

Factors affecting translation at the programmed –1 ribosomal frameshifting site of Cocksfoot mottle virus RNA *in vivo*

Katri Mäkeläinen^{1,2} and Kristiina Mäkinen^{1,2,*}

¹Department of Applied Biology, PO Box 27 and ²Institute of Biotechnology, PO Box 56, University of Helsinki, FIN-00014 Helsinki, Finland

Received January 11, 2005; Revised and Accepted March 31, 2005

ABSTRACT

The ratio between proteins P27 and replicase of Cocksfoot mottle virus (CfMV) is regulated via a –1 programmed ribosomal frameshift (–1 PRF). A minimal frameshift signal with a slippery U UUA AAC heptamer and a downstream stem–loop structure was inserted into a dual reporter vector and directed –1 PRF with an efficiency of $14.4 \pm 1.9\%$ in yeast and $2.4 \pm 0.7\%$ in bacteria. P27-encoding CfMV sequence flanking the minimal frameshift signal caused ~2-fold increase in the –1 PRF efficiencies both in yeast and in bacteria. In addition to the expected fusion proteins, termination products ending putatively at the frameshift site were found in yeast cells. We propose that the amount of premature translation termination from control mRNAs played a role in determining the calculated –1PRF efficiency. Co-expression of CfMV P27 with the dual reporter vector containing the minimal frameshift signal reduced the production of the downstream reporter, whereas replicase co-expression had no pronounced effect. This finding allows us to propose that CfMV protein P27 may influence translation at the frameshift site but the mechanism needs to be elucidated.

INTRODUCTION

The principal mechanism of translation is the accurate decoding of the triplet codon sequences in one reading frame of mRNA. Specific signals built into the mRNA sequences can cause deviations from this rule. Viruses exploit several translational ‘recoding’ mechanisms, including translational hopping, stop codon readthrough and programmed ribosomal frameshifting (PRF) [reviewed in (1,2)], for regulating the amount of proteins produced from their

polyproteins. For positive-stranded RNA viruses, –1 PRF is the prevailing recoding mechanism and an essential determinant of the stoichiometry of synthesized viral proteins. Most viral –1 PRF signals are regulating the production of replication-associated proteins. Depending on the virus, the efficiency of –1 PRF can vary between 1 and 40% (3), and changes in the efficiency can inhibit virus assembly and replication (4–6). Therefore, –1 PRF can be regarded as a potential target for antiviral agents (4,7). However, the development of efficient antiviral drugs is still hindered, since little is known about the *trans*-acting factors and the biophysical parameters affecting the –1 PRF efficiencies. Database searches have identified putative frameshift signals from a substantial number of chromosomally encoded eukaryotic mRNAs (8). Thus, –1 PRF may also have an impact on the complexity of the proteome of several eukaryotic organisms.

Two *cis*-acting signals, a slippery heptamer X XXY YYZ (the incoming reading frame indicated) and a downstream secondary structure, direct the slippage and are therefore essential for this event (9). –1 PRF takes place after the accommodation step in the slippery sequence by simultaneous slippage of both tRNAs into the overlapping –1 frame XXX YYY (9,10). The sequence of the heptamer allows post-slippage base-pairing between the non-wobble bases of the tRNAs and the new –1 frame codons of the mRNA. Downstream RNA secondary structures [reviewed in (11)] force the ribosomes to pause, and place the ribosomal A- and P-sites correctly over the slippery sequence (12). However, the pausing of the ribosomes is not sufficient for –1 PRF to occur (13); in fact, the duration of the halt does not necessarily correlate with the level of the –1 PRF observed (12). Crystallographic, molecular, biochemical and genetic studies suggest that a pseudoknot restricts the movement of the mRNA during the tRNA accommodation step of elongation by filling the entrance of the ribosomal mRNA tunnel (14). This restriction can be eased either by unwinding the pseudoknot, which allows the mRNA to move forward, or by a slippage of the mRNA one nucleotide backwards. Chemical agents such as

*To whom correspondence should be addressed. Tel: +358 9 19158342; Fax: +358 9 19158633; Email: kristiina.makinen@helsinki.fi

antibiotics, certain mutations in the translation apparatus, and in translation elongation factors that change the translation fidelity and kinetics, have been shown to influence -1 PRF efficiency [(10,15); reviewed in (16)].

