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Analysis of the role of the pseudoknot component in the SRV-1 *gag-pro* ribosomal frameshift signal: Loop lengths and stability of the stem regions

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ABSTRACT

The simian retrovirus-1 (SRV-1) *gag-pro* frameshift signal was identified in previous work, and the overall structure of the pseudoknot involved was confirmed (ten Dam E, Brierley I, Inglis S, Pleij C, 1994, *Nucleic Acids Res 22*:2304–2310). Here we report on the importance of specific elements within the pseudoknot. Some mutations in stem S1 that maintain base pairing have reduced frameshift efficiencies. This indicates that base pairing in itself is not sufficient. In contrast, frameshifting correlates qualitatively with the calculated stability of mutations in S2. The stems thus play different roles in the frameshift event. The nature of the base in L1 has little influence on frameshift efficiency. It is however required to bridge S2; deleting it lowers frameshifting from 23 to 9%. In L2, frameshift efficiency was not affected in a mutant that changed 10 of 12 bases. This makes it unlikely that the primary sequence of L2 plays a role in −1 frameshifting, in contrast to readthrough in Moloney murine leukemia virus (Wills N, Gesteland R, Atkins J, 1994, *EMBO J 13*:4137–4144). Deletions of 2 and 3 bases gave more frameshifting than the wild type, probably reflecting the increased stability of the pseudoknot due to a shorter loop L2. Deleting even more bases reduces frameshifting compared to wild-type levels. At this point, stress will build up in L2, and this will reduce overall pseudoknot stability.

Keywords: pseudoknot; RNA structure; stability; translation

INTRODUCTION

Over the last few years it has been shown that a large number of eukaryotic viruses use −1 ribosomal frameshifting as a translational control mechanism to regulate the expression of their polymerase genes (see Atkins et al., 1990; ten Dam et al., 1990; Hatfield et al., 1991; Farabaugh, 1993). The signal that prompts the translating ribosome to change reading frame is in general composed of two separate elements: a seven-base slip site and an RNA structure element located downstream, separated by a spacer region of a defined length. The slip site is the sequence where the actual −1 ribosomal shift takes place. It conforms to the consensus motif X XXY YYN (the initial reading frame is indicated by the triplets; the bases X and Y can be identical) as formulated in the "simultaneous slippage" model proposed by Jacks and co-workers (1988). The structure element can be formed by a simple hairpin (Parkin et al., 1992; Falk et al., 1993; Nam et al., 1993), or a pseudoknot (Brierley et al., 1989, 1991; Dinman et al., 1991; Chamorro et al., 1992; ten Dam et al., 1994). A classic pseudoknot can be considered to consist of two double-stranded stem regions (S1 and S2, numbering is from the 5′ end) and two connecting loops (L1 and L2) (see Fig. 1, and Puglisi et al. [1991], Westhof & Jaeger [1992], and ten Dam et al. [1992] for reviews on RNA pseudoknots). We have previously shown that the *gag-pro* overlap of the type D simian retrovirus-1 (SRV-1) RNA contains a *cis*-acting −1 ribosomal frameshift signal (ten Dam et al., 1994). It has an efficiency of 23% in rabbit reticulocyte lysate and wheat germ extracts and consists of a G GGA AAC slip site (as in feline immunodeficiency virus [FIV] [Morikawa & Bishop, 1992]) and beet western yellows virus [BWYV] [Garcia et al., 1993]) followed after seven nucleotides by a pseudoknot (see Fig. 1). Spacing between the slip site and the pseudoknot is critical, as is the presence of the two stems. Interestingly, mutants that had three base pairs in stem S1 reversed did not fully return to *wild-*

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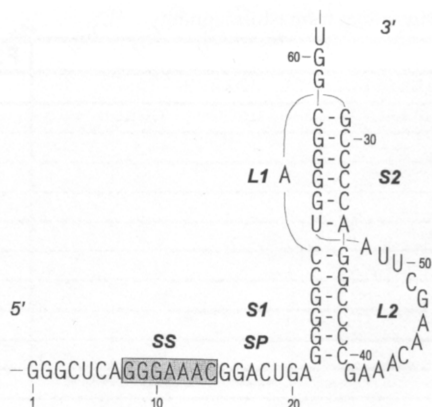

FIGURE 1. SRV-1 *gag-pro* frameshift signal. Indicated are the slip site (*SS*), spacer region (*SP*), and the stems and loops (*S1, S2, L1,* and *L2*) of the pseudoknot. Numbering is from the start of the T7 RNA polymerase transcript.

type frameshift efficiency, contrary to mutations in stem 2 (ten Dam et al., 1994).

