor groove of stem 1 involving base–base and base–sugar contacts. A mutational analysis was performed to test the functional importance of the triple helix for −1 frameshifting *in vitro*. Changing bases in L2 or base pairs in S1 involved in a base triple resulted in a 2- to 5-fold decrease in frameshifting efficiency. Alterations in the length of L2 had adverse effects on frameshifting. The *in vitro* effects were well reproduced *in vivo*, although the effect of enlarging L2 was more dramatic *in vivo*. The putative role of refolding kinetics of frameshifter pseudoknots is discussed. Overall, the data emphasize the role of the triple helix in −1 frameshifting.

INTRODUCTION

Ribosomal frameshifting is a translational recoding mechanism that allows the synthesis of multiple proteins from a single mRNA. During this process a certain proportion of the ribosomes is forced to move one or two nucleotides backwards (–1 or −2 frameshift) or forwards (+1 or +2 frameshift) whereafter they continue translation in the new reading frame. As a result the stop codon of the first open-reading frame is bypassed and a fusion protein is synthesized [reviewed in (1,2)]

Frameshifting is frequently used by RNA viruses, in particular by those with a single genome, and is thought to lead to precise ratios of viral proteins, which is crucial for successful infection (3,4). Frameshifting is occasionally used by the eukaryotic cell, e.g., to regulate expression of antizyme (5) or by the prokaryotic cell to regulate production of release factor RF2 (6) and synthesis of the gamma subunit of DNA polymerase (7).

The signal that makes a ribosome shift comprises two elements: a slippery sequence, where the ribosome switches the reading frame, and an adjacent stimulatory signal, usually a specific RNA structure. For −1 frameshifting, the slippery sequence usually consists of a heptanucleotide motif X XXY YYZ, where X can be any three identical nucleotides, Y can be three A's or U's, and Z is not G (8). The slippery sequence has been shown to be shifty on its own *in vitro*, up to 2%, but is strongly stimulated, up to 40-fold, by the presence of a hairpin, a pseudoknot, a three-way junction (9) or an antisense oligonucleotide (10,11), located 5 to 8 nt downstream of the slippery sequence.

Despite recent progress (12–14), the exact mechanism by which a downstream RNA structure stimulates −1 frameshifting remains unclear. The current view is that the downstream RNA element forms a physical barrier that causes a fraction of ribosomes to stall in their translocation step and puts tension on the mRNA–tRNA interaction (15). This tension is relieved by realignment of A-site and P-site tRNAs in the 5′-direction, whereafter the ribosome resumes translation in the −1 reading frame. Several data also point to a role for the E-site tRNA in stimulating frameshifting (16,17).

In general, a pseudoknot is more efficient in stimulating frameshifting than a hairpin of the same sequence (18, R.C.L. Olsthoorn unpublished data). This difference is likely related to a higher thermodynamic stability of the pseudoknot. Indeed, from thermodynamic analyses it appears that pseudoknots are more stable than their hairpin counterparts (19–21). Recent studies employing mechanical 'pulling' of frameshifter pseudoknots have shown a correlation between the mechanical strength of a pseudoknot and its frameshifting capacity (13,14) and influence of major groove and minor groove triplex structures (22). The higher strength of a pseudoknot can be primarily attributed to the formation of base triples between the lower stem S1 and loop 2 (Figure 1), making it more resistant against unwinding by an elongating ribosome (15,23). Base triples in the pseudoknots of *Beet western yellows virus* (BWYV) (24), *Pea enation mosaic virus* (PEMV-1) (21) and *Sugarcane yellow leaf virus* (ScYLV) (25) have been shown to play an essential role in frameshifting.

We previously solved the solution structure of the pseudoknot present in the overlapping region of gag and pol genes of Simian retrovirus type-1 (SRV-1) by NMR (26). The structure has a classical H-type fold and is further characterized by interactions between the minor groove of stem 1 and loop 2, which forms a triple helix by various tertiary interactions and extensive stacking (Figure 1A).

