

Identification and analysis of the pseudoknot-containing *gag-pro* ribosomal frameshift signal of simian retrovirus-1

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ABSTRACT

The *pro* and *pol* genes of simian retrovirus-1 (SRV-1) are expressed as parts of a fusion protein generated by -1 ribosomal frameshifting. To investigate the requirements for frameshifting at the *gag-pro* overlap, we have inserted a stretch of 58 nucleotides containing the proposed frameshift signal into a plasmid that allows monitoring of translation in all three reading frames. *In vitro* translation of mRNAs derived from this plasmid indicated that the 58 nucleotides from the SRV-1 *gag-pro* overlap were sufficient to induce an efficient -1 shift in a heterologous context. Mutational analysis demonstrated that the slip site is formed at the heptanucleotide G GGA AAC. The frameshift efficiency of the *wild type* sequence in rabbit reticulocyte lysate was 23%. A second component of the frameshift signal is formed by a pseudoknot seven bases downstream of the slip site. The presence of this pseudoknot was confirmed by mutational analysis, employing complementary and compensatory base changes, and by probing the structure of short RNA transcripts containing the frameshift signal. Adding increasing amounts of an SRV-1 pseudoknot containing RNA transcript to a translation reaction programmed with an SRV-1 frameshift reporter mRNA had no effect on the frameshift efficiency, arguing against the role of a specific pseudoknot-recognising factor in the frameshifting process.

INTRODUCTION

Ribosomal frameshifting is a recoding mechanism that regulates and co-ordinates the expression of multiple open reading frames (ORFs) from a single messenger RNA during translation (for a review see [1]). A single fusion protein is synthesised from two or more overlapping ORFs through alteration of the reading frame; this change of frame can occur by movement of the ribosome by one nucleotide in either the 5' (-1) or 3' ($+1$) direction. Most examples of -1 frameshifting come from eukaryotic RNA virus genomes. It was first observed as the mechanism used by Rous sarcoma virus to express the *pol* gene

that encodes three essential enzymatic proteins: integrase, protease and reverse transcriptase [2, 3]. These are expressed as a single fusion protein, together with the internal structural proteins of the virus core (encoded in the upstream *gag* reading frame). This multi-protein precursor is later processed into the mature products. The structural proteins (core proteins) are needed in much larger amounts than the catalytic proteins (polymerase and protease), and to produce these gene products in the correct ratio, the virus maintains a fixed amount of ribosomal frameshifting. The relative expression levels of these proteins is thought to be important for the efficient assembly of the virus particles [4].

-1 Frameshifting has now been demonstrated to occur not only in retroviruses, but also in coronaviruses [5–7], yeast viruses [8, 9], *E. coli* [10–12], the torovirus BEV [13], plant viruses [14–17], astroviruses [18] and giardiaviruses [19].

Previous analysis of viral -1 frameshift signals has shown that they generally consist of two elements: a) a heptanucleotide sequence that forms the actual frameshift site, and b) an RNA structure element downstream of the slip site, usually a pseudoknot [3, 20, 21]. Although various sequences can function as shift sites, they nearly all conform to the consensus motif X XXY YYN (the initial reading frame is indicated by the triplets, the bases X and Y can be identical). This motif suggests a mechanism as formulated in the 'simultaneous slippage' model as proposed by Jacks *et al.* [3]. The two tRNAs on the ribosome, in the A and P sites respectively, move at the same time one base in the 5' direction, thereby breaking away from the 0-frame and pairing again in the -1 frame. In this way, at least two out of three bases of each anticodon will remain paired after the slippage has occurred.

Several authors have noticed that potential stem-loop structures could form almost immediately downstream of potential -1 frameshift sites [22–24], and in fact almost all of the potential slip sites in retroviruses are followed within nine nucleotides by stable hairpins. The evidence for the need for such structures to obtain efficient frameshifting came firstly from the study of the RSV *gag-pol* frameshift site. Mutational analysis showed that a double stranded region 6 nucleotides downstream of the slip site was necessary for efficient frameshifting [3]. However, an

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additional 23 nucleotides downstream of this hairpin were also essential for frameshifting, suggesting the presence of a pseudoknot [25]. In a more comprehensive analysis of the ORF 1a–1b overlap of the infectious bronchitis virus (IBV) RNA, an avian coronavirus, Brierley and colleagues demonstrated that the –1 frameshift event is pseudoknot-dependent [20]. Mutational analysis showed that both stems forming the pseudoknot were necessary, and that mutations in one strand of a stem could be compensated for by complementary mutations in the other strand. Moreover, the pseudoknot could not be replaced by a hairpin of the same length and composition as the two stacked stems forming the pseudoknot, indicating that some specific feature of the pseudoknot structure is required [20, 26]. Pseudoknot-dependent frameshifting has now been shown to occur in a number of other viruses: e.g. mouse mammary tumor virus [27], and the lentivirus feline immunodeficiency virus (FIV) [28].