The parameters known to contribute to the efficiency of -1 PRF are the sequence of the slippery heptamer, the downstream secondary structure, and the length and sequence of the spacer between the two *cis*-acting signals. Up- and downstream sequences such as termination codons in the vicinity of the -1 PRF signals, or even several kilobases away from them, can affect the -1 PRF efficiencies (3,17–22). A specific sequence in the Barley yellow dwarf virus (BYDV) 3' untranslated region (UTR), 4 kb downstream from the slippage site, is vital for -1 PRF (6,19). A stimulating effect is achieved through the formation of a tertiary structure, where complementary nucleotides from the 3' UTR base pair with a single-stranded bulge in the *cis*-acting stem-loop (6). Human immunodeficiency virus (HIV) was also shown to require a more complex secondary structure instead of a simple stem-loop for optimal -1 PRF *in vivo* (21,22). These investigations suggest that -1 PRF studies carried out with minimal frameshift signals may lead to inaccurate estimates of the stoichiometry of synthesized viral protein products during infection.

Cocksfoot mottle virus (CfMV; genus *Sobemovirus*) infects a few monocotyledonous plant species such as barley, oats and wheat. It has a monopartite, single-stranded, 4082 nt long, positive-sense RNA genome (23,24). The polyprotein of CfMV is translated from two overlapping open reading frames (ORFs) 2A and 2B by a -1 PRF mechanism (25). In this study, we wanted to determine the *in vivo* -1 PRF efficiency guided by the CfMV U UUA AAC heptamer and the stem-loop structure. In addition to the minimal signal (18), we decided to test the effect of flanking CfMV sequences for their ability to contribute to -1 PRF. We found that the surrounding viral sequences promoted more efficient -1 PRF than the minimal signal sequence *in vivo* when measured with the dual reporter vector system developed by Stahl *et al.* (26). Therefore, we carried out an expression pattern and deletion analysis to understand the molecular basis of the observed upregulation. In addition, we critically analysed the suitability of the implemented experimental system for this type of a recoding study. An interesting possibility is that the viral proteins produced via -1 PRF could regulate -1 PRF. This hypothesis was tested by co-expressing the CfMV proteins P27 and replicase together with the dual reporter vectors.

MATERIALS AND METHODS

Plasmid constructs

Three regions from the CfMV polyprotein ORFs (Figure 1A) were cloned into the NheI and BclI sites between the *lacZ* and the *luc* ORFs in pAC74 (26). This dual reporter vector was a generous gift from Dr J. Rousset of the Universite Paris-Sud, France. The inserted sequences 1602–1720 (A region), 1386–2137 (B region) and 1551–1900 (C region), were amplified by PCR using pAB-21 as a template (18). Primers were used to introduce NheI and BglII sites to the flanking ends of the inserts. Since NheI digestion removed *lacZ* ORF, it was reintroduced into the plasmids as a final cloning step. The resulting plasmids were named pAC-A, pAC-B and pAC-C.

Corresponding inframe controls, where one nucleotide was added in front of the slippery heptamer, were generated by PCR-based mutagenesis (Exsite, Stratagene) and named as pAC-Am, pAC-Bm and pAC-Cm, respectively. Deletion plasmids pAC-AB/ABm (1602–2137), pAC-AC/ACm (1602–1900), pAC-BA/BAm (1386–1720) and pAC-CA/CAm (1551–1720) were also generated. The target sequences are shown in Figure 1B. The base numbering refers to the CfMV genome as in (23). Transcription was driven from SV40 promoter. Plasmids encoded leucine (*LEU2*) and β -lactamase (ampicillin resistance) as selective markers. Plasmids were transformed into *Saccharomyces cerevisiae* H23 [MAT α hsp150::URA3 ura3-1 his3-11 15leu2-3 112trp1-1 ade2-1 can100]. Dual reporter plasmid pAC1789 and the inframe control pAC1790 containing a 53 bp sequence from the HIV-1 frameshift region (26) were used as a positive control for monitoring the -1 PRF efficiency.

