Structural diversity of frameshifting signals : reprogramming the programmed

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Chapter V

Antisense oligonucleotides that mimic a pseudoknot are highly efficient in stimulating –1 ribosomal frameshifting

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Abstract

Programmed –1 ribosomal frameshifting (–1 PRF) is stimulated by RNA structures like pseudoknots or hairpins. Previously, it was shown that antisense oligonucleotides (ONs) annealing downstream of the slippery sequence and mimicking the stem of a hairpin are capable of inducing efficient PRF. Pseudoknots generally induce higher levels of frameshifting as compared to hairpin structures partly due to the formation of triple interactions between bases in loop 2 (L2) and stem 1 (S1). Based on our knowledge of the *Simian Retrovirus type 1* (SRV-1) gag-pro frameshifting pseudoknot, we here designed ONs that after binding to mRNA would mimic pseudoknots. Our data demonstrate that pseudoknot-forming ONs do induce more frameshifting than duplex-forming ONs. Depending on the length of S1, this enhancement was affected by the identity of bases in L2. This finding was corroborated by testing the corresponding *in cis* pseudoknots, i.e. the frameshift-inducing ability of pseudoknots with longer S1 are less affected by the identity of L2 in a length dependent manner. The greater flexibility of using small ONs to study –1 PRF allows the use of non-natural modifications. For instance it was found that 2'ACE protected ONs carrying a bulky bis(2-hydroxyethoxy) methyl orthoester group at their 2' hydroxyls are fully capable of inducing frameshifting, implying functional extensions of this type of modification in gene regulation by ONs. Our findings are discussed in relation to natural frameshifter pseudoknots and other antisense induced frameshifting studies.

Introduction

The genetic information has to be decoded into functional polypeptides through translation. Although the genetic code has been deciphered, it is still far from perfect to extrapolate protein sequences from the DNA information of the genome in living organisms. One of the reasons is that in certain genes standard rules of decoding are overruled by alternative ways of translation, named recoding, which are stimulated by various kinds of signals embedded in the mRNA (1). To date, several biologically important recoding events, including stop codon-redefinition, translational hopping, and programmed ribosomal frameshifting (PRF) have been characterized in all three kingdoms [for reviews, see (2, 3)].

During PRF, elongating ribosomes are re-directed at a defined frequency by specific RNA elements into alternative reading frames either one nucleotide (nt) into the 3' direction (+1 PRF), or one (–1 PRF) or two nts (–2 PRF) into the 5' direction. –1 PRF, among the most inviting and best characterized frameshifting events, is promoted by two *in cis* RNA signals: a heptameric slippery sequence, X.XX Y.YY Z., where the dots indicate the original reading frame and spaces the frame after –1 PRF; and a structural element, either a simple stem-loop or a pseudoknot structure generally located 5-7 nts downstream of the slippery sequence [for a review, see (4)]. Most cases of –1 PRF have been found in the genomes of eukaryotic RNA viruses and prokaryotic insertional sequences [for reviews, see (5, 6)] whereas one example of a cellular gene in *Escherichia coli* (7) and three cellular genes in mammalians (8–10) are known to be expressed through –1 PRF.

It is generally believed that –1 PRF is promoted by downstream secondary structures which stall elongating ribosomes over the slippery sequence followed by tandem or single tRNA slippage into the –1 reading frame. A recent study using cryo-electron microscopy to image mammalian ribosomes stalled by the *Infectious bronchitis virus* (IBV) frameshifting pseudoknot or its inactive hairpin derivative showed several interesting features that further elucidate the mechanism of –1 PRF (11). First, the pseudoknot, as expected, resides in the mRNA entrance channel and makes direct contact with the putative ribosomal helicase in agreement with previous assumptions (12). Strikingly, the A-site is occupied by eEF-2 while the P-site tRNA is strongly bent toward the 3' direction probably due to the opposing forces raised between translocation and the hard-to-melt pseudoknot during translation elongation. However, tRNA bending is not observed in the control experiment using a frameshift-inactive hairpin construct. These findings suggest that the P-site tRNA dissociates and re-pairs into the –1 reading frame to release the tension built up by the frameshifting pseudoknot that resists unwinding by the ribosomal helicase. Therefore,

it is assumed that the better frameshifter RNAs can more resist unwinding by ribosomal helicase. Recently developed methods using optical tweezers to probe mechanical stability of RNA structures have shown a promising correlation between stability and frameshifting efficiency (13, 14).