Simian retrovirus-1 (SRV-1) is a type D retrovirus related to Mason–Pfizer monkey virus (MPMV). It causes simian acquired immune deficiency syndrome (SAIDS) in rhesus macaques, inducing a number of symptoms including depletion of B- and T-lymphoid cells [29]. In an earlier study employing a computer analysis of the secondary structure of portions of the viral RNA, we predicted that SRV-1 uses ribosomal frameshifting as a strategy for expression of the *pro* and *pol* genes and that the *gag-pro* overlap contains a frameshift signal similar to that of IBV and of other retroviruses [21]. More specifically, the slip site was predicted to be formed by the heptanucleotide G GGA AAC, followed by a seven base spacer region and a pseudoknot (see Fig. 1). This proposed frameshift signal is located in the 181 base overlap between the *gag* and *pro* coding regions. The three elements of the frameshift signal appear to be conserved in the closely related viruses simian retrovirus-2, MPMV and Jaagsiekte retrovirus, with sequence divergence occurring only in regions proposed to be single stranded [30–32]. Here we describe the structure of the RNA of the proposed *gag-pro* frameshift signal and its capability of inducing ribosomal frameshifting in the –1 direction when placed in a heterologous context.

MATERIALS AND METHODS

(a) Plasmids used in this study

The proposed SRV-1 frameshift signal was inserted into a vector that allows analysis of frameshifting into all three possible reading frames; i.e. one that allows detection of –1, 0, and +1 frame products formed after the ribosomes pass the frameshift site. It is based on the plasmid pFScass5 described earlier [33]. First, the IBV sequences were deleted from pFScass5 by site-directed mutagenesis using a procedure based on the method of Kunkel as described [20, 34]. Subsequently, 58 nucleotides containing the proposed frameshift signal (corresponding to nt. 2320–2377 in the SRV-1 sequence [29]) were introduced downstream of the T7 promoter in pFScass5 in the 0-reading frame in two rounds of mutagenesis to generate pSF0 and pSF1 (See Table 1). Plasmid pSF2 was constructed by changing the G directly downstream of the slip site to a T, introducing a UGA stop codon in the 0-frame (see mutational analysis section and Fig. 2). Plasmids with the correct mutations were identified by colony hybridisation and confirmed by dideoxy sequencing. For sequences of the mutants see Table 1.

Enzymes and biochemicals were purchased from Pharmacia except where indicated otherwise. Oligonucleotides were synthesised on a Applied Biosystems 381A DNA synthesiser.

(b) Site directed mutagenesis

Further mutants were made in pSF2 and its derivatives by site-directed mutagenesis based on the method of Kunkel as described before [33, 34]. Mutagenic oligonucleotides contained at least ten bases complementary to the single stranded template on each side of the mutations to be introduced. All mutations were identified and verified by dideoxy sequencing.

(c) *In vitro* transcription and translation

Plasmid preparation and linearization, SP6 RNA polymerase transcriptions, rabbit reticulocyte lysate translations and SDS-polyacrylamide gel electrophoresis were as described [33]. Rabbit reticulocyte lysate was obtained from Promega. The relative amounts of the translation products were calculated by quantifying the ³⁵S-methionine incorporation in the bands on a Betagen Betascope 603 blot analyser and correcting for background and differential methionine content of the products.

(d) RNA structure probing

Short RNA fragments were obtained as follows: plasmids pSF1 and pSF2 were purified on CsCl gradients, digested with PvuII and then transcribed using T7 RNA polymerase. Transcription and 5' end labelling were as described before [35], except that rGMP was added to the transcription reaction to 2 mM final concentration to obtain a high yield of transcription product. ³²P-labelled transcripts were purified on 12% polyacrylamide/urea gels and used for structure probing as described [36].