So far, it is not clear what makes a pseudoknot an efficient stimulator of ribosomal frameshifting (Atkins et al., 1990; Farabaugh, 1993). For some viruses, e.g., human immunodeficiency virus (HIV-1), human T-cell leukemia virus-1 (HTLV-1), a simple stem-loop structure or hairpin is sufficient for frameshifting (Parkin et al., 1992; Falk et al., 1993; Nam et al., 1993). The requirement for a hairpin or pseudoknot may depend on the nature of the slip site. It is not possible to replace the pseudoknot with a hairpin with an identical number of base pairs in the pseudoknot-dependent frameshift signal of infectious bronchitis virus (IBV) (Brierley et al., 1991). Pseudoknots involved in ribosomal frameshifting show great variation in stem and loop size and stability (ten Dam et al., 1990), but it is not directly apparent how these properties relate to the overall efficiency of the frameshift signal. In general, there seems to be little requirement for the base composition of the stems. However, for a number of viruses, S1 revertants described do not fully revert to wild-type efficiencies (Chamorro et al., 1992; ten Dam et al., 1994). Loop 2 of the pseudoknot in the IBV frameshift signal can be shortened from 32 to 8 bases without effect on frameshift efficiency, and the remaining 8 bases in L2 can be changed to others without affecting frameshifting (Brierley et al., 1991). No detailed analysis of the length requirements of loops has been carried out. Interestingly, in the pseudoknot-containing readthrough signal of Moloney murine leukemia virus (Mo-MuLV), sequences in loop 2 seem to be important for readthrough (Wills et al., 1994). Here we will investigate the pseudoknot-component of the *gag-pro* −1 ribosomal frameshift signal of SRV-1 RNA in more detail. To test the role of stability, stem composition and loop lengths of the pseudoknot in the translational frameshifting process, we have tested mutants made in these areas in an in vitro translation system for their capability to induce −1 frameshifting (Brierley et al., 1992; ten Dam et al., 1994).

RESULTS

The SRV-1 *gag-pro* frameshift signal was identified in previous work, and the overall structure of the pseudoknot was studied and confirmed (ten Dam et al., 1994). Here we report on the importance of individual specific elements in the pseudoknot structure. The structure of the pseudoknot was analyzed by direct structure mapping and mutational analysis. Mutants with changes in either of the two stem regions or the two loops of the pseudoknot were tested as described before (ten Dam et al., 1994; see Materials and methods).

Mutants in the stem regions

Because a revertant mutant in stem S1 restored frameshifting only partly (10% versus 23% for the wild type and 6 and 3% for the stem-disrupting mutations), and changes in the stem regions were made as crude three-base block mutations (ten Dam et al., 1994), we set out to investigate the role of the stems in more detail. In particular we were interested to see if there was a correlation between the stability of S1 and S2 and the level of frameshifting induced. To analyze this, we designed mutations in the base pairs in the middle of stem 1 and stem 2, respectively. No parameters are established to allow calculation of the overall thermodynamic stability of pseudoknots, due to the unknown energy contributions made by the two loops and their potential interactions with the two stems. Having the base changes in the middle of the double-stranded regions, however, allows the calculation of the change in thermodynamic stability caused by the mutations using the standard rules for calculation of the stability of double-stranded RNA structures (Turner et al., 1988). This is valid only on the assumption that the overall structure of the pseudoknot is maintained, which means that only mildly destabilizing mutations could be introduced, mainly changes in a single base pair. Numbering of the bases starts at the first base of the T7 RNA polymerase transcript (see Materials and methods, and Fig. 1).

S1 single base pair mutants

To test the importance of stem S1 for the frameshifting efficiency, we designed a set of mutants that changes base pairing in the middle two base pairs of S1 (mutants pSF38–40 and pSF44–pSF52, see Table 1). Disrupting G_{24}-C_{38} reduces the level of frameshifting, as seen in mutants pSF38, pSF39, pSF44, and pSF45 (see Fig. 2; Table 1). However, mutant pSF40 (A_{24}-U_{38}),