To analyse the proteins produced during -1 PRF, *lacZ*-A/Am/B/Bm/C/Cm-*Fluc* fragments were cloned inframe with the N-terminal 6xhistidine-tag in pYES2/NT KpnI and XhoI sites (Invitrogen). Reporter fusions were amplified by PCR using pAC-A/Am, pAC-B/Bm or pAC-C/Cm as templates. The resulting plasmids were named pYES2/NT-A/Am, B/Bm and C/Cm. Protein expression was regulated from GAL1 promoter. Two CfMV encoded proteins, P27 (C-terminal end of ORF2A) and replicase (ORF2B), were cloned into pYES2 (Invitrogen). Translation initiation codons were introduced within the oligonucleotides during PCR. The resulting plasmids were named pYES-P27 and pYES-Rep. Control plasmids, which lacked the translation initiation codons were prepared by PCR-based mutagenesis (pYES-P27 Δ AUG and pYES-Rep Δ AUG) and the resulting plasmids were verified by sequencing. Plasmids encoded auxotrophic marker for uracil (URA3).

All cloning steps were performed using standard protocols. Plasmids were amplified either in *Escherichia coli* DH5 α or JM110, and purified with Qiagen columns. Inserts were verified by sequencing. Yeast transformations were done using the LiAc method (27), and transformants were selected on a synthetic minimal defined medium (SC) lacking the corresponding auxotrophic marker(s) encoded by the used plasmid(s). Bacteria (*E.coli* DH5 α) were grown in LB-medium containing ampicillin, whereas yeast cells were grown either in YPD, or in an SC medium. Protein expression from GAL1 promoter was repressed during growth at SC medium containing 2% glucose. Expression was induced by replacing glucose with 2% galactose and 1% raffinose.

Protein purification and analysis

Reporter fusions were expressed in *S.cerevisiae* INVSc1 (his3 Δ 1/his3 Δ 1, leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52) (Invitrogen) overnight. Protein fusions were purified in denaturing conditions using Ni-NTA agarose (Qiagen), and analysed in 6% SDS-PAGE gels. Proteins were visualized either by Coomassie staining, or by using antisera raised against the CfMV polyprotein region 1386–1724 encoding CfMV VPg (28). Protein antibody complexes were visualized with horseradish peroxidase-conjugated anti-rabbit antibodies (Sigma) and ECL chemiluminescent reagents (Amersham).