In addition to the mentioned –1 PRF inducing RNA secondary structures, antisense ONs annealing downstream of the slippery sequence and mimicking the stem of a hairpin were recently found to be capable of inducing efficient PRF (15, 16). Since pseudoknots generally induce higher levels of frameshifting as compared to stem-loop structures, we here attempted to design ONs that would mimic a pseudoknot using our knowledge of the SRV-1 gag-pro pseudoknot (17, 18). Our data demonstrate that pseudoknot-forming ONs do induce more frameshifting than duplex-forming ONs. Interestingly, depending on the length of stem 1 (S1), this enhancement is affected by the identity of bases in loop 2 (L2) of the pseudoknot. The latter result is discussed in relation to natural frameshifter pseudoknots.

Materials and Methods

Frameshift reporter construction and oligonucleotides

The –1 PRF reporter constructs in this report were based on plasmid pSF208 (19). Briefly, pSF208 was digested by SpeI and NcoI followed by ligation of sets of complementary oligonucleotides (Eurogentec, Liege, Belgium) with designed mutations. A list of oligonucleotides is available upon request. All constructs were verified by sequencing (LGTC, Leiden, The Netherlands).

Antisense RNA oligonucleotides were all purchased from Dharmacon (Lafayette, USA). Of the delivered RNA oligonucleotides, which carry bis(2-hydroxyethoxy) methyl orthoester protection groups on their 2'OH, half the amount was de-protected by incubating with 100 mM acetic acid pH 3.8 and N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) at 60°C for 30 minutes.

In vitro transcription

In vitro transcription reactions using a RiboMAX Large Scale RNA Production Systems kit (Promega, The Netherlands) were carried out as described before (19).

In vitro translation

In vitro translations were carried out in nuclease-treated rabbit reticulocyte lysate (RRL) (Promega). Prior to translation transcripts (0.025 pmoles) were incubated without or with 15.625 pmoles of ONs for 20 minutes at room temperature. After

incubation, 4 µL of RRL, 0.01 mM amino acids mixture except methionine, 2 µCi of ^{35}S methionine (10 mCi/ml, MP Biomedicals, *in vitro* translational grade) were added in a total volume of 10 µL and incubated at 28°C for 1 hour. After translation, samples were resolved by gel electrophoresis and frameshift percentages determined as described before (19).

Determination of the melting temperature (*T*m) of oligonucleotide duplexes

RNA oligonucleotide 18RNA (5'GCGCGCUGGAGGCCAUGG3') with and without 2'ACE modifications was mixed in a 1:1 molar ratio with RNA18 (5'CCAUGGCCUCCAGCGCGC3') also with and without 2'ACE modifications, in UV-melting buffer (100 mM NaCl, 10 mM Na-cacodylate, pH 6.8). The measurements were performed on a Varian Cary 300 spectrophotometer with a heating rate of 0.25°C/min over a temperature gradient of 30°C to 90°C. The absorbance at 260 nm was recorded and normalized to the blank control. Data was analyzed by fitting the transition to a two-state model with correcting sloping baselines using a nonlinear least-squares program to estimate *T*m.

Results

***In trans* re-creation of the SRV-1 frameshifter pseudoknot by structured ONs**

It has previously been shown that linear ONs that bind downstream of a slippery sequence and mimic the double-stranded stem region of frameshifter hairpins, efficiently stimulate ribosomal frameshifting (15, 16). Since pseudoknots are better frameshifters than their hairpin derivatives (19, 20), we attempted to design pseudoknot-mimicking ONs based on structural (Fig. 1A) (17) and functional studies (18) with the aim to enhance ON-induced frameshifting. We first compared the frameshifting efficiency induced by a linear (R6b, hairpin-mimicking) and a structured (R28, pseudoknot-mimicking) RNA ONs (Fig. 1B). Figure 1C shows that R28 promoted 1.6% of ribosomes to switch frame at the UUUAAAC slippery sequence, compared to 0.8% by R6b. This 2-fold increase may be due to a more stabilized ON-mRNA interaction contributed by tertiary interactions between L2 and minor groove of S1 as in SRV-1 frameshifting pseudoknots (Fig. 1A). To support this idea we designed another two mutants (Figure 1B): M28C, which is reminiscent of the A26C mutation shown previously to reduce frameshifting more than 3 fold in the context of the wild-type *in cis* pseudoknot (18), and M28, in which most of the adenosines were replaced by uridines to disrupt potential triple interactions. The reduction in frameshifting obtained with M28C (Fig. 1C, lane 5) and M28 (lane 4), by