TABLE 1. Summary of mutants made in the pseudoknot of the SRV-1 *gag-pro* ribosomal frameshift signal.[a]

clone	sequence								FS (%)
	SSSSSSS	111111	L	222222	111111	LLLLLLLLLLL	222222		
pSF2	gggcucagggaaacugacuga	ggGGcc	A	gcCCca	ggCCcc	GAAACAAGCUua	ugGGgc	GGucuucag	23
pSF21			-						9
pSF22					*A*				5
pSF23			-		*AAUU*				5
pSF24			-		*AAUUUAA*				7
pSF33			C						18
pSF34			U						23
pSF35			G						21
pSF37						-----			10
pSF38		A							2
pSF39					U				6
pSF40		A			U				5
pSF41				U					19
pSF42							A		9
pSF43				U			A		25
pSF44		C							3
pSF45					G				2
pSF46		C			G				12
pSF47		A							1
pSF48					U				12
pSF49		A			U				15
pSF50		C							2
pSF51					G				4
pSF52		C			G				22
pSF53				UU					5
pSF54							AA		6
pSF55				UU			AA		13
pSF56				UU			A		2
pSF57				U			AA		6
pSF61						AUCGAUGAAA			20
pSF66						----			18
pSF67						---			30
pSF68						--			34
pSF69						--			21
pSF71						*A*			28
pSF76				*AAA*					6
pSF77				*ACA*					5
pSF78				*AAC*					11

[a] This table lists the RNA sequence and frameshift efficiencies of transcripts of the clones used in this study. The error in determining frameshift efficiencies (FS) is estimated to be 1% from several independent translations. Slip site, stem S1, stem S2, and the loops are indicated above the pSF2 sequence by S, 1, 2, and L, respectively. Sequence changes are indicated with respect to pSF2; bases changed in the variants are indicated in capitals. Deletions of nucleotides are indicated by a dash (-); insertions in the sequence are indicated by italics. The sequence given under pSF2 corresponds to the 67-base RNA fragment generated after *Pvu* II digestion and T7 RNA polymerase transcription of plasmid pSF2 (see Materials and methods). The reading frame is corrected for the −1 product to be 22 kDa in clones pSF66, 68, and 71 by insertion or deletion of a single C 19 bases downstream of the last base of the pseudoknot in pSF2.

where base pairing is restored, gives only 5% frameshifting. And although there is a slight increase in pSF46 (C_{24}-G_{38}) to 12%, it is still well below wild-type levels. Structure probing shows that this mutant still forms the pseudoknot structure (see Fig. 3 for nuclease S1 data; RNase T1 and cobra venom RNase analysis not shown). For example, it has the bands characteristic of the pseudoknot conformation near the full-length RNA species, which are absent in the non-pseudoknotted constructs pSF21, pSF23, pSF24, and pSF45. Mutations in base pair G_{25}-C_{37} (clones pSF47–PSF52) have a different effect; mismatches reduce frameshifting, but G-U and A-U base pairs gave rise to intermediate frameshift levels (12% and 15%, respectively). Furthermore, pSF52, which reverses the G-C base pair to C-G, has wild-type efficiency again with 22% −1 frameshifting (see Fig. 2; Table 1) and folds into the pseudoknot (see Fig. 3).

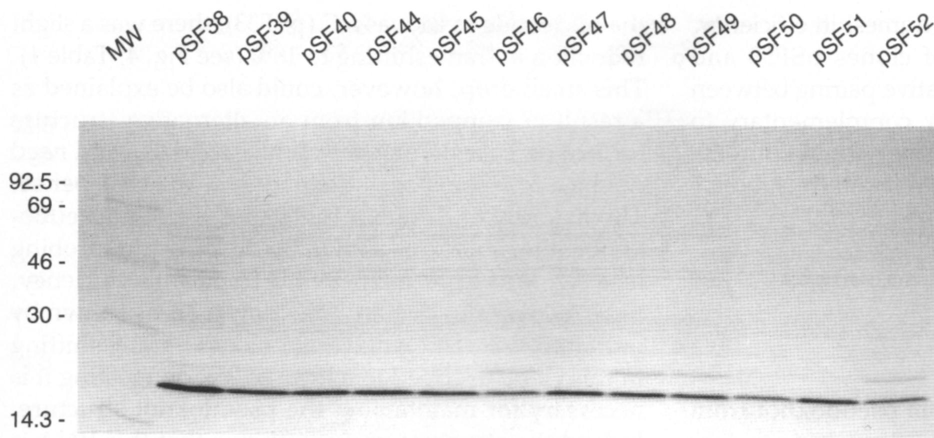

FIGURE 2. S1 single base pair mutants. Analysis of mutants in the stem S1 region of the pseudoknot. Reticulocyte lysate translation products of mRNAs derived from *Bam* HI-digested pSF templates were separated on a 17.5% SDS–polyacrylamide gel and detected by fluorography. pSF clones translated (bottom) and the approximate size of the polypeptides (left) are indicated. See also Table 1.