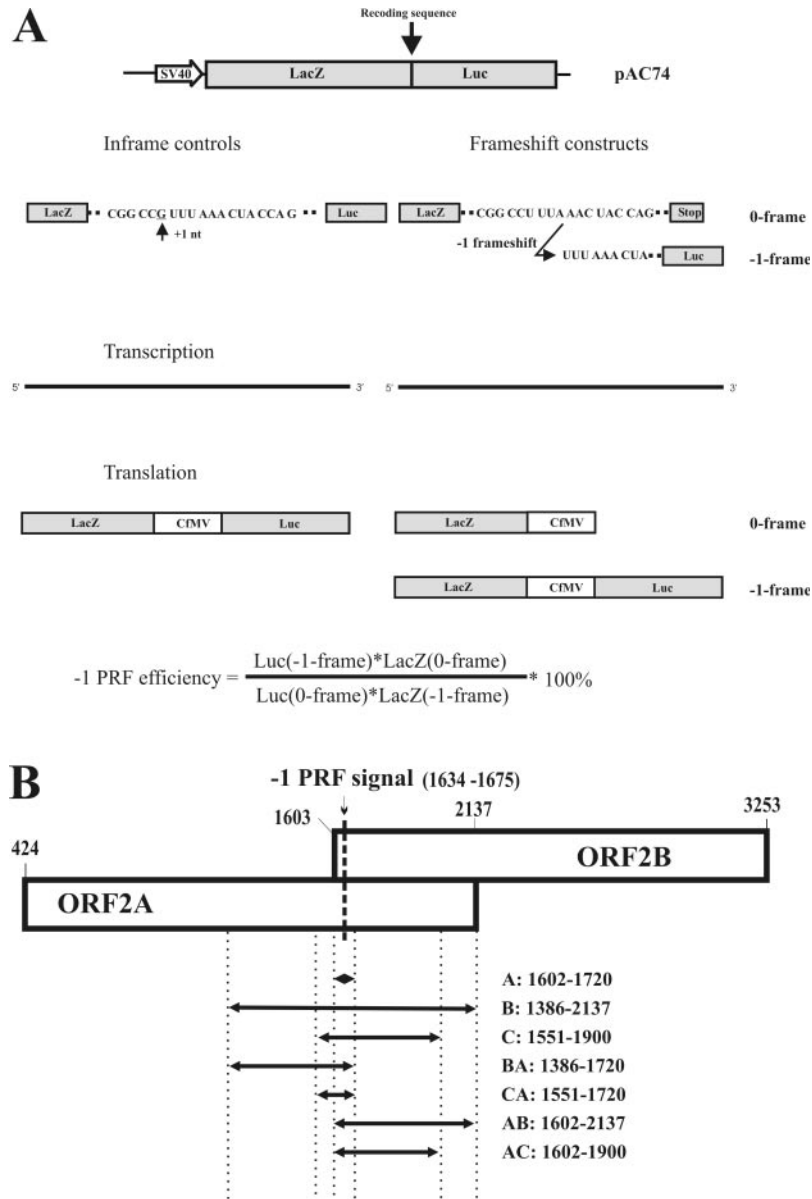


Figure 1. -1 PRF constructs based on the CfmV frameshift signal. (A) CfmV -1 PRF test and control sequences were cloned between the β -galactosidase (*LacZ*) and firefly luciferase (*Luc*) genes into a dual reporter vector pAC74 (26). Inframe control constructs had one extra nucleotide inserted in front of the slippery heptamer, which fused the reporters into the same reading frame. Thus, translation of the inframe control results in the production of a β -galactosidase-CfmV-firefly luciferase fusion. Translation of the test constructs in the incoming 0-frame yields a β -galactosidase-CfmV fusion, whereas -1 PRF produces a β -galactosidase-CfmV-firefly luciferase fusions identical to those produced from the inframe controls. -1 PRF efficiencies were calculated from the firefly luciferase activities after β -galactosidase normalization with the given formula. (B) CfmV polyprotein is encoded by two overlapping ORFs, 2A and 2B via -1 PRF. Sequence regions tested in the dual reporter vectors for their activity to promote -1 PRF are indicated. The numbering refers to the CfmV RNA sequence as published in (23).

Plasmids pYES-P27, pYES-Rep, pYES-P27 Δ AUG, pYES-Rep Δ AUG, or empty pYES2 were co-expressed with pAC-A or with the corresponding pAC-Am inframe control in *S.cerevisiae* EGY48 strain (MAT α , *ura3*, *trp1*, *his3*, 6lexAop-LEU2) (Invitrogen). Transformants were grown overnight in SC-Leu-Ura media in non-inducing conditions, and used to inoculate induction medium. Cells were harvested at late logarithmic phase. Expression of the CfmV proteins was confirmed by western blotting using polyclonal antisera against the CfmV ORF 2a and 2b proteins (28). Determining the enzymatic activities as described below monitored the effect of CfmV P27 and replicase on -1 PRF.