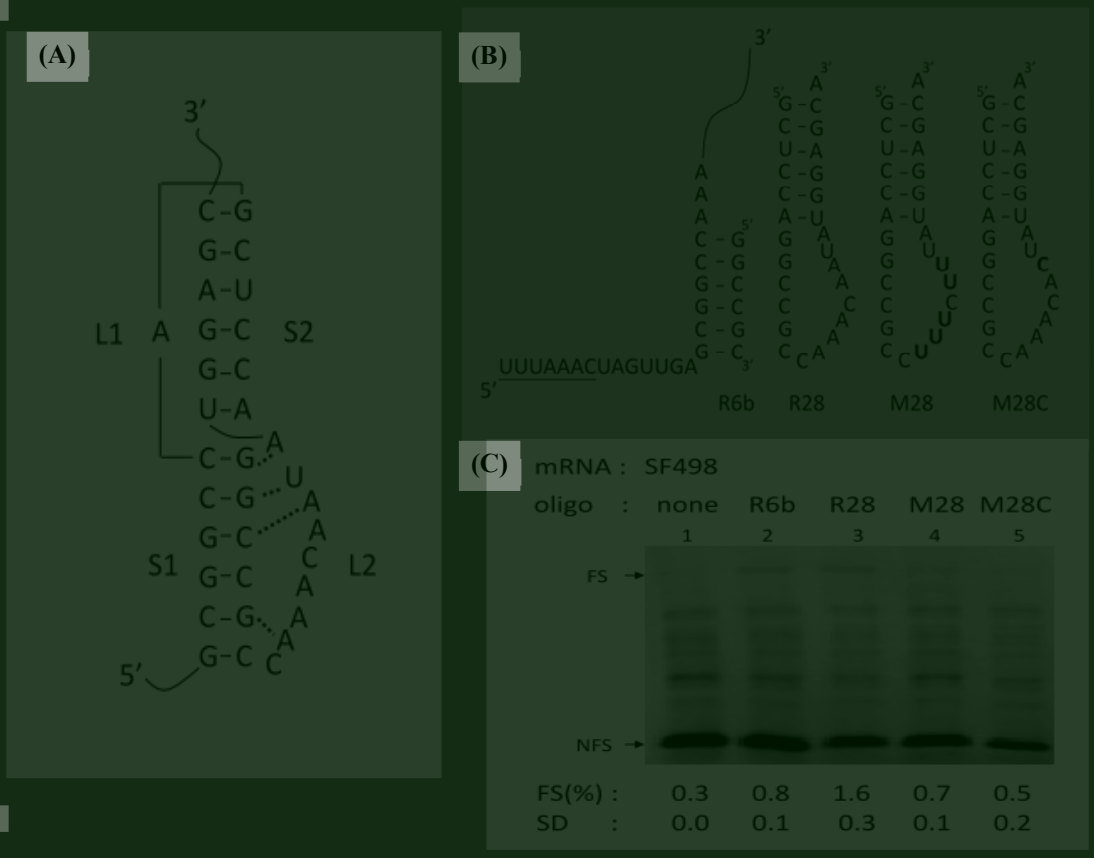

Figure 1. –1 PRF induced by ONs mimicking the SRV-1 frameshifter pseudoknot. (A) Secondary structure of the SRV-1 frameshifting pseudoknot (18). Dashed lines represent base triples. The annotation of stems and loops is indicated. (B) Sequences of a linear ON mimicking the S1 region of the SRV-1 pseudoknot (R6b) and structured ONs mimicking the entire SRV-1 pseudoknot (R28, M28 and M28C) and binding 7 nts downstream of the UUUAAAC slip site (underlined) are shown. Changes with respect to R28 are shown in bold. (C) SDS-PAGE analysis of 35S-methionine labeled translation products in rabbit reticulocyte lysate. –1 PRF is monitored by appearance of a 65 kD shifted product (FS). The non-shifted in-frame 19 kD products are indicated by NFS. Quantitative analysis of frameshifting efficiency is described in Materials and Methods. The standard deviation (SD) shows the variation of the averaged frameshifting efficiency from at least three independent measurements.