(e) Competition experiment

The short competitor RNAs used in this experiment were prepared by *in vitro* transcription from pSF2 templates as described under (d). Following transcription, the reaction mixture was phenol:chloroform extracted, ethanol precipitated and the RNA taken up in a buffer containing 40 mM Tris–HCl pH 7.5, 10 mM NaCl, 5 mM DTT and 6 mM MgCl₂. 50 Units RNA Guard and 40 units DNase I were added per 100 μl and incubated for 15 minutes at 37 °C. The reaction mixture was then extracted with phenol:chloroform, the RNA passed over a Sephadex G-50 spun column (equilibrated with water) and concentrated by ethanol precipitation. The RNA was dissolved in water and quantitated by UV spectroscopy. In the competition experiments, the short competitor RNA was added to the reticulocyte lysate and the reaction pre-incubated for 5 minutes at 30 °C. After this time, a capped frameshift reporter mRNA was added and the incubation continued for a further 55 minutes. Translation products were analysed as described in (b).

RESULTS

Mutational analysis

Basic constructs. Construction of the reporter plasmid containing an 58 base pair insert corresponding to nt. 2320–2377 in the SRV-1 sequence [29] was done in two steps. First, sequences containing the slip site, the spacer region and the first 13 nucleotides of the pseudoknot (up till the A in S2, see Fig. 1) were introduced, resulting in plasmid pSF0. This plasmid codes for a 85 kD 0-frame product, and, in the case of a –1 frameshift at the SRV-1 sequence, a 28 kD protein. The latter product however, was not observed, indicating that additional sequences are needed (see Fig. 3). Upon insertion of the remaining part of the proposed frameshift site (clone pSF1), in addition to the

predicted 28 kD 0-frame product, a second translation product of 22 kD appears, resulting from the -1 frameshift. The frameshift efficiency of the *wild type* sequence of pSF1 is 23%. Frameshifting was exclusively in the -1 direction as no +1 products were detected. Plasmid pSF1, however, was not considered to be the ideal construct for the analysis of frameshift efficiency, since the -1 frameshift product is smaller in size than the non-frameshifted 0-frame product. In the case of pSF1, the

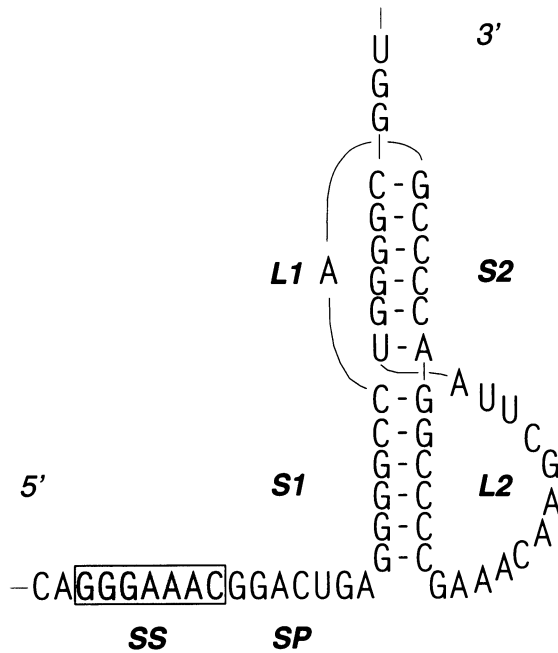


Figure 1. The proposed SRV-1 frameshift signal. Indicated are the slip site (SS), spacer region (SP) and the stems and loops (S1, S2, L1, and L2) of the pseudoknot.

accuracy of quantitation of the frameshifted protein could well be influenced by co-migrating products arising from premature translation termination events during *in vitro* translation. For this reason, an additional construct was made, pSF2, in which the first codon following the slip sequence in the 0-frame was changed to a stop codon (GGA → UGA). As can be seen in Fig. 3, the frameshift efficiency of pSF2 was identical to that of pSF1, the *wild type* sequence. This indicates that the frameshift must take place upstream of the inserted stop codon of pSF2 as expected if the frameshift would occur at the slippery sequence. In pSF2, the inserted stop codon reduces the size of the 0-frame product from 28 kD to 19 kD, which is smaller than the 22 kD frameshift product. Both proteins are now similar in mass and methionine content and premature termination events during translation or degradation of the products are less of a concern. All other mutants in this study are based upon this clone (pSF2) and have the 0-frame stop codon directly after the slip site. See Table 1 for a summary.

Two other mutants were made that have either a deletion or an insertion of a single C residue 19 nucleotides downstream of the pseudoknot. These clones have different -1 'exit' frames, and the corresponding products now have sizes of 28 kD (for pSF14) and 85 kD for pSF19, respectively. Antibodies raised against the N-terminal part of the PB2 gene react with both the 19 kD and the 85 kD products, while the 85 kD protein is only recognised by the C-terminal specific antibody (results not shown). This confirms that the 85 kD protein is a -1 frameshift product.