In vitro transcription and translation

For the *in vitro* analysis, the *lacZ*-gene of pAC-A/Am, -B/Bm and -C/Cm vectors was replaced with PCR-amplified *Renilla* luciferase (*Rluc*) gene from pRLnull vector (Promega). The resulting pACRF plasmids were used as templates for PCR in order to add T7 promoter upstream of the *Rluc* gene. These PCR products were used for RNA synthesis with RiboMax kit (Promega). Transcripts were treated with RQ1-DNase (Promega), purified with Qiagen RNeasy columns, and quantified spectrophotometrically. The integrity of the transcripts was checked in agarose gels. *In vitro* translations were carried

out in wheat germ extract (WGE) according to the manufacturer's protocols (Promega). Reactions were incubated in room temperature for 60 min, and stopped on ice prior to enzymatic measurements.

Enzymatic measurements

Cell cultures were started from at least three independent clones and grown until the late exponential phase. Cells were collected by centrifugation, frozen in liquid nitrogen and stored at -70°C . Bacterial cells were lysed by sonication (3×15 s), and yeasts by vortexing with glass beads (0.5 vol) in $+4^{\circ}\text{C}$ for 30 min. Lysates were cleared by centrifugation, and enzymatic activities were determined immediately. Total protein concentrations were measured by using a Bradford protein assay reagent (Bio-Rad). β -Galactosidase (LacZ) and firefly or *Renilla* luciferase (LUC or RUC) activities were measured with commercial kits from Promega according to the manufacturer's instructions. LacZ activity was determined as the colour intensity at A414 nm. Luciferase activities were measured as relative light units (RLUs) with luminometer (Biohit or ThermoLabsystems). -1 PRF efficiencies were calculated from normalized firefly luciferase activities with the following formula: $[(\text{LUC activity from the test construct})/(\text{LacZ or RUC activity from the test construct})]/[(\text{LUC activity from the inframe control})/(\text{LacZ or RUC activity from the inframe control})] \times 100\%$.

RESULTS

Dual reporter vectors

In CfMV, the motif for -1 PRF is the slippery heptamer U UUA AAC and a stem-loop structure 7 nt downstream (25). The efficiency of -1 PRF directed by CfMV *cis*-acting signals was assayed *in vivo* using a dual reporter vector system (Figure 1A). Since reporters are produced from one single mRNA, factors that affect the stability of the mRNA as well as the rate of translation initiation have a similar influence on the expression of both reporters, and these variations can be monitored as changes in the activity of the upstream reporter. We quantified -1 PRF by comparing the β -galactosidase normalized firefly luciferase activities derived from the test constructs via -1 PRF to those obtained from the inframe controls, in which identical β -galactosidase-CfMV-firefly luciferase fusions are produced without -1 PRF due to the added nucleotide in front of the slippery heptamer (see Figure 1A). Similar vectors have been shown to detect even small changes in the recoding efficiencies resulting from alterations in the *cis*- or *trans*-acting factors (26,29–32).

-1 PRF in yeast and bacteria

Three inserts of varied lengths from the CfMV polyprotein-encoding region (ORF2A/2B) were introduced between the two reporters (Figure 1B). The A-region, which at 119 bp was the shortest, represented approximately the minimal frameshift signal proven to be functional *in vitro* (18). The longest region was the B-insert. At 752 bp, it started from the 5'-terminus of the 12 kDa viral genome-linked protein (VPg) gene and continued to the end of ORF2A. This region encodes CfMV protein P27 with an unknown function (28). Since the minimal

requirements for the functional frameshift signal *in vivo* were not known, an intermediate 349 bp C-sequence was also selected for the analysis. A well-characterized 53 bp frameshift cassette derived from HIV-1 RNA was used as a positive control. Our results regarding the HIV -1 PRF efficiency, $0.7 \pm 0.1\%$ in bacteria, and $4.5 \pm 1.1\%$ in yeast (Figure 2), are corroborated by those published earlier (26,33,34) indicating that our dual reporter system was fully functional.