3.2 and 2.3 fold, respectively, is comparable to the effect of these mutations in the wild-type pseudoknot (18). These data suggest that binding of structured ONs can mimic stem-loop tertiary interactions in the minor groove of a pseudoknot stem 1, thereby enhancing frameshifting efficiency.

Improving ON-induced frameshifting efficiency by structured oligonucleotides – a longer S1 version

We previously reported that a linear ON of 12-18 nts has the optimal length to induce

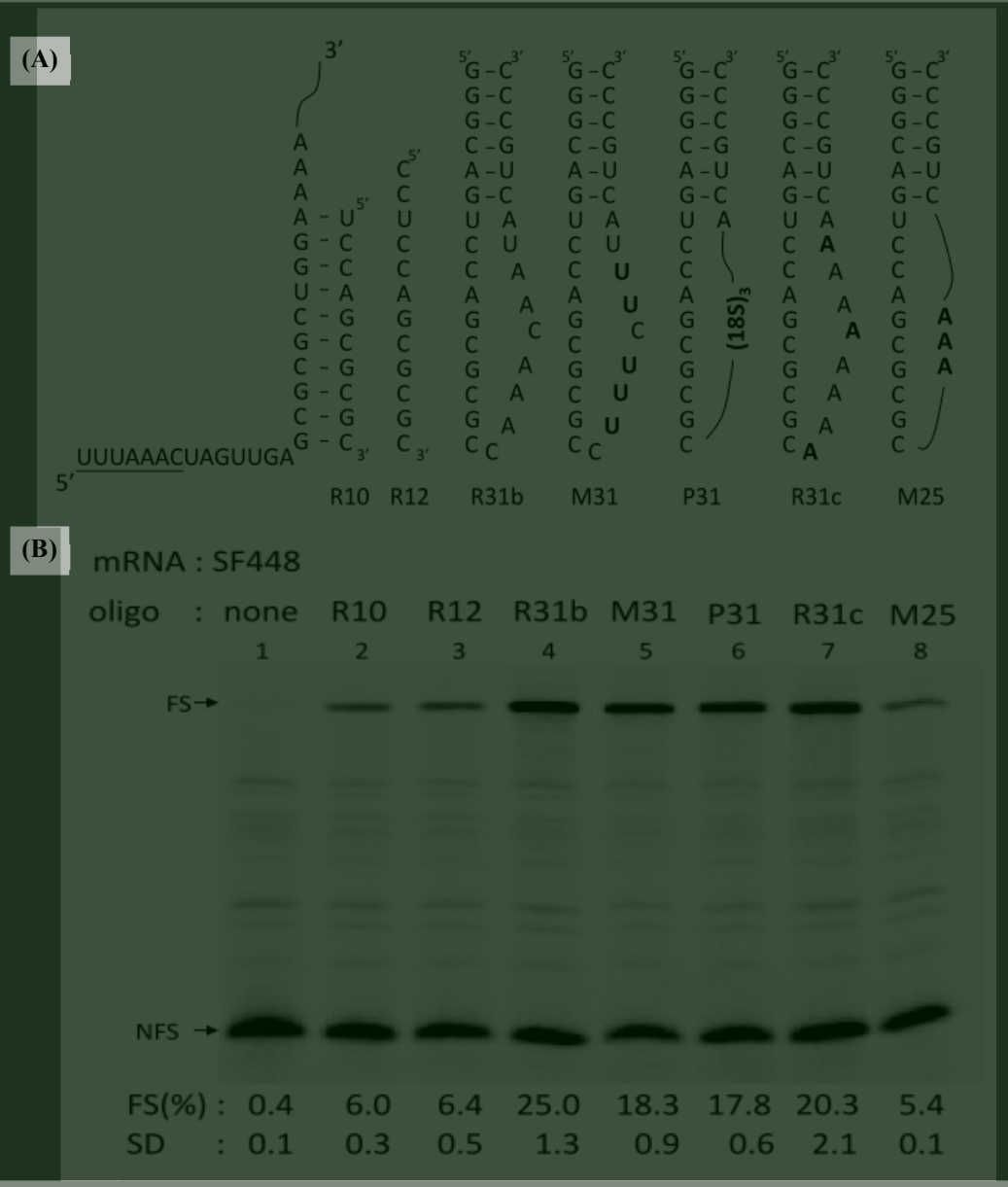

Figure 2. –1 PRF induced by structured ONs mimicking a frameshifter pseudoknot with a 10 bp stem S1 and the effect of ACE modifications at the 2'-OH of the ribose in these ONs. (A) Sequences of linear ONs (R10 and R12) and structured ONs (R31b, M31, R31c, and M25) with 10 base complementarity to a region 7 nts downstream of UUUAAAC slip site (underlined) are shown. Changes with respect to R31b are shown in bold. (18S)3 is the abbreviation of three 18-atom spacers, which are composed of consecutive hexaethylene glycols. (B) SDS-PAGE analysis of 35S-methionine labeled translation products. The RNA molecules of each ON are de-protected by the protocol provided by the manufacturer. See legend to Figure 1C and Materials and Methods for more details. (C) SDS-PAGE analysis of 35S-methionine labeled translation products in RRL. The RNA molecules of each ON are protected by ACE groups at the 2'-OH of the ribose. See Figure 1C and Materials and Methods for more details.

frameshifting, yielding an efficiency of 13% (21). It was worthwhile to test whether

we could increase this efficiency by introducing base-triples using structured ONs in a longer stem construct in R10 (Fig. 2A) similar to the R6 and R28 as described above. Building upon the data obtained with R28 and M28, we designed R31b, M31, and M25 structural ONs hybridized to an mRNA template used previously (19) to investigate the effect of base triples with an S1 of 10 bp (Fig. 2A). The results shown in Figure 2B indicate that R31b induces 25.0% of frameshifting, which is about 4-fold more efficient than linear R10 (6.0%) or R12 (6.4%). Next, we investigated what are the critical components of this "pseudo-pseudoknot" for frameshifting. Reducing the loop length to three nts (M25) was predicted to disrupt the pseudoknot-like structure since three nts are not sufficient to cross the minor groove of a 10 bp stem (22). Indeed, frameshifting was strongly reduced to a level (5.4%) that was close to that of R10 (Fig. 2A, lane 8). Interestingly, when the loop was replaced by a U-rich sequence (M31) with the aim to abrogate triple interactions as with