S2 single base pair mutants

When the C_{31}-G_{56} base pair in stem 2 was mutated to a C·A mismatch (pSF42), a reduction in frameshifting efficiency to 9% was observed (see Table 1). Changing it to the more stable U-G (pSF41) or U-A (pSF43) base pairs increased it again, with the U-G giving 19% and the U-A even 25% frameshifting. So, contrary to S1, in these S2 mutants frameshifting efficiency correlates with the thermodynamic stability of S2, at least qualitatively. Starting from mutants pSF41 and pSF43, extra mutations were introduced by changing base pair C_{32}-G_{55} to further destabilize S2, resulting in mutants pSF53–pSF57. All these give lower percentages of frameshifting than their "parent" clones (see Table 1), again in agreement with a correlation between the sta-

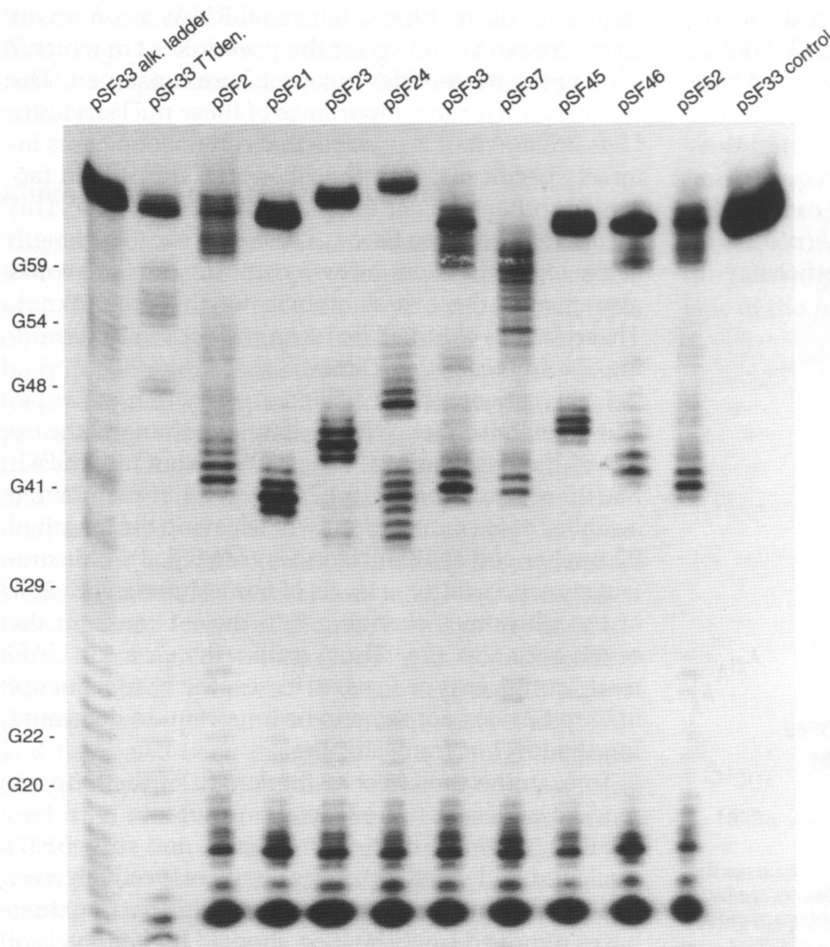

FIGURE 3. Structure mapping with nuclease S1. 5′ ^{32}P-labeled RNA fragments derived by T7 RNA polymerase transcription from *Pvu* II-digested pSF-plasmid DNA (see the Materials and methods) were treated as follows (from left to right): alkaline hydrolysis ladder (pSF33 transcripts); RNase T1 sequencing reaction under denaturing conditions (pSF33); nuclease S1 structure probing performed at 37 °C on transcripts from pSF2, pSF21, pSF23, pSF24, pSF33, pSF 37, pSF45, pSF46, and pSF52; untreated pSF33 transcripts.

bility of S2 of the pseudoknot and frameshift efficiency. The low frameshift efficiency of clones pSF53 and pSF56 may be a result of an alternative pairing between bases 30 and 37, which are now complementary to bases 16–23. This alternative pairing would compete with the formation of the pseudoknot and thus reduce frameshifting.

Mutants in the two loop regions

Loop L1

The first loop in the SRV-1 *gag-pro* pseudoknot from the 5′ end is formed by a single adenosine residue. This base has to cross the deep (major) groove formed by the six base pairs in stem S2. Based on the coordinates of an A-type RNA double helix, it is possible to calculate the distance to be spanned by the loops. The shortest distance between the two phosphates to be connected by loop L1 at the opposite ends of stem S2 occurs when S2 consists of six or seven base pairs, and it might be just possible to bridge this distance with a single base (Pleij et al., 1985). Pseudoknots that have a single base in L1 have been described and proposed before, e.g., those in the 3′ noncoding region of tobamoviral RNAs (van Belkum et al., 1985), the leader of gene 32 mRNA (McPheeters et al., 1988), and the one at the S15 repressor binding site (Philippe et al., 1990). In pseudoknots from the stalk region of the 3′ UTR of tobamoviral RNAs, a G seems to be the preferred base in this position, and in the S15 pseudoknot, an A is found, but it could be replaced by a G. It would thus seem that a purine in one base of L1 is required for some unknown structural reason. In the case of the pseudoknot of SRV-1, changing A_{28} to a U (pSF34) or a G (pSF35) had no effect on frameshift efficiency. If