β -Galactosidase has been shown to retain its specific activity well, irrespective of the C-terminal fusions (35). This is important, since the first reporter serves to control

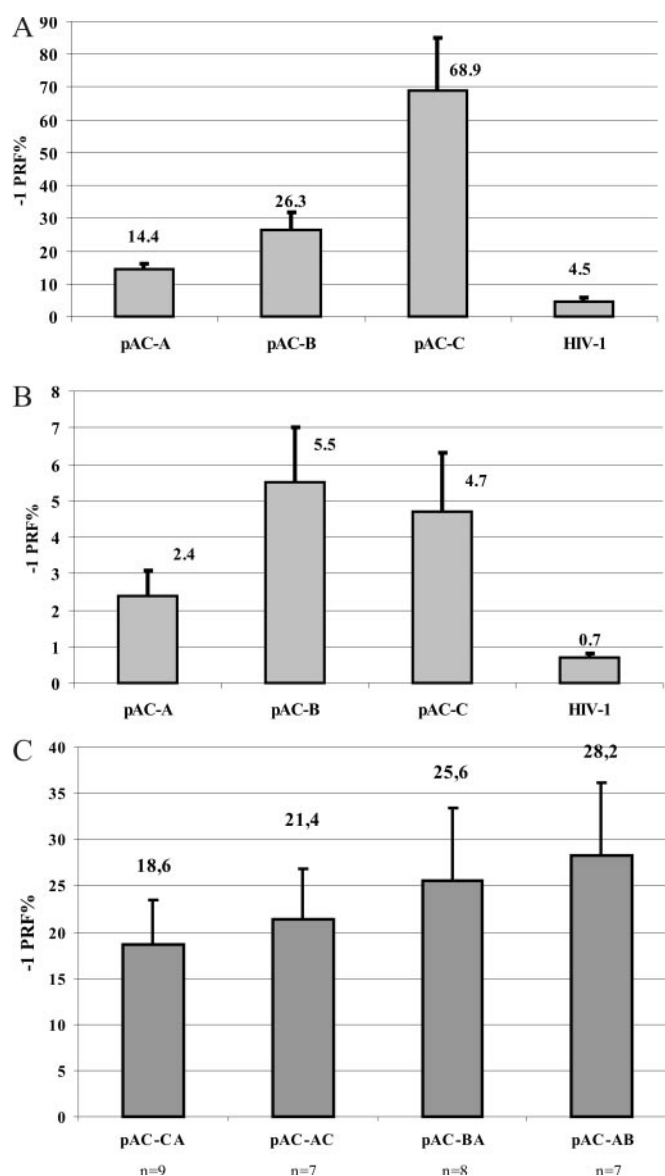


Figure 2. -1 PRF efficiencies in yeast and bacteria. -1 PRF efficiencies were calculated with enzymatic activities measured from (A) yeast and (B) bacteria. Dual reporters containing HIV-1 frameshift region (26) were used as a positive control. (C) To identify sequence(s) involved in directing the enhanced -1 PRF, up- or downstream regions flanking the minimal -1 PRF signal were deleted from pAC-B and pAC-C as indicated in Figure 1B. Deletion plasmids were analysed only in yeast. The mean values of -1 PRF efficiencies calculated from at least four independent experiments are presented. The error bars indicate the SD calculated from the mean values.

the variations among the abundance and translation rates of the studied mRNAs (26,30). In addition to changes in specific activities, heterologous fusions can cause alterations in the solubility and conformation, which can expose cryptic protease target sites and reduce the stability of the proteins (36). Therefore, for a reliable quantification of -1 PRF, it was important to test that equimolar amounts of fusions produced from the corresponding test and control constructs had similar enzymatic activities. Most inframe controls and the analogous test constructs had equal absolute β -galactosidase activities (Table 1). Comparable results were obtained, if activities were normalized with total protein concentration (data not shown). These results indicated that the length of the fusion as such did not affect the specific activities. The β -galactosidase activity from pAC-Am inframe control was also comparable to activity obtained from an empty pAC74, where this enzyme has no fusion (data not shown). This further supported the view that the few observed variations in the β -galactosidase activities more likely resulted from the changes in translatability or stability of the transcripts. In addition to pAC-Cm, two inframe controls pAC-Am and pAC-ACm showed $\sim 25\%$ lower β -galactosidase activities when compared to the equivalent test constructs (Table 1), indicating that the productivity from these constructs was reduced. Taken together, β -galactosidase seemed to fit well to be used as the first reporter and thus normalization factor in the *in vivo* experiments of this study.