M28, frameshifting decreased 1.4-fold compared to R31b (Fig. 2B, lanes 4 and 5), much less than the difference between R28 and M28 (2.3 fold). Although learning from natural pseudoknots that L2 is generally A-rich because triple interactions mainly occur through the amino groups of adenosines, we could not exclude the possibility of interactions through the U-rich loop. Therefore, we designed P31 of which the bases and riboses of L2 were replaced by polyethylene glycol (PEG) linkers to completely rule out the possibility of triplex formation (Fig. 2A). The frameshifting efficiency of P31 was also 1.4 fold less than that of R31b (Fig. 2B, lanes 4 and 6) further indicating the role of triple interactions of structured ONs in enhancing frameshifting. However, replacing all bases in L2 to adenines (R31c) caused a 5% decrease in frameshifting (Fig. 2B, lanes 4 and 7). Native gel electrophoresis of this ON showed a large fraction of R31c to form dimers, thereby reducing its effective concentration (data not shown).

Effect of 2' bulky groups on frameshifting efficiency by affecting hairpin formation

The ONs used in our assays were purchased with ACE [bis(2-acetoxyethoxy) methyl orthoester] protective groups at the 2'OH, which were removed according to manufacturer's instructions (see Materials and Methods). Since only half the amount of each ON was deprotected, the other half allowed us to investigate the effect of bulky 2' moieties on complex stability and frameshifting efficiency. It has been suggested that the 2' bulky group may prevent the formation of intra-molecular structure (23). The ACE version of RNA31b when used in the frameshift assay was nearly 4-fold less efficient than its non-modified form (Fig. 2C, lane 4). This suggested that the ACE side group interfered with the formation of the second stem. The same effect was observed for ONs M31, M25, and R31c, which all induced less

*T*m (°C)	RNA18 with 2'ACE	RNA18 w/o 2'ACE
18RNA with 2'ACE	78	81
18RNA w/o 2'ACE	80	82

Table 1. *T*m measurements of AONs with and without 2'ACE modification.

frameshifting than their non-protected counterparts (Fig. 2C, lanes 5, 7, and 8). Intriguingly, the P31 with ACE modification (Fig. 2C, lane 6) was as effective as its counterpart without protection. The reason for this is still under investigation. UV-melting experiments indeed showed that ACE-ACE duplexes were less stable than ACE-RNA and RNA-ACE duplexes (Table 1). Surprisingly, linear R10 and R12 ONs with ACE modification, although forming a less stable duplex with RNA, were ~1.6 fold more efficient than their non-modified versions (Fig. 2C, lane 1 and 2),