We will now present results for mutants in the three elements of the frameshift signal: slip site, spacer and pseudoknot.

Slip site mutants. The slippery sequence is one of the major determinants of the overall frameshift efficiency. To analyse its role in the SRV-1 *gag-pro* frameshift signal, we made mutations that either disrupted or modified the X XXY YYN motif. By changing G GGA AAC to G CGA AAC or G GGA CAC (clones

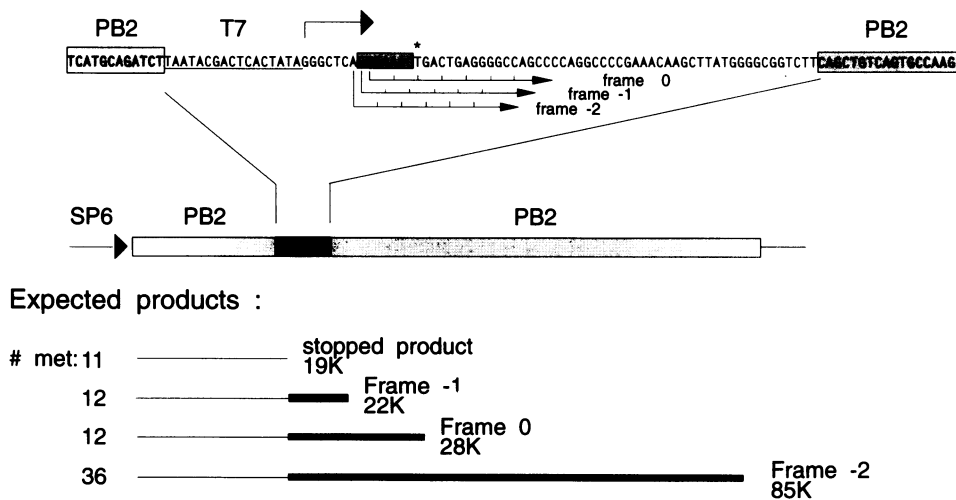


Figure 2. Features of plasmid pSF2. The plasmid contains the PB2 gene from influenza virus A/PR8/34 under the control of a SP6 RNA polymerase promoter [33]. In the PB2 gene is inserted the SRV-1 frameshift signal, immediately preceded by a T7 RNA polymerase promoter. The sequence is shown in the top of the figure. SP6 and T7 RNA polymerase promoters are shown as arrows, and the three reading frames are indicated. The stop codon introduced in pSF2 directly downstream of the slip site is indicated with an asterisk. Arrangement of stop codons downstream of the SRV-1 sequences is such that termination products in all three reading frames can be discerned. In the bottom half of the figure the size and the methionine content of each of the products is indicated. The plasmid also contains a β -lactamase gene conferring resistance to the antibiotic ampicillin, and the f1 origin of replication to allow the production of single stranded DNA for sequencing or mutagenesis following superinfection of cells harbouring the plasmid with phage R408 [38].

pSF28 and 29) frameshifting was reduced to 7 and 4% respectively, in agreement with the simultaneous slippage model (see Fig. 4 and Table 1). Changing the slip site to U UUA AAC (clone pSF18), the sequence of the IBV shifty heptanucleotide, increased frameshifting to 40%. This is in accordance with the higher efficiency found for the IBV frameshift signal (30%) and pFScass5 (40%, [33]). The IBV slip site is thus functional at a similar level in the SRV-1 pseudoknot context. It also shows that the IBV slip site is 'shifter' than that of SRV-1, and can be

supported by the SRV-1 pseudoknot to induce efficient frameshifting [20, 33].

The base at position 7 of the slip site (N in the X XXY YYN motif) was changed from C to the three other bases. This changes the type of tRNA as well as the interactions between the tRNA and messenger RNA in the A-site both before and after the slip. Changes here are thus likely to affect the frameshift efficiency. Indeed different frameshifting frequencies were found for all four bases, the efficiency decreasing from C (23%, pSF2) via A (11%, pSF17) and U (9%, pSF15) to G (6%, pSF16) (see Fig. 4 and Discussion).