Here we present a detailed mutational analysis of the SRV-1 pseudoknot addressing the role of the triple helix for −1 frameshifting *in vitro* and *in vivo*. The data not only emphasize the functional role of the triple helix in −1 frameshifting but also suggest a role for refolding kinetics of frameshifter pseudoknots.

MATERIALS AND METHODS

Constructs

Mutations in the SRV-1 frameshifting signal were made in plasmid SF2 that is derivative of pSFCASS5 (8), a frameshift reporter construct used in earlier phenotypic studies. SF105–113 [except SF110 (A28G) see SF210] were obtained by a two-step PCR mutagenesis procedure using degenerated primers on plasmid pSF103 that contains the sequence of the 'NMR pseudoknot' (26). The appropriate mutants were selected by dideoxy sequencing.

SF202–206 and SF209–211 were constructed as follows. First a BglII-NcoI fragment from the SF2 vector was replaced by a short DNA fragment obtained by hybridization of oligonucleotides (5′-GATCTTAATACGACT CACTATAGGGCTCAGGGAAACTGATCA-CGTGG C-3′) and 5′-CATGGCCACGTGATCAGTTTCCCTGA GCCCTATAGTGAGTC-GTATTAA-3′) creating a unique BclI restriction site just downstream of the slippery sequence. The resulting plasmid pSF201 was opened at the BclI and NcoI sites and sets of complementary oligonucleotides corresponding to mutants SF202–206 were inserted. In mutant SF207, which codes only for the −1 reading frame product, the BglII-NcoI fragment was replaced by two short complementary oligonucleotides: SF218/SF219: 5′GATCTGGCCACTAGTAC/5′CATGG TACTAGTGGCCA). Note that this in-frame product is 21 aa shorter than the frameshifted −1 product.

Figure 1. Structure of the 'NMR' SRV-1 frameshift pseudoknot and the effect of substitutions in L2 on its frameshift-inducing capacity. This pseudoknot differs from the viral pseudoknot by having G2C18-to-CG, C10G32-to-UA, G20-to-C and deletion of GCU between C24 and A25 substitutions (26). (**A**) The 3D model of the NMR pseudoknot (PDB 1E95); L2 bases, magenta; S1 and S2 base pairs in cyan, L1 base in red, 3′ single-stranded sequence AC in yellow. (**B**) Substitutions in the NMR pseudoknot sequence. Dashed lines illustrate base triples. (**C**) Rabbit reticulocyte lysate translation products of mRNAs derived from BamHI-digested templates were separated on a 17.5% SDS polyacrylamide gel and detected by fluorography. The migration of the 19 kDa 0-frame product (NFS) and the 22 kDa 'frameshift' product (FS) are indicated. SF103, wild-type; see Table 1 for the explanation of other constructs.

SF213–224 were constructed as follows. First the entire BglII-NcoI fragment of pSF2 was replaced by a synthetic dsDNA fragment (5′-GATCTTAATACGACTCAC TATA-GGGCTCATTTAA<u>ACTAGT</u>TGAGGGGCCA TATTTCGC-3′ and 5′-CATGGCGAAATATGGCCC CTCA<u>ACTAGT</u>TTAAATGAGCCCTATAGTGAGTC GTATTAA-3′, sequences forming a SpeI restriction site are underlined). The resulting plasmid pSF208 was opened at the BglII and SpeI sites and oligonucleotides SF226 (5′-GATCTTAATACGACTCACT-ATAGGGCTCAGG GAAA-3′) and SF227 (5′-CTAGTTTCCCTGAGCCCT ATA-GTGAGTCGTATTAA-3′) were inserted. This yielded pSF212, which was subsequently digested with SpeI and NcoI to insert sets of complementary oligonucleotides corresponding to mutants SF213–224. The sequences of these oligonucleotides are available on request. All mutants were checked by dideoxy sequencing and are listed in Table 1.

In vitro transcription

DNA templates were linearized by BamHI digestion and purified by successive phenol/chloroform extraction and column filtration (Qiagen). SP6 polymerase directed transcription was carried out in a 50-µl reaction containing 1–3 µg linearized DNA, 1 mM NTPs, 40 mM Tris–HCl (pH 7.9), 10 mM NaCl, 10 mM DTT, 6 mM MgCl$_2$, 2 mM spermidine, 6 units of Rnase inhibitor (RNAguard, Pharmacia) and 15 units of SP6 polymerase (Promega). 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 desalted and sterilized water were directly used for *in vitro* translations. Alternatively, transcripts were purified by phenol/chloroform extraction and isopropanol precipitation as described earlier (27).