The relation between S1 length and S1-L2 triple interactions in frameshifter pseudoknots

Although the loop-stem interactions further stabilized the ON-mRNA interaction, their effect was less significant when the length of S1 was increased. This observation fits with the general belief that frameshifter pseudoknots with a long S1 (10-11 bp) are not dependent on the L2 sequence to induce efficient frameshifting; short S1 (4-6 bp) pseudoknots, however, rely on S1-L2 tertiary interactions to be efficient frameshifters (4, 24). To investigate the relation between S1 length and triple interactions in our experiments, we designed pseudoknots with different lengths of S1 (Fig. 3A) based on the above data with *in trans* ONs. We first modified the L2 sequence of the SRV-1 pseudoknot from A-rich (SF520) to U-rich (SF522) to disrupt triple interactions (Fig. 3A). This resulted in a 2.6-fold (from 37.6% to 14.6%) decrease in frameshifting efficiency (Fig. 3B, lanes 1 and 2), in accordance with our data using structured ONs (Fig. 1C, lane 3 and 4). In the context of another S1 sequence based on our previous publication (21), a more dramatic difference (about 3.6-fold) was observed as a result of the U-rich loop sequence (Fig. 3B, lanes 3 and 4). In the latter construct, extending S1 to 7 bp the difference in frameshift activity between the A-rich and U-rich L2 constructs was less than 3-fold [SF482 and SF484 (Fig. 3B, lanes 5 and 6)]. Further increasing S1 to 8 bp (Fig. 3B, lane 7 and 8) and 9 bp (Fig. 3B, lane 9 and 10) reduced the difference to 1.4-fold and 1.6-fold, respectively. Interestingly, at a stem length of 10 bp, there was no difference any more in the frameshifting efficiency between the two different kinds of L2 sequence (Fig. 3B, lanes 11 and 12). These *in cis* pseudoknot data correlate well with the *in trans* structured ON data and also