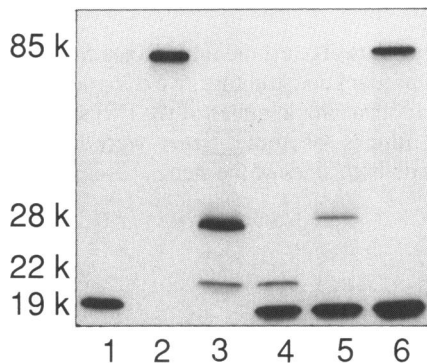


Figure 3. Analysis of basic pSF-clones containing half or the complete SRV-1 frameshift signal. Rabbit reticulocyte lysate translation products of mRNAs derived from BamHI digested pSF templates were separated on a 17.5% SDS-polyacrylamide gel and detected by fluorography. Lane 1: pSF30; lane 2: pSF0; lane 3: pSF1; lane 4: pSF2; lane 5: pSF14; lane 6: pSF19. The approximate size of the polypeptides is indicated.

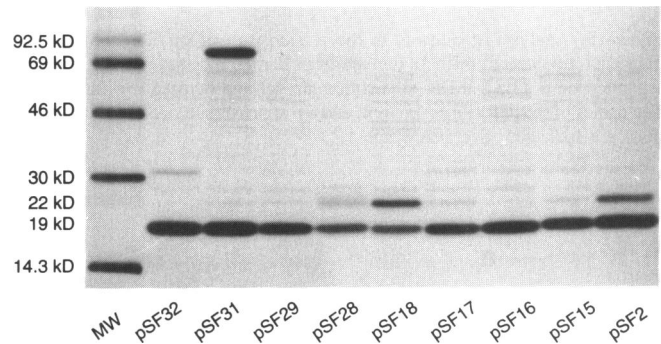


Figure 4. Analysis of mutants in the slip site and spacer region. The figure shows a fluorogram of reticulocyte lysate translation products of mRNAs derived from BamHI digested pSF templates separated on a 17.5% SDS-polyacrylamide gel. The names of the clones (bottom) and the approximate size of the polypeptides (MW, left) are indicated. See also Table 1.

Table 1.

name of clone	sequence	FS (%)
pSF0	SSSSSSS 11111 1L22222211 1111LLLLLL LLLLLL2222 22	0
pSF1	G	23
pSF2	uauagggcuc aGGGaAaCUg acugaggGGC caGcCCCagG CCcgaaca agcuuauGGG gCggucuca gcuguca	23
pSF4	CCG	6
pSF5	C GG	3
pSF6	CCG C GG	10
pSF7	GGG	3
pSF8	CCC	6
pSF9	GGG CCC	22
pSF10	C	12
pSF11	G	10
pSF12	C G	20
pSF14	see legend	22
pSF15	A	11
pSF16	U	9
pSF17	G	6
pSF18	UUU	40
pSF19	see legend	18
pSF28	C	7
pSF29	C	4
pSF31	UG ACUCGAGGGG	11
pSF32	-	12

In Table 1 the RNA sequence and frameshifting efficiencies of transcripts of the clones used in this study are given. The error in frameshifting efficiencies given is estimated to be 1%. Sequence changes are given with respect to pSF2, bases changed in the variants are indicated in capitals. Slip site, stem S1, stem S2 and the loops are indicated by S, 1, 2 and L respectively. Nucleotides not present in clone pSF0 and the deletion in pSF32 are indicated with a dash. The insertion of a C in pSF31 is shown in bold. Clones pSF14 and pSF19 were made by a deletion and an insertion respectively of a single C residue 19 bases downstream of the last base of the pseudoknot in pSF2.

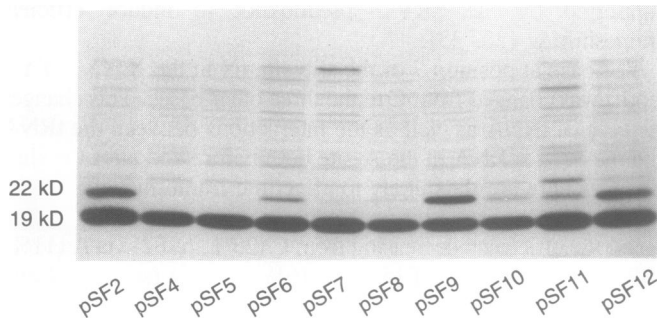


Figure 5. Analysis of mutants in the pseudoknot region. Reticulocyte lysate translation products of mRNAs derived from BamHI digested pSF templates were separated on a 17.5% SDS-polyacrylamide gel and detected by fluorography. The names of the pSF clones (bottom) and the approximate size of the polypeptides (left) are indicated. See also Table 1.