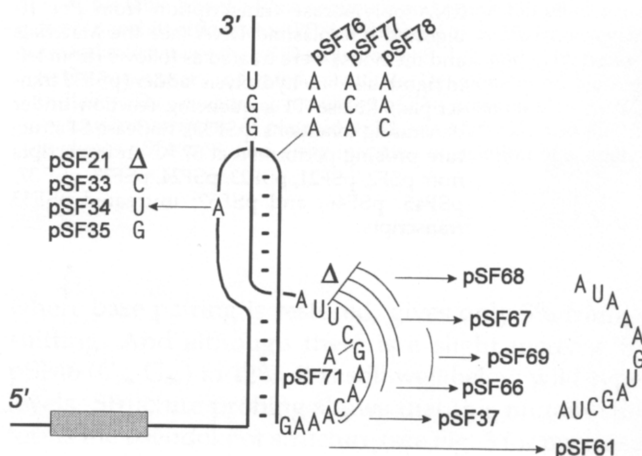

FIGURE 4. Summary of loop mutants. Shown are the mutants in the loop regions of the SRV-1 *gag-pro* pseudoknot. Mutations are indicated in the pSF2 (wild-type) background. Deletions of bases are indicated by Δ.

the nucleotide in L1 was a C (pSF33), there was a slight reduction in frameshifting to 18% (see Fig. 4; Table 1). This small drop, however, could also be explained as a result of competition from an alternative structure formed on base pairing between C_{28} and G_{59} and need not be a consequence of the nature of the base per se. The majority of the RNA molecules is in the pseudoknot conformation as shown by the structure probing (see Fig. 3) as expected from the frameshift efficiency. Base A_{28} was not dispensable, however, as shown by mutant pSF21. Upon deleting this base, frameshifting in this clone dropped to a level of 9%, suggesting it is necessary for maintaining the pseudoknot structure. Indeed the structure probing shows that this RNA is in a different conformation (see Fig. 3). Surprisingly, when the length of L1 was extended by three bases, a drop in frameshift efficiency was observed. Mutants pSF76–pSF78 have frameshift efficiencies of 5–11%, well below wild-type levels (see Table 1).

Loop L2

In the case of the pseudoknot-dependent translational readthrough of the type C retroviruses as exemplified by Mo-MuLV, the overall level of readthrough is influenced by the nature of some of the nucleotides in loop L2 (Wills et al., 1991, 1994). On comparison of the L2 sequences of the type C retroviral RNAs a consensus structure can be drawn for the pseudoknot in which 7 of 18 of the nucleotides in loop L2 are conserved. The exact reason for the importance of these nucleotides in L2 is unknown. It is possible that certain loop bases interact specifically with the ribosome, translation factors, or other parts of the pseudoknot structure. This would require those bases to be conserved. The length of the loop influences its own conformation and could also change the overall stability of the pseudoknot. These factors could all be of importance in determining the frameshift efficiency.

To investigate the possible necessity to have a specific primary sequence in the loop, we changed the order of the bases in L2. The resulting mutant, pSF61, had 10 of the 12 bases in L2 changed but maintained a similar base composition, while leaving the length of L2 unchanged at 12 nucleotides (see Fig. 4). This mutant showed wild-type levels of frameshifting with 20% of the ribosomes changing into the −1 frame at the seven-base slip site. Thus, unlike the case for UAG readthrough in type C retroviruses, the base sequence in loop L2 does not seem to be important in determining the level of frameshifting.

To examine the effect of the length of the loop we made a series of nested deletion mutants with two (pSF68), three (pSF67), four (pSF66), and six (pSF37) nucleotides deleted from the loop, respectively (see Fig. 4). Surprisingly, the mutants with two or three bases removed from the loop showed higher levels of