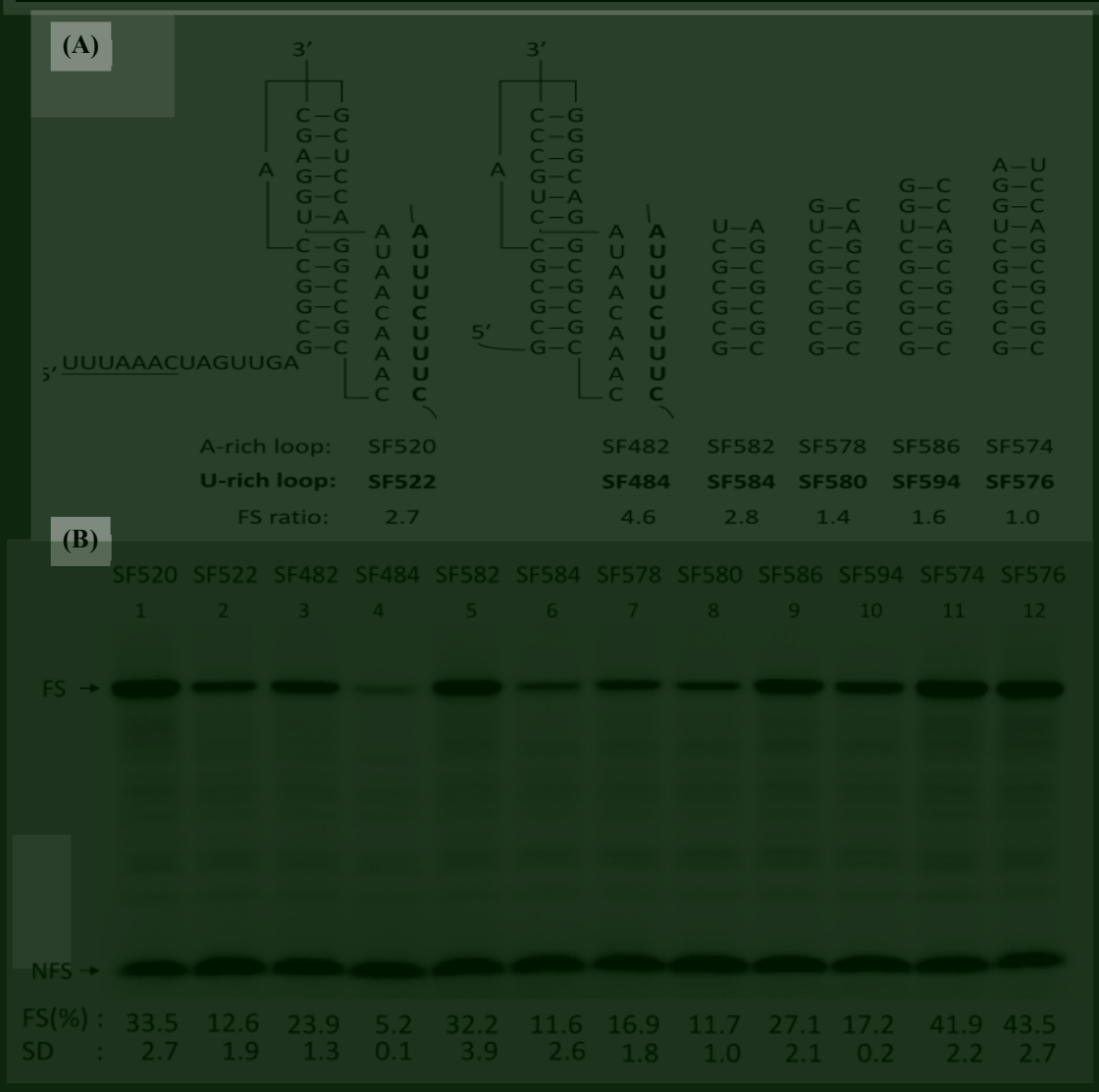

Figure 3. The correlation between S1 length and S1-L1 interactions in frameshifter pseudoknots. (A) SF520 represents the SRV-1 frameshifter pseudoknot shown in Figure 1A. SF522 is a mutant of SF520 with a U-rich L2 sequence (in bold). SF574 is the in cis frameshifter pseudoknot that is equivalent to the one formed in trans by RNA R31b, shown in Figure 2A. SF576 is a mutant of SF574 with a U-rich L2 like SF520. The length of stem 1 of SF574 and SF576 is reduced sequentially one base-pair from top of the S1 producing constructs with 9, 8, 7, and 6 bp, respectively, with either A-rich L2 or U-rich L2. The FS (frameshifting) ratio is calculated by dividing the averaged frameshifting efficiency of A-rich loop by the averaged frameshifting efficiency of U-rich loop of constructs with the same S1 length from Figure 3B. (B) SDS-PAGE analysis of 35S-methionine labeled translation products in rabbit reticulocyte lysate. See legend to Figure 1C and Materials and Methods for more details

demonstrate that the contribution of triple interactions between S1 and L2 is inversely correlated with the length of S1.

Discussion

In the present study, we have demonstrated how structured ONs mimicking pseudoknots can enhance antisense-induced –1 PRF efficiency through stem stacking and tertiary loop-stem interactions. The dissection of the pseudoknot into two parts also allowed us to investigate the effect of base or sugar modifications on ribosomal frameshifting. Moreover, construction of the pseudoknot-like structures in sense supports the observation in antisense, and further demonstrates that there exists an inverse correlation between the S1 length and the contribution of S1-L2 triple interactions to frameshifting. Our findings provide a way to enhance antisense ON-induced ribosomal frameshifting and lend further support for the notion that "longer S1" frameshifting pseudoknots are not sensitive to L2 sequences while ones with a "shorter S1" are.

A pioneering study on the formation of "pseudo-half-knots" by binding of ONs to the HIV-1 TAR RNA loop opened the way to reconstruct pseudoknot structures *in trans* by ONs (25). There are three reports in which a similar idea was applied to study ribosomal frameshifting. Plant *et al.* (26) created pseudo-pseudoknots by hybridizing linear DNA ONs to the loop of a hairpin to restrict loop rotation in order to test their torsional restraint model for frameshifting. Fayet's group (27) restored a novel "kissing loop" frameshifting signal of bacterial insertional sequence (IS) *3411* by expressing part of the required structure *in trans* as a fusion with tRNA. In another study Chou *et al.* (28) designed linear RNA ONs mimicking human telomerase hTPK-Du177 pseudoknot to investigate the importance of triplex structures spanning the helical junction and triple interactions between the major groove of S2 and L1. Here, we demonstrate that a distinct type of antisense ON, namely structured ones, can also mimic pseudoknots and enhance antisense-induced frameshifting through triple interactions between the minor groove of S1 and L2. The results are in agreement with our previous data in that building up the stability in the proximal end of an mRNA-ON duplex can enhance ribosomal frameshifting (21). Note that in our pseudo-pseudoknots, in contrast to the work of Plant *et al.* (26), there is no torsional strain built-up since the antisense ON can freely rotate around the mRNA during ribosomal encounter. Yet, they are highly efficient stimulators of frameshifting.