Spacer. In our computer analysis of frameshift signals we found that pseudoknots are usually located 5 to 8 bases downstream of the slip site. In the SRV-1 *gag-pro* frameshift signal the spacing is 7 bases [21]. To test the proximity requirements for this system we have made mutants with an insertion (pSF31) or deletion (pSF32) of one base in the spacer region. In both cases frameshift efficiency was lowered in comparison with the *wild type*; pSF31 gave 11 % and pSF32 12% frameshifting (see Fig. 4). Thus, for the SRV-1 *gag-pro* frameshift signal the optimal distance between slip site and pseudoknot is 7 bases as found in the *wild type*, although it can not be excluded that primary sequence effects also play a role (see Discussion).

Pseudoknot mutants. To test the importance for frameshifting of the predicted pseudoknot structure, we designed a set of mutants that changes basepairing in either of the two stem regions of the pseudoknot. Blocks of three bases were changed to their complements on both sides of the stems, resulting in clones pSF4

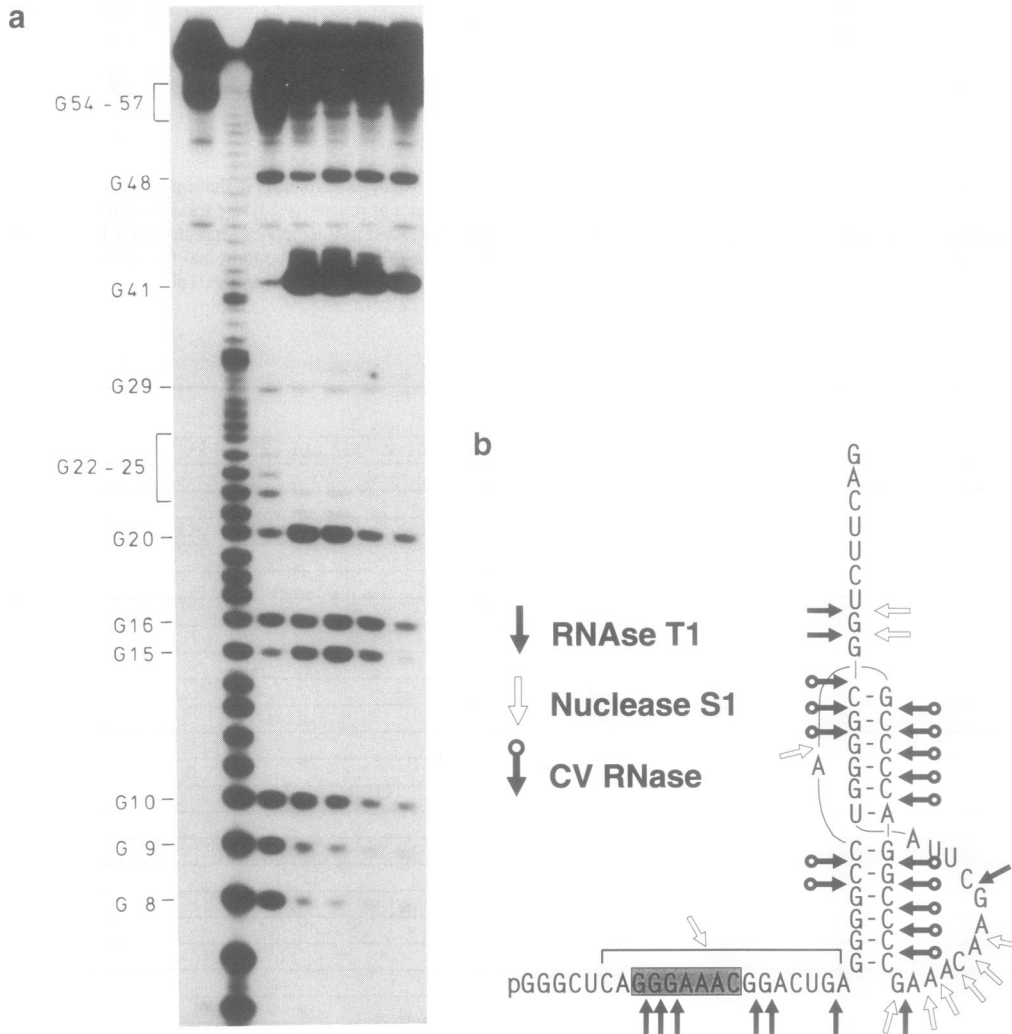


Figure 6. Probing results. **a.** RNase T1 analysis. Shown on the autoradiogram are 5' ³²P-labelled RNA fragments derived by T7 RNA polymerase transcription from PvuII digested pSF1 plasmid DNA (see Methods), and treated as follows: (from left to right) control lane, alkaline hydrolysis ladder, RNase T1 sequencing reaction under denaturing conditions, RNase T1 reactions at 0, 20, 37 and 50 °C. **b.** Summary of the probing results. Sites sensitive to endonucleases at 20 °C are indicated by arrows.