In vitro translation

Reactions contained 4 µl of RNA solution, 4.5 µl of reticulocyte lysate (Promega), 1 µl of ^{35}S methionine (ICN, *in vitro* translation grade), 0.5 µl of 1 mM amino acids (−Met) and were incubated for 60 min at 28°C. Samples were boiled for 3 min in the Laemmli buffer and loaded onto 12% SDS polyacrylamide gels. Gels were dried and exposed to phosphoimager screens. Band intensity of 0 frame and −1 frame products was measured using Molecular Imager FX (Biorad) and Quantity One software. Frameshift percentages were calculated as the amount of −1 frame product divided by the sum of 0 and −1 frame products, corrected for the number of methionines, multiplied by 100.

In vivo assay

Pseudoknot mutants were cloned in KpnI/BamHI digested pDUAL-HIV(0) (4). The GGGAAAC slippery sequence was changed to the more efficient UUUAAAC (28) to obtain a better read-out. In these constructs the stopcodon of the first open-reading frame, Renilla luciferase, is located downstream of the pseudoknot. A non-frameshifting control was constructed by changing

Table 1. Summary of all mutants and their relative frameshifting efficiency compared to wild-type ('NMR pseudoknot')

Construct	Mutation	Frameshifting efficiency relative to the wild-type[a] *In vitro* (%)	Frameshifting efficiency relative to the wild-type[a] *In vivo* (%)
SF103, SF206[b]	Wild-type	100	100
SF105	C20G	121	
SF106	C20A	151	
SF107	C19G	42	
SF108	A28U	58	
SF109	A28C	27	
SF210	A28G	63	
SF111	C24U	98	
SF112	C24G	91	
SF113	C24A	124	
SF114	C19iUUU	88	
SF202	C24del	100	69
SF203	C5U, G15A	27	20
SF204	G4A, C16U	13	
SF205	C6U, G14A	40	
SF342	G3A, C17U	40	
SF209	G1C, C19G	73	
SF211	G1A, C19U	37	
SF213	A21U	63	
SF214	A21C	60	
SF215	A21G	107	
SF216	A26U	33	
SF217	A26C	31	43
SF218	A26G	71	
SF219	U27A	69	
SF220	U27C	48	
SF221	U27G	40	
SF222	C2G, G18C, C20G, delC24, U10C, A32G	33	
SF223	C2G, G18C, C20G, delC24	59	
SF224	C2G, G18C, delC24, C20G, U10G, A32C	111	
SF229	L2 = **CUUCUCGUG**	12	18
SF348	L2 = AAA	6	
SF350	L2 = CAACAUGAAU CAACAUCUA GACAAAUA	40	17

[a]All assays were performed at least three times, at non-saturating mRNA concentrations. SD ± 10%.
[b]SF200–SF350 series were constructed in a modified version of the SF103 construct ('Materials and Methods' section).

the slippery sequence to UUUAAGC, while in the in-frame control a C-residue was added directly downstream of this heptamer. In both constructs the pseudoknot was the NMR-pseudoknot. A list of oligonucleotide sequences is available on request. All constructs were confirmed by DNA sequencing. HeLa cells (24-well plate) were transfected with 250 ng of plasmid using lipofectamine-2000 (Invitrogen). Cells were lysed 20 h after transfection and luciferase activities were measured in a Glomax-multidetector (Promega) according to manufacturer's protocol.

RESULTS AND DISCUSSION

The NMR structure of the SRV-1 pseudoknot (26) revealed a number of interesting features that may be

relevant to the process of frameshifting. These features include stacking of adenosines A21–A26 in loop L2 and flipping out of cytosine 24, base–base and/or base–sugar interactions of A21, A26, U27 and A28 from L2 with the minor groove of stem S1 (Figure 1A). To test the importance of these interactions in frameshifting, mutations were introduced in these regions of the pseudoknot and their frameshifting capacity was measured in rabbit reticulocyte lysates and compared to the NMR wild-type pseudoknot (Table 1).