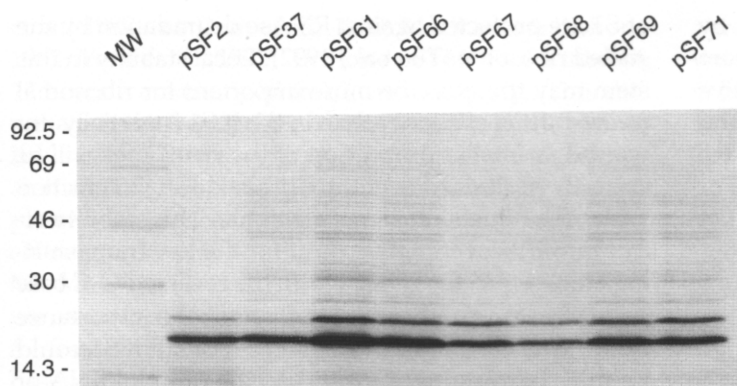

FIGURE 5. L2 mutants and alternative pseudoknots. Analysis of mutants in loop L2 of the pseudoknot. Reticulocyte lysate translation products of mRNAs derived from *Bam* HI-digested pSF templates were separated on a 17.5% SDS–polyacrylamide gel and detected by fluorography. pSF clones (top) and the approximate size of the polypeptides (left) are indicated. See also Table 1.

frameshifting than the wild type, pSF2, up to 34%. Mutants pSF66 and pSF37, with deletions of four and six bases respectively, had reduced levels of frameshifting (see Fig. 5; Table 1). RNA structure probing showed that mutant pSF37 still adopts a pseudoknot conformation (see Fig. 3). However, when a second mutant with a two-base deletion at another position in L2 was made (pSF69), it showed only 21% frameshifting. This might be a result of competition from an alternative structure. The two-base deletion generates a sequence complementary to PB2 sequences in the vector 44–54 bases downstream of the pseudoknot. Mutant pSF71, which has an A inserted between G_{48} and C_{49}, shows a relatively high level of frameshifting, 28%. Currently, we have no explanation why this is 5% higher than the wild type.

Alternative pseudoknotted structures

The pseudoknot of the SRV-1 frameshift signal is of the so-called classic or H-type (Pleij & Bosch, 1989; Pleij et al., 1992). This relatively simple and widespread type of pseudoknot is formed when bases of the loop of a hairpin pair with bases outside the loop. A possibility for an alternative way of stacking the two stems of the pseudoknot arises on deleting A_{28}, the single residue that forms L1, and inserting nucleotides between A_{34} and G_{35} (see Fig. 1). If S2 stacks on S1 with its other end, as compared to the classic pseudoknot, L2 will be outside the helical cylinder formed by the combined stems S1 and S2. This will result in L2 having to bridge the combined length of the two stems of the pseudoknot, 12 base pairs in total (see Fig. 6). Structures like this have been proposed to occur in group II introns (Michel et al., 1989) and 16S rRNA (Kössel et al., 1990) and have also been described on a theoretical basis (Abrahams et al., 1990; Mans & Pleij, 1993). We were interested to see if such structures could form, and if so, whether they would support frameshifting. As already shown above, in mutant pSF21 A_{28} was deleted leading to a drop in frameshifting to 9% (see Table 1). Insertion of a single A base between A_{34} and G_{35} (pSF22) also severely reduced frameshifting, this time to 5%. This mutation disrupts the configuration in the stacking region between S1 and S2. Mutants pSF23 and pSF24 combine the deletion of A_{28} with insertions between A_{34} and G_{35} of four and seven bases, respectively (see Table 1). Frameshifting was low in both of these mutants, which could be due either to the fact that the predicted structures did not form or, alternatively, that these structures are not capable of supporting the slip site in inducing a −1 shift of the ribosome. To investigate this, we analyzed the structure of the mutants pSF23 and pSF24 directly. Structure mapping with nuclease S1 (and RNAse T1, not shown) was used to determine the overall structure of RNA fragments derived from these mutants (see Fig. 3). Although the bands in the gels show intense compression due to the very high G-content of these RNA fragments, it is clear from the gel that the clones pSF23 and pSF24 fold into structures different from the predicted alternative pseudoknot. For example, bands having a size close to the full-length RNA species and which are characteristic of the pseudoknot conformation are absent. This

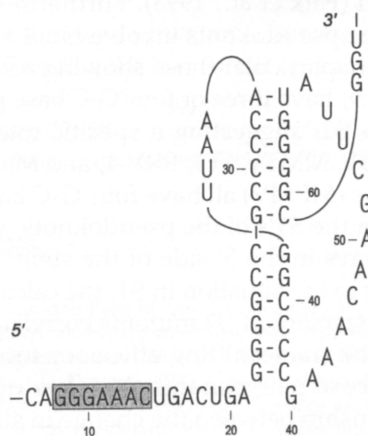

FIGURE 6. Alternative pseudoknot structure. Shown here is the possible alternative structure for mutant pSF23. Note that the 3′ end is leaving the pseudoknot at a different position compared to the wild-type SRV-1 pseudoknot (see Fig. 1).

alternative folding was subsequently confirmed by computer structure analysis using the STAR program (Abrahams et al., 1990), which predicted an alternative base-pairing interaction between nucleotides 22–33 and 59–70. Destabilizing this structure by changing the two G bases directly after the pseudoknot to either A's or C's increased frameshift efficiency only marginally (not shown), so the question remains whether these alternative pseudoknot structures as proposed in Figure 6 actually form in this case.