CfMV frameshift signals generated significant -1 PRF in yeast. -1 PRF level measured from pAC-A was 3-fold higher than from HIV RNA (Figure 2A and B). The extent of -1 PRF directed by the minimal region A in yeast, $14.4 \pm 1.9\%$, was at the same level as that reported for the CfMV minimal frameshift signal *in vitro* (12.7%) (18). In contrast to our earlier *in vitro* observations (18), the longer CfMV sequences upregulated -1 PRF *in vivo*. In yeast, the level of upregulation was 2-fold for pAC-B, the -1 PRF frequency being 26.3%, and almost 5-fold for pAC-C resulting in efficiency close to 70% (Figure 2A), which is an extremely high value, if compared to the other values published earlier (3). CfMV frameshift signals directed -1 PRF at a lower level in bacteria than in yeast (Figure 2B). The extent of -1 PRF directed by region A in bacteria was $2.4 \pm 0.7\%$. As in yeast, the longest B region stimulated -1 PRF 2-fold in bacteria when compared with pAC-A. However, region C did not further improve -1 PRF, but programmed -1 PRF to similar levels as pAC-B, the percentages being $4.7 \pm 1.6\%$ for pAC-C and $5.5 \pm 1.5\%$ for pAC-B.

Table 1. β -Galactosidase activities derived from *S.cerevisiae* cells expressing -1 PRF test and inframe control dual reporter vectors

CfMV region	Frameshift construct	<i>n</i>	Inframe control	<i>n</i>
A	1.14 ± 0.27	9	0.89 ± 0.30	9
CA	0.89 ± 0.24	11	0.81 ± 0.22	11
AC	0.99 ± 0.39	11	0.73 ± 0.33	11
BA	1.13 ± 0.46	11	1.05 ± 0.34	11
C	0.74 ± 0.28	6	0.55 ± 0.28	6
AB	0.75 ± 0.26	11	0.77 ± 0.31	11
B	0.87 ± 0.32	7	0.85 ± 0.30	7

β -Galactosidase activities were determined from yeast lysates as colour intensity at A414 nm. Given are the mean \pm SD values from a given number of experiments ($=n$), each of which contained 2–4 replicates.

To identify the sequence(s) responsible for the enhancement of -1 PRF *in vivo*, a deletion analysis was carried out. The 5'- or the 3'-sequences flanking the A-region were deleted from pAC-B/Bm or pAC-C/Cm as indicated in Figure 1B, which generated vectors pAC-AB/ABm, pAC-BA/BAm, pAC-AC/ACm and pAC-CA/CAm. -1 PRF frequencies were determined in yeast (Figure 2C). Increased -1 PRF was observed in all deletion constructs in comparison to the -1 PRF directed by the A region. The BA and AB regions promoted -1 PRF as efficiently as the B region, whereas regions CA and AC were better than region A, but not as good as region B. In other words, the presence of nucleotides 1386–1720, or downstream nucleotides 1602–2137, was sufficient to increase -1 PRF to the level directed by the region B. Thus, the deletion analysis did not identify single specific sequence region as being responsible for the increased -1 PRF frequencies.