Changes in L2

C20. For NMR purposes the wild-type G-residue at this position was replaced by a C (26). Back mutation to a G (SF105) resulted in a 1.2-fold increase in frameshifting (Figure 1C). An adenosine at this position (SF106) was even more beneficial for frameshifting showing a 1.5-fold increase relative to the control NMR pseudoknot (Figure 1C, compare lanes 'SF103' and '106'). A uracil at this position was not tested since this would lead to a premature stopcodon in the −1 frame. Insertion of UUU (SF114) between C19 and C20 led to a slight decrease in frameshifting: 88% relative to the NMR wild-type (SF103). Even though in the latter mutant two additional base pairs might be formed with the 5′ single-stranded sequence (AU and GU) these are probably melted when the ribosome is stalled over the slippery sequence.

A21. This nucleotide forms a base triple with the C2–G18 base pair. Mutation of A21 to G did not affect frameshifting, but replacement with C or U decreased the relative frameshifting activity to 60 and 63%, respectively (Table 1). Although a guanine cannot substitute for the triple interaction with the C2–G18 base pair, its stacking properties may compensate for this. The two pyrimidines may be too small to bridge the distance here to form an interaction with C2–G18.

C24. This nucleotide is extruded from loop 2 by the stacking interaction of the continuous run of adenines and is not involved in any interaction. Thus, from a structural point of view, the presence of C24 in loop L2 seems superfluous or even a nuisance by hindering the stacking of the A21–A26 adenosines. Changing C24 to U (SF111) did not affect frameshifting (Figure 1C), suggesting that also a uridine at this position is flipped out. Replacing C24 by G (SF112) or deleting it (SF202) had no significant effect on frameshifting while an adenosine at this position slightly enhanced frameshifting activity (SF113), possibly as a result of extended adenosine stacking. These results show, as expected, that C24 is irrelevant and dispensable for frameshifting.

A26. A26 forms three hydrogen bonds with stem 2. Two hydrogen bonds from the N3 and hydroxyl group of A23 to the C16 2′OH together form a single ribose-zipper motif. The third hydrogen bond is between the N1 and amino group of G4. A pyrimidine seems too small to fulfil these interactions and indeed replacing A26 by C or U showed an approximately 3-fold lower frameshifting activity (Table 1, SF217 and SF216). G would be

capable of forming the ribose zipper motif though a considerable clash between its N1 and the G4 amino group can be expected. The relatively high frameshifting activity, 70% of the A26G pseudoknot, suggests that, at this position in L2, stacking properties assisted by the ribose interaction may be more important than formation of the zipper motif itself.

U27. U27 forms only a single hydrogen bond via O2 to the amino group of G15, putting less structural restraints on frameshifting. Still mutation of this residue reduced frameshifting 1.4- to 2.5-fold (Table 1). The 2-fold reduction seen by substituting C for U27 is not easily explained as this pyrimidine has an identically positioned O_2 to hydrogen bond to the amino group of G15.

A28. A28 is a key nucleotide in stabilization of the pseudoknot fold by anchoring L2 to the S1/S2 junction through formation of two hydrogen bonds with the 2′-hydroxyl group of G14. Replacement by U (SF108) or G (SF210) resulted in almost 2-fold decrease whereas a C (SF109), though having a similarly positioned N1 and amino groups as an adenosine, resulted in ∼4-fold drop in frameshifting (Figure 1C). Apparently, the relatively small pyrimidine ring is too far away to form interactions with the 2′OH of G14. In addition, the lower frameshift activity of the C-mutant could also be caused by formation of a base pair with G14, which could change the overall structure around the S1/S2 junction.