DISCUSSION

What is it exactly that makes a pseudoknot, compared to hairpins, so efficient as a stimulator in −1 frameshift signals? Is it just their stability or are more specific interactions with the translational machinery involved? In this study we have investigated each of the four elements of the SRV-1 *gag-pro* pseudoknot: the two stems and both loop regions.

Stem stability and composition

Mutations in base pairs G_{24}-C_{38} and G_{25}-C_{37} in the SRV-1 *gag-pro* pseudoknot show different effects on the level of frameshifting. Although the upper base pair shows its effects only through stability, for the lower base pair additional factors must play a role because mutants of identical predicted stability show reduced levels of frameshifting. This in spite of the fact that for instance mutant pSF46 still forms a pseudoknot. This behavior is not unique to SRV-1; a mutant that reverses three of the four G-C base pairs in stem S1 of the mouse mammary tumor virus (MMTV) *gag-pro* pseudoknot restores frameshifting to only 50% of the wild-type efficiency (Chamorro et al., 1992). Similarly, stem S1 revertants show only partial recovery in the case of the yeast ds RNA virus L1 (Tzeng et al., 1992) or in the hairpin containing a −1 frameshift signal of HTLV type II (Falk et al., 1993). Furthermore, a large number of the pseudoknots involved in −1 ribosomal frameshifts, in particular those showing a high frameshift efficiency, have three or four G-C base pairs at the start of stem S1, suggesting a specific role for these base pairs; IBV, MMTV, FIV, SRV-1, and Mason–Pfizer monkey virus (MPMV) all have four G-C base pairs at the bottom of the S1's of the pseudoknots, with the G residues always in the 5′ side of the stem.

In contrast to the situation in S1, the calculated thermodynamic stability of S2 mutants correlates qualitatively with the frameshifting efficiency for the single and double base pair mutants. No clear quantitative linear relationship between the change in stability and the frameshift level was apparent however (data not shown).

Translating ribosomes are paused by pseudoknots (Somogyi et al., 1993); and six nucleotides at the 5′ end of S1 are protected against RNAse degradation by the stalled ribosome (Tu et al., 1992). Local stability in this stem may therefore be more important for ribosomal frameshifting efficiency than in S2. If S1 is partially unwound, a smaller proportion of the ribosomes will be correctly positioned over the slip site during interaction with both frameshift signal elements. This stability effect can, however, not account for the low frameshifting efficiency in the mutant with the reversed G-C base pair (pSF46). Another explanation for the importance of the G-C base pairs at the bottom of stem S1 could be that they are involved in specific protein–RNA or RNA–RNA interactions with the translational machinery, or that a double helix formed by base pairing between an oligo G and oligo C stretch adopts a special conformation, more resistant to unwinding. We are currently investigating the latter possibility.

Loop length and composition

The nature of the base in L1 seems to have little influence on frameshift efficiency in the case of the SRV-1 *gag-pro* pseudoknot. Frameshift levels of mutants where the A is changed are equal or close to wild-type efficiencies. This is similar to the situation in the tRNA-like structure of turnip yellow mosaic virus, where changes in loop L1 have little effect on the aminoacylation of the RNAs (Mans et al., 1992). The L1 nucleotide, however, is necessary to maintain the pseudoknot conformation (as shown by structure probing); removing it reduces frameshifting to 9%. Unexpectedly, different three-base insertions in L1 all reduce frameshifting compared to wild-type levels. This is probably a consequence of the energetically less favorable conformation of the now larger loop 1. This effect may be partially compensated for in mutant pSF78, which showed slightly higher levels than pSF76 and pSF77, possibly due to an extension of S2 using the newly inserted nucleotides in L1.