to pSF9 (see Fig. 5). Mutants pSF4 and pSF5 have lost the possibility of basepairing in stem S1 of the pseudoknot, and frameshifting is reduced from 23% to 6% and 3% respectively. In mutant pSF6 however, basepairing should be restored and stem S1 formed again through introduction of compensatory complementary base changes on the other side of the stem. Indeed in this case frameshifting was increased, although only modestly, to 10%. For stem S2, mutants pSF7 and pSF8 reduced frameshifting to 3% and 6% respectively, but in the double mutant pSF9 frameshifting was restored to a level of 22%, the *wild type* level. Mutants pSF10, pSF11 and pSF12 (see Fig. 5) were designed to test the presence of the top basepair in stem S2, and to see if the single A in loop L1 is sufficient to cross this stem. Indeed, the single mutants pSF10 and pSF11 have reduced frameshift efficiencies of 12% and 10% respectively. In the double mutant pSF12, once again frameshifting was restored, this time nearly to the *wild type* level (20%).

Structure probing. To confirm the proposed pseudoknot, we have directly analysed the secondary structure of RNA fragments transcribed from the pSF plasmids. The plasmids were linearized with PvuII and transcribed using the T7 RNA polymerase promoter positioned directly upstream of the inserted SRV-1 sequences. The transcripts thus obtained begin seven bases upstream of the shift site, and end nine bases after the pseudoknot. The results, as summarised in Fig. 6b, are in good agreement with the presence of the proposed pseudoknot. G residues proposed to be unpaired are sensitive towards the single strand specific RNase T1, but those involved in the formation of the two double helical regions are not attacked by the enzyme (see Fig. 6a). Also, nuclease S1, another single strand specific enzyme, gives cuts at positions that are supposed to be unpaired. The double-strand specific RNase V1 gives cuts in the two stems, again in good agreement with the structure prediction and the mutational analysis (data not shown). In Fig. 6b the structure analysis is shown for transcripts derived from the plasmid pSF1. Similar results were obtained when pSF2 derived RNAs were used as substrates (not shown).

Competition experiment. A possible role for the pseudoknot in the frameshift signal could be the binding of, or the interaction with a specific factor involved in the frameshift process. In an attempt to detect the presence of such a factor in the translational system we set up the following competition experiment. If -1 frameshifting is induced through binding of a hypothetical factor to the pseudoknot in the mRNA, adding increasing amounts of a short RNA fragment containing the SRV-1 frameshift signal to the translation mixture would compete for this factor and perhaps reduce the formation of the 22 kD frameshifted product.

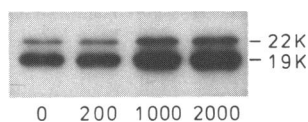


Figure 7. Competition experiment. Fluorogram of reticulocyte lysate translation products of mRNAs derived from BamHI digested pSF2 templates separated on a 17.5% SDS-polyacrylamide gel. Translations were done in the presence of increasing amounts of short RNA fragments containing the SRV-1 frameshift signal (see text). Molar excess of short RNA over mRNA (bottom) and the approximate size of the polypeptides (left) are indicated.

Alternatively, a possible interaction site on the ribosome for the pseudoknot part of the frameshift signal might be saturated by the competitor. The competitor RNA added was the same pSF2 derived T7 transcript as that used in the structure probing experiments and it was added in 200, 1000 and 2000-fold molar excess over the frameshift reporter (pSF2) mRNA in the translation mixture. However, even at the highest concentration we found no effect on frameshift efficiency, arguing against the involvement of such a factor (see Fig. 7). In contrast, a slight increase in overall translational efficiency was seen, possibly due to protection of the mRNA from RNases in the lysate by the large amount of added RNA.

DISCUSSION

In this study we have analysed the ribosomal frameshift signal in the *gag-pro* overlap region of SRV-1 *in vitro* in rabbit reticulocyte lysate. We have shown that 58 nucleotides from the SRV-1 overlap are sufficient to induce efficient translational frameshifting into the -1 frame ('leftward') within the heterologous context of the influenza PB2 gene, and that slip site, spacer and pseudoknot each play a role in determining the overall frequency of ribosomal frameshifting. The frameshift signal was also shown to be active in a wheat germ translation system with similar efficiency [16].