In summary, the effect of single-nucleotide mutations in L2 show the functional, critical importance of the A26:G4–C16 and A28:C5–G15 triples that were previously identified by NMR spectroscopy (26). Base triples at these positions, stabilizing the S1/S2 junction have been recognized to be essential for frameshifting in other pseudoknots as well, be it with different hydrogen bond patterns (21,24,25,29). To provide additional evidence for the importance of the specific interactions between L2 and S1 in frameshifting, we accumulated a number of the adverse mutations into one construct. Therefore, a construct was designed with a pyrimidine-rich L2 sequence CUCUCGUG (SF229, 'L2-mut') that included the A21U, C24U, A26G and A28G mutations. SF229 showed an 8-fold lower activity in frameshifting (Table 1), which is approximately the sum of the single-nucleotide changes.

Changes in S1

G1–C19. A previous sequence comparison showed that most frameshifter pseudoknots start with a 5′G–C3′ base pair (30). Changing this base pair, which is not involved in any loop interaction, to C–G (SF209) affected frameshifting efficiency somewhat (73%). As stacking energies of this base pair are also lower (−2.4 versus −3.3 kcal/mol) this confers to the general notion that helix stability is an important determinant in frameshifting. Changing G1–C19 into an A–U base pair (SF211) or a G–G mismatch (SF107) reduced the efficiency even further to about 40%, again emphasizing the role of S1 stability.

G3–C17. This pair is also not involved in any triple interaction, but replacing it with A–U (SF342) resulted in a

60% drop in frameshifting. Again this indicates that the stability of S1 plays an important role in the frameshifting capacity of the pseudoknot. Previously, changing G3–C17 in the wild-type pseudoknot to A–U resulted in an almost 5-fold drop in frameshifting (31). Since we do not have high-resolution structural information of the wild-type pseudoknot (L2: GAAACAAGCUUA), we cannot exclude that the G3–C17 base pair forms a base triple in this context, which could further attribute to the enhanced effect of mutating this base pair.

G4–C16. This base pair is involved in a base triple with A26, mutation of which had quite some impact on frameshifting (see above). Changing G4–C16 into A–U (SF204) is predicted to result in the loss of one hydrogen bond (G24N2H–A26N1), similar to changing A26 to G (SF218), which was still 71% active in frameshifting. The observed dramatic low activity of the A–U base pair mutant (13%) cannot be solely attributed to the loss of one hydrogen bond and must therefore also be due to compromising the stability of the S1 stem.

C5–G15. This pair forms a triple interaction with U27, mutation of which had at most a one-third lower frameshifting activity. Changing the base pair to U–A (SF203) caused an almost 4-fold drop in frameshifting activity, again showing the importance of stem stability.

C6–G14. This pair forms a base triple with A28 via the hydroxyl group of G14. This interaction is not expected to change by mutating it to a U–A bp (SF205). The 2.5-fold decrease must therefore be a consequence of reduced stem stability and/or stacking properties with the S2 U–A pair

at the junction. This large decrease again illustrates the critical importance of the A28:C6–G15 triple interaction.

As with the single-nucleotide substitutions, the effect of the base pair mutations also correlates with the NMR structure, thereby illustrating the functional importance of the S1–L2 interactions. Also the stability of S1 as a primary determinant of frameshifting is clearly confirmed.

Supershifter

Using the above knowledge we attempted to create a supershifting pseudoknot (Figure 2). To this end we substituted G for C20, removed C24 and restored the G2–C18 and C10–G32 base pairs, as present in the SRV-1 pseudoknot. To our surprise this mutant was 3-fold less active in frameshifting than the parent SF206 pseudoknot. Close inspection of the sequence indicated a possible alternative structure containing two hairpins competing with pseudoknot formation (Figure 2). To prevent this alternative structure, we mutated C10–G32 to U–A (SF223) or G–C (SF224). In these constructs the U–A pair is predicted to destabilize the 4- and 7-bp hairpins and the G–C base pair is predicted to completely disrupt the 4-bp hairpin and destabilize the 7-bp hairpin. Frameshift levels of SF224 increased approximately 2-fold compared to SF222, while that of SF223 increased more than 3-fold suggesting that the role of alternative structure formation should not be underestimated.