Our data show that frameshifting induced by structured ONs is sensitive to the sequence identity of L2 when they form a 6 bp stem 1 but less so when they form a 10 bp S1 (Fig. 1C and Fig. 2B). A similar effect was observed with the corresponding *in cis* pseudoknots (Fig. 3B). One explanation for this observation is that a longer stem obviates the need for triple interactions, in other words they may be forming but they are not contributing to the stability of the structure. Unfortunately, there is not (yet) a high-resolution structure of a frameshifter pseudoknot possessing an S1 larger than 6

bp available. Therefore, it is hard to know the specific interactions, if any, between L2 and S1 in a large pseudoknot.

In the present study we addressed another important question about the impact of triple helix formation in pseudoknots with various S1 sizes. Our data show that there exists a good inverse correlation between S1 size and the effect of triplex formation. Upon reviewing viral frameshift-inducing pseudoknots (5), we can categorize them into two major groups based on S1 length: one group has an S1 length between 4 and 6 bp and the other has an S1 length of 11 to 14 bp. Interestingly, those long S1 pseudoknots have a relatively long L2 with more than 30 nts except for two frameshifting signals in *S. cerevisiae* viruses (ScV) whose L2 length is 11 nts. Pseudoknots with short S1 feature an L2 of less than 12 nts. Moreover, the long L2s are either have no apparent secondary structures (29) or a structure that is not important for frameshifting (30, 31). This may imply that the less stable short S1 pseudoknots have "evolved" specific triples to induce significant levels of frameshifting, while for pseudoknots with longer S1 the extra stabilization, contributed by base triples, may be dispensable. Here the highly flexible L2 is probably used to store genetic (protein-coding) information or perform other unknown functions rather than to stabilize the pseudoknot conformation.

Our *in cis* pseudoknot data are actually in conflict with previous findings from Brierley's lab who showed that variants of the IBV pseudoknot with less than 11 bp in S1 were largely inactive in frameshifting (32). Their pKA13 pseudoknot with a 10 bp S1 induced merely 7% of frameshifting while our SF574 and SF576 pseudoknots which have 6 out of 10 bp in common with pKA13's S1 showed 41.9% and 43.5% frameshifting, respectively (Fig. 3A and 3B). Moreover, their other pseudoknots showed background levels of frameshifting when S1 became shorter than 9 bp whereas we still detect significant levels of frameshifting with our constructs possessing an S1 of 6 to 8 bp (Fig. 3A and 3B). To elucidate why a pseudoknot whose global structure is indistinguishable from pKA13 but is just 1 bp shorter showed almost 7-fold drop in frameshifting, they separately modified the spacer length, L2 length, and S1 sequence. These changes in the context of pKA13 led to a 1.7-fold, 1.6-fold, and 2.4-fold increase in frameshifting, respectively. Yet, a construct combining all these changes was not tested. It would be interesting to know the activity of this "evolved" pseudoknot to further understand the role of S1 length in promoting frameshifting.

The linear R10 and R12 ONs with ACE modification are surprisingly efficient in inducing frameshifting (Fig. 2C) taking into account that their duplex stability is lower than the standard RNA-RNA duplex. This suggests that the 2'ACE modification may be a poor substrate for the ribosomal helicase or interferes with the

translocation step. Although the specific reason needs further investigation, the 2'ACE-modified RNAs were shown, for the first time, to be functional in inducing frameshifting and may be applied in other antisense applications such as exon skipping or microRNA inhibition.

In conclusion, our data demonstrate that pseudoknot-mimicking ONs stabilized by loop-stem | interactions are better frameshifters than hairpin-mimicking ONs. Moreover, these tertiary interactions were shown to be dependent on the length of stem S1. Finally, the use of small ONs that are amenable to chemical modification opens a new way to study ribosomal frameshifting and may ultimately lead to applications of ONs in curing defects caused by frameshift mutations.

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