Comparing the loop 2 sequences from pseudoknots found at −1 ribosomal frameshift sites, the loop is in general larger than appears necessary on theoretical grounds. Using the known coordinates of an RNA-A helix (Arnott et al., 1972), one can calculate that four bases should be sufficient to cross an S1 of six bases as found in the SRV-1 *gag-pro* pseudoknot (Pleij et al., 1985). However, the length of L2 is often much larger: e.g., 32 bases in IBV, 11 in yeast L-A ds RNA virus, 11 in FIV, and 12 in SRV-1 (ten Dam et al., 1990). We set out to investigate if any particular features of L2 were involved in the frameshifting process. Comparing loop L2 sequences from SRV-1 to those of other viruses having a G GGA AAC slip site and similar pseudoknots as part of their frameshift signals, one can see little conservation. Only two residues, A_{46} and A_{52}, occur at the same position in SRV-1, SRV-2, MPMV, FIV, and jaagsiekte sheep retrovirus (Sonigo et al., 1986; Thayer

et al., 1987; ten Dam et al., 1990; Morikawa & Bishop, 1992; York et al., 1992). In clone pSF61 10 of a total of 12 bases were changed in L2, including A_{46}. This did not affect the frequency of frameshifting. In the case of IBV, none of the bases in loop 2 are essential for frameshifting (Brierley et al., 1991). It is thus unlikely that the primary sequence of loop 2 plays a role in −1 frameshifting, in contrast to the situation for readthrough in Mo-MuLV (Wills et al., 1994). However, at the moment we cannot exclude the possibility that A_{52} is necessary for frameshifting. The fact that deletions of two or three bases in loop L2 give a higher level of frameshifting than the wild type shows that the level of frameshifting is not maximized, but that it is probably optimized for viral replication. On reducing the length of loop 2, it is likely that the interaction forming S2 is stabilized. This is true until a point is reached where the length of loop L2 is no longer adequate to bridge S1. At this point, the chain of nucleotides that forms L2 will have to stretch, and this will come at a cost in terms of overall pseudoknot stability. In addition, the conformation of loop L2 will change upon variations in its length and this might influence frameshifting through alteration of its interaction with the ribosome. NMR studies on model pseudoknots and the pseudoknot from the MMTV *gag-pro* frameshift signal showed that bases at the 5′ end of L2 continue stacking on S2 (Puglisi et al., 1990). This stacking is likely to be affected by the length of loop L2. It is unknown if this is a feature of all pseudoknots, or indeed if it occurs in the SRV-1 *gag-pro* pseudoknot.

In summary, viruses have many possibilities to fine-tune the level of frameshifting by varying their frameshift signals. Besides changing the actual slip site or the length of the spacer region, the stability of the pseudoknot is a major determinant of overall frameshift efficiency. Its stability can be modified not only by changing the stability of the stem regions, but also by varying the loop length, especially loop L2. This makes the question why viruses show such a broad range of frameshift efficiencies for functionally similar genes (e.g., HIV 5% [Parkin et al., 1992], FIV 25% [Morikawa & Bishop, 1992]) even more interesting.

MATERIALS AND METHODS

Plasmids used in this study

Analysis of the SRV-1 frameshift signal and mutant variants was done using plasmid pSF2 and derivatives thereof mutated in the frameshifting region (ten Dam et al., 1994; see Table 1). Briefly, the minimal SRV-1 *gag-pro* frameshifting signal was inserted in plasmid pFScass5 into the influenza PB2 polymerase gene used as a reporter gene (Brierley et al., 1992). Capped SP6 RNA polymerase transcripts were then translated in rabbit reticulocyte lysate (Promega), and normal (19 kDa) and −1 frameshifted (22 kDa) translation products were identified by their size. Mutants in the frameshift region were made by site-directed mutagenesis based on the method of Kunkel as described before (Kunkel, 1985; Brierley et al., 1992). Mutagenic oligonucleotides were designed to contain at least 10 bases complementary to the single-stranded template on each side of the mutation to be introduced. All mutations were identified and verified by dideoxy sequencing. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. Enzymes and biochemicals were purchased from Pharmacia except where indicated otherwise.

In vitro transcription and translation

Plasmid preparation and linearization, transcriptions with SP6 RNA polymerase, rabbit reticulocyte lysate translations, and SDS-polyacrylamide gel electrophoresis were as described (Brierley et al., 1992). Rabbit reticulocyte lysate was obtained from Promega. The relative amounts of the in-frame and frameshifted translation products were calculated by quantifying the ^{35}S-methionine (Amersham) incorporation in the peptide bands on a Packard Instant Imager 2024 and corrected for background activity and differential methionine content of the products (ten Dam et al., 1994).

RNA structure probing

Short RNA fragments harboring the slip site and pseudoknot were obtained as follows: plasmids from the pSF series were purified on CsCl gradients following standard protocols (Sambrook et al., 1989), digested with *Pvu* II, and then transcribed using T7 RNA polymerase yielding 67 base long fragments (see Table 1). Transcription conditions and 5′ end labeling were as described before (Mans et al., 1992). 5′ ^{32}P-end-labeled RNA fragments were subsequently purified on 10% polyacrylamide/8 M urea gels and used for structure probing experiments as described before (van Belkum et al., 1988).

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