Although SF224 is a slightly better shifter than SF206 (1.11-fold) with an absolute frameshift frequency of 24% it still cannot be considered a supershifter. Previous experiments with SRV-1 pseudoknot mutants yielded better shifters (31). One of these, SF67, which differed from

SF222: 33%
(WT=100%)
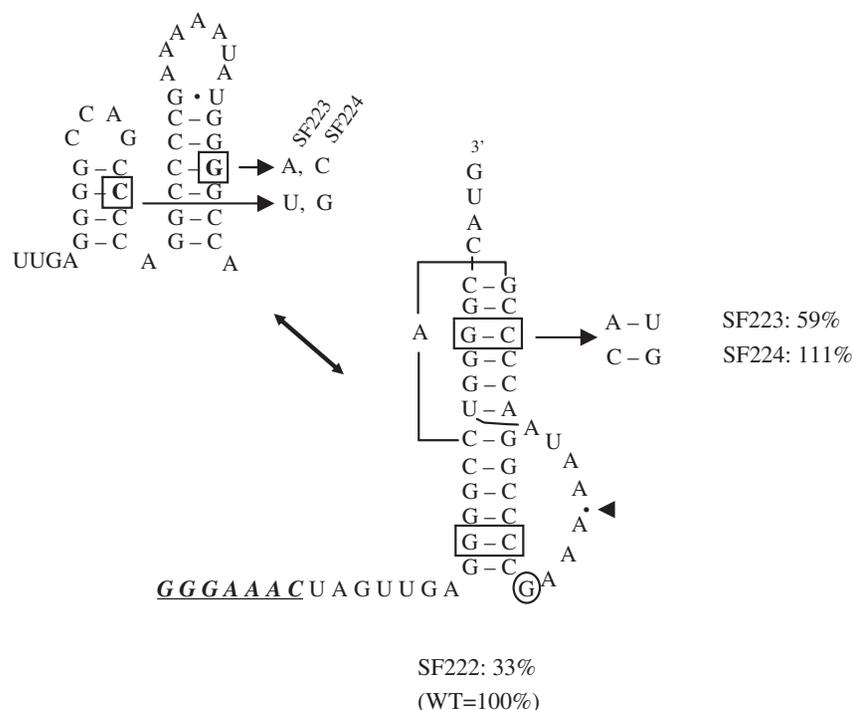
Figure 2. Putative alternative structure of the 'supershifter' pseudoknots. The boxed G–C base pairs and the circled G are also present in the wild-type SRV-1 pseudoknot. The position of the C24 deletion is indicated by the dot and arrowhead.

SF222 by still having C24, was capable of shifting 30% of the translating ribosomes. Thus, it appears that depending on the context deleting C24 is not always beneficial for frameshifting.

At present it is difficult to interpret these results. One explanation could be that C24 or other L2 nucleotides also play a role in refolding of the pseudoknot after ribosome passage. It can be envisaged that a faster refolding pseudoknot might be a more efficient frameshifter, because in order to cause a frameshift, a stable pseudoknot needs to be regenerated before it encounters the next ribosome.

If refolding kinetics of the pseudoknot is a determinant in frameshift efficiency the length of L2 is also expected to have an effect on frameshifting. To test this possibility we changed the length of L2 to 3 and 27 nt in mutants SF348 and SF350, respectively. The absolute frameshifting activity of SF348 was only 1.3%, i.e. a 16-fold decrease compared to SF206. SF350 showed 8.4% frameshifting, a 2.5-fold drop. Of course, when introducing such dramatic changes, one should also consider their effects on the pseudoknot structure and stability. In SF348 most of the base triples are presumably lost and a 3-nt loop crossing the minor groove of stem S1 may not be sterically and thermodynamically favorable. In fact, the very large—16-fold—reduction in frameshifting suggests that the pseudoknot is not formed at all (the presence of a pseudoknot structure was not checked by e.g. structure probing).