Slip site

The frameshift signal is formed by two elements: a seven base slip site and a pseudoknot structure located seven nucleotides downstream of that. In, 1988, Jacks and co-workers proposed the simultaneous slippage model for -1 ribosomal frameshifting in retroviruses [3], which also seems to hold for SRV-1 and other viruses with similar expression strategies for *pol*-like genes, such as the coronaviruses and a yeast ds RNA virus [5, 8]. Frameshift sites consist in general of a 7 nucleotide X XXY YYN motif, where X can be any base, Y is A or U and N is A, U or C. In the SRV-1 *gag-pro* overlap there is a G GGA AAC sequence that conforms to this consensus. Mutations that disrupt this motif (pSF28 and 29) abolish frameshifting, and modifying it affects frameshifting in a positive (pSF18) or negative way (pSF15, 16 and 17). Furthermore, the introduction of a stopcodon in the 0-frame in pSF1 leading to pSF2 shows that the actual shift site must lie upstream of that termination codon. Taken together, this strongly supports the likelihood that the G GGA AAC sequence is the *gag-pro* slip site in SRV-1. The frameshift takes place exclusively into the -1 direction, since the sizes of the synthesised proteins in each case correspond to 0 and -1 frame products, and no bands are observed at positions corresponding to +1 (or maybe -2) frameshift products. G GGA AAC has previously been shown to be the slip site in FIV with a comparable efficiency in a baculovirus expression system [28]. In contrast, in the context of the beet western yellows virus orf2-orf3 frameshift signal, G GGA AAC is only a weak signal [16].

The results for the mutants at position 7 of the slip site can be explained in terms of stability of the pre-slip codon-anticodon interactions. AAN codes for two amino acids: AAC and AAU are decoded by tRNA^{Asn}, AAA and AAG by tRNA^{Lys}. In mammals there are two different lysine-acceptor tRNAs present: tRNA^{Lys}_{U9UU} (U9=5-methoxycarbonylmethyl-2-thiouridine), which decodes AAA, and tRNA^{Lys}_{CUU} which decodes AAG. The codon AAG has a stronger interaction with its cognate tRNA,

tRNA, tRNA^{lys}_{CUU}, and thus is less slippery than AAA, in agreement with the experimental observations. The codons AAC and AAU are decoded by one tRNA, tRNA^{asn}_{QUU} (Q=queosine), which has a preference for AAU ([33] and references therein). AAC is thus expected to be more slippery, and indeed this is the case comparing clones pSF2 and pSF15. The frameshift efficiency in position 7 variants can therefore be explained by pre-slippage codon-anticodon stability, in agreement with the observations of others [33, 37].

Spacing

Spacing between slip site and pseudoknot is also important: pseudoknots are found four to eight bases after the heptanucleotide slip site [21]. Changing the distance from the original six bases found in the viral RNA reduced frameshift efficiency in the coronavirus IBV [20, 33]. For SRV-1, the optimal spacing seems to be the natural occurring seven bases, since changing this distance by only one base lowered the amount of frameshifting. It is not clear why there is a difference between the spacing requirements for these two viruses. A possible explanation might be that the primary sequence of the spacer determines its effective length, and that this is different for the two viruses. However, changing the nucleotide directly downstream of the slip site from G to U did not affect frameshifting efficiency. Furthermore, sequence comparison with the spacers from the slip sites of related viruses such as SRV-2, MPMV and Jaagsiekte retrovirus show that there is little primary sequence conservation in this region [30–32].

Pseudoknot

Analysis of –1 overlaps in viral RNAs (using computer structure predictions) showed that for a majority of the (potential) frameshift sites it was possible to form a pseudoknot four to eight bases downstream of the slip site. In this study, we have tested the structure as proposed for SRV-1 [21]. The necessity and existence of a pseudoknot is supported by the mutational analysis and structure probing experiments. The double mutant in stem S1, pSF6, which has the possibility to form the pseudoknot again, does not revert fully to *wild type* efficiency. The reason for this is unknown, but might be caused by specific structural features of S1.

Competition experiment

The variety in size and composition of the pseudoknots found at –1 frameshift sites is unsupportive of a specific cellular factor involved in the frameshift process. The lack of any effect of competing short pseudoknotted RNAs on frameshifting in the competition experiment described (see Results) is perhaps not surprising. The competing RNAs used however, did not include translation initiation signals and would not have been translated. Hence this type of assay cannot rule out the possibility that a specific factor is included in the frameshift process, but only in the context of the elongating ribosome. Further studies are required before the role of the pseudoknot in the frameshift process is understood fully.

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