With SF350, containing the 27-nt L2, the situation is different. In this mutant some of the triples may still form but the additional unstructured 18 nt are expected to introduce a substantial entropic penalty into the system, which increases ΔG and therefore reduces frameshifting. In addition, a large loop may lead to a longer refolding time of the pseudoknot once a ribosome has passed through this structure.
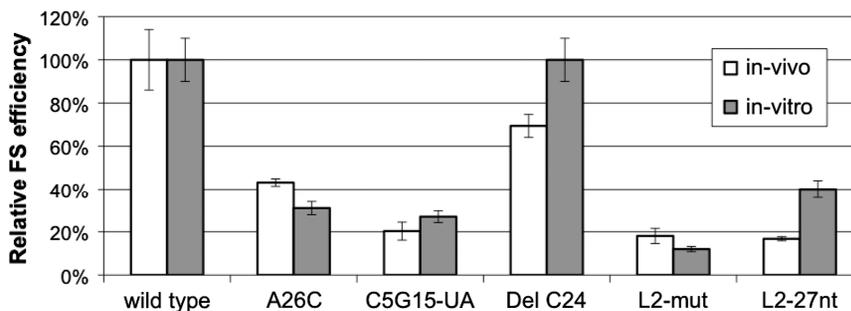
In vivo frameshifting

To investigate whether the mutations that affected frameshifting efficiency *in vitro* would also exert their effect *in vivo*, we introduced some of the above mutations into a dual luciferase reporter plasmid and assayed their activity *in vivo* ('Materials and Methods' section). The frameshifting efficiency of the wild-type SRV-1

pseudoknot and GGGAAAC slippery sequence (SF400) was only ~8%, against a background of 2% of a non-frameshifting control (data not shown). This is a factor of 3 lower than that reported *in vitro* (28). The NMR pseudoknot showed ~11% of frameshifting (SF402, data not shown) that increased to ~19% in combination with the UUUAAAC slippery sequence (SF404). This 1.7-fold increase is very similar to the previously reported increase from 23 to 40% *in vitro* for changing the slippery sequence (28). Mutations were made in the pseudoknot of plasmid SF404. As can be seen in Figure 3, the substitutions had almost the same effect on frameshifting *in vivo* as *in vitro*. The only exception seems to be the effect of enlarging L2, which is consistently more detrimental for frameshifting *in vivo* than *in vitro*. This could be due to refolding kinetics of the pseudoknot, what in combination with heavier ribosomal traffic *in vivo* would result in a lower fraction of properly folded pseudoknots. However, other possibilities, like enhanced RNase susceptibility or alternative folds involving L2, may also account for this effect.

CONCLUSIONS

The interactions between L2 and S1 in the SRV-1 pseudoknot as previously determined by NMR (26) turn out to be important for frameshifting. Although single alterations of the base triples found in the NMR study had at most a 3-fold deleterious effect on frameshifting, combining several of these changes into one mutant, L2-mut, led to an 8-fold decrease *in vitro* and a 6-fold decrease *in vivo*. This is quite remarkable as the mutated sequence should still be able to fold as a pseudoknot, but apparently critical stem–loop interactions are lacking. As found for other frameshifter pseudoknots, the composition and length of L2 can greatly influence frameshifting. Most frameshifter pseudoknots have adenine-rich L2 loops, presumably because of the need for stable triple helix formation, which is best performed by adenosine's N1 and C6-amino groups (2).

Deletion of the bulging C24 from the loop L2 did not affect frameshifting significantly *in vitro* and *in vivo*. This adds to the notion that this nucleotide can be safely squeezed out to allow stacking of adenosines in L2. It is noteworthy that other frameshifter pseudoknots also have a cytosine flanked by adenines in L2: GAACAAA in

Figure 3. Comparison of *in vivo* and *in vitro* frameshifting activities relative to that of the NMR pseudoknot. White bars, *in vivo*; gray bars, *in vitro*. The efficiency of the NMR pseudoknot ('wild-type') is set at 100%. Experiments were done at least three times in triplicate.

BWYV (29), UAAAAACAC in ScYLV (25), ACUCAAA A in MMTV (32) and AAACAA in HIV-1 type O (33). Interestingly, L2 of the human telomerase pseudoknot, not involved in frameshifting, consists of CAAACAAA. This pseudoknot has recently been shown to function in frameshifting *in vitro* (22). Also here the adenosines formed triple interactions with base pairs in stem S1 and were essential for frameshifting (22,34). The effect of substituting the second C in L2 has not been investigated; its presence may be required to prevent slippage of the RNA polymerase.

The main role of the triple interactions seems to be in stabilizing the pseudoknot such that high frequencies of frameshifting are possible. Mutations that remove triple interactions in general lead to lower frameshifting. Conversely, it seems that frameshifting pseudoknots with weaker stems need to be stabilized by such triples. For instance in the BWYV and PEMV-1 pseudoknots, S2 is only three base pairs but this is compensated by an additional triple between L1 and S2. The high number of triples in luteovirus pseudoknots may explain why for instance the ScLYV pseudoknot with a relatively high number of A–U base pairs in the bottom of S1 is still capable of inducing ~15% of frameshifting (25). On the other hand, when S1 is increased to 11 base pairs, triple interactions do not seem to contribute anymore as frameshifting becomes independent of the sequence of L2 (C-H. Yu and R.C.L Olsthoorn, unpublished data).

If we assume that ribosomes first sense the bottom of stem S1 we would expect base triples to be present at the bottom of the stem or in the 5′ end of L2. This would be in line with the observation that the first three base pairs of a frameshift pseudoknot are usually G–C (30). However, most triples are found near the 3′ end of L2 close to the junction with S2. Possibly, triples at the junction render the pseudoknot more resistant to forced unwinding by the ribosomal helicase, which would otherwise serve as a pivot.

Because the mere presence of a pseudoknot structure with a certain minimal thermodynamic stability might not be the sole primary determinant of frameshifting a number of alternative models have been proposed in which mechanical resistance of the pseudoknot to forced unwinding is the key (12,15,23). In these models a pseudoknot trapped in the mRNA entry tunnel resists mechanical unwinding, which causes the ribosome to pause. Indeed, recent optical tweezers experiments, in which rupture forces of RNAs are measured by pulling 5′ and 3′ ends apart, showed a correlation between frameshifting and mechanical strength of the pseudoknot and confirmed a positive effect of base triple interactions on frame shift efficiency (13,14,22). Frameshifting by the trapped pseudoknot is thought to be either passive, by stalling the ribosome for a sufficient amount of time, or active, by building up tension at the mRNA–tRNA interface by a counteracting ribosome, which can be relieved by a −1 frameshift (15). Observations that ribosomes can stall for seconds to minutes without frameshifting to occur (35) and pausing appears to be necessary, but not sufficient for frameshifting (36,37), rather point to an active role. Although it now seems mechanical resistance

to RNA unfolding is the key to ribosomal frameshifting, how the ribosome exactly 'chokes' on such a structure is still far from being understood. Remaining questions are for instance if torsional restraint of the pseudoknot resisting ribosomal unwinding is essential (23), whether the pseudoknot truly plays an active role in for instance overbending the tRNA in the P-site (12) and at which stage of translational elongation frameshifting actually occurs.

One should also keep in mind the possible formation of alternative structures that can affect the frameshifting efficiency by lowering the fraction of properly folded pseudoknots. This possibility has not been investigated in detail but our own data (Figure 2) suggest that this may be a relevant issue. Especially during heavy ribosomal traffic, as may be the case *in vivo*, fast and correct refolding of pseudoknots could be an important parameter for frameshifting. Under such circumstances it is conceivable that a hairpin is more preferred as it probably refolds faster than a pseudoknot. A recent study on the HIV-1 frameshift signal has shown that a decrease in the translation initiation frequency can lead to an increase in the frequency of frameshifting (38). Also a previous study in yeast has shown a correlation between frameshifting and translation initiation frequency (36). Our data with the enlargement of L2 also hint at a possible role for folding kinetics.

Although the time scale of folding (milliseconds) may at first seem irrelevant compared to the time scale of translation elongation (seconds) it has recently been reported that the conversion rate between two comparatively stable hairpins ranges from a few seconds to several minutes (39). Also, slow-folding pseudoknots, 5–350 s, limit the activity of the Hepatitis Delta virus ribozyme (40). Thus the efficiency of a frameshifter pseudoknot may not be solely dictated by its stability but also by its kinetic properties.

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