Protein analysis

The expression pattern of the test and control constructs was analysed to understand the basis for the observed upregulation in yeast. Cassettes containing the reporters and the studied intercistronic sequences were expressed and purified as N-terminal histidine fusions. This allowed us to capture all the N-terminally intact products. The affinity-purified proteins were separated in SDS-PAGE gels, and visualized either by Coomassie staining (data not shown), or by western blotting with the CfMV-specific anti-VPg antibodies. The expected β -galactosidase-CfMV fusions terminating at the end of the 0-frame in the test constructs were detected. Also, the longer transframe β -galactosidase-CfMV-firefly luciferase fusion proteins were present in both the test and the inframe constructs (Figure 3). Comparison of the Coomassie-stained gels with the western blots revealed that the antisera recognized the products terminating at the CfMV-encoding regions better than the transframe products. Furthermore, the small size of the CfMV-specific region in the pYES2/NT-Am decreased the

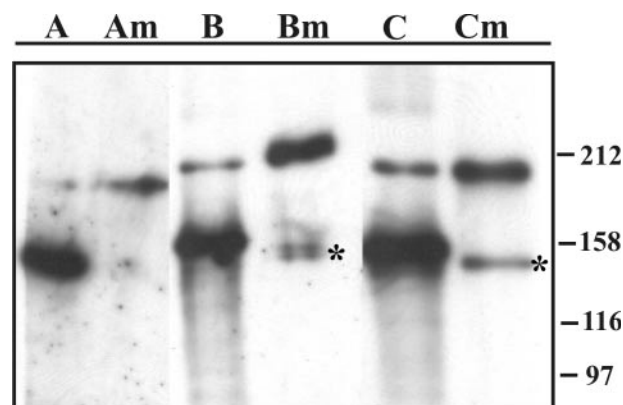


Figure 3. Proteins produced from -1 PRF test and control constructs. Western blot analysis of fusion proteins produced via -1 PRF recognized proteins terminating at the CfMV-encoding region. N-terminally tagged fusion proteins were purified from yeast cells expressing -1 PRF test and inframe control constructs by using Ni-NTA agarose and detected with antisera raised against CfMV polyprotein region 1386–1724 (28). The calculated molecular weights of the β -galactosidase-CfMV-firefly luciferase fusion proteins were 183 kDa (A/Am), 206 kDa (B/Bm) and 191 kDa (C/Cm). β -Galactosidase-CfMV 0-frame products had calculated sizes of 122 kDa (A), 145 kDa (B) and 130 kDa (C). Asterisks indicate the putative premature termination products.

binding of the antibodies to these inframe control fusions. Thus, this data were not suitable for quantitative analysis of -1 PRF. Interestingly, an additional protein, which reacted with CfMV-specific antisera, was co-purified from the cells expressing pYES2/NT-Bm and pYES2/NT-Cm inframe controls (Figure 3). The size of these fusions suggested that translation had terminated approximately at the site for -1 PRF signals. If such putative termination products were also present in cells expressing the test constructs, the correctly terminated 0-frame products in the western blots masked these products.

A closer look at the absolute β -galactosidase and firefly luciferase activities revealed that firefly luciferase expression from pAC-Cm was clearly reduced (data not shown). In fact, expression from the inframe control was comparable to the corresponding pAC-C test construct. This was also obvious when the firefly luciferase activities were normalized with the total protein amount. After setting the activity from pAC-Am to a relative value of one, the corresponding values from pAC-Bm and pAC-Cm were 0.80 and 0.28. Although the β -galactosidase measurements (Table 1) suggested that the overall translatability of the pAC-Cm mRNA was also reduced to some extent, it explained the decrease in firefly luciferase expression only partially. In the light of these findings, the extremely high -1 PRF frequency estimate calculated for the C-region could be explained with more frequent translation termination at the frameshift signals of the pAC-Cm mRNA, which reduced firefly luciferase activity in relation to β -galactosidase.

-1 PRF *in vitro*

-1 PRF was also assayed *in vitro* in WGE. Although *LacZ*-encoding gene is suitable for the *in vivo* studies, it is an unsuitable first reporter for the *in vitro* determination of -1 PRF efficiencies due to its big size (30). In good agreement with this, we observed several unexpected products in the *in vitro* translations programmed with *LacZ*-CfMV-*luc* mRNAs (data not shown). *Renilla* luciferase has been shown to retain its specific activity irrespective of the C-terminal fusions (30). Therefore, we decided to use *Rluc*-CfMV-*luc* transcripts to determine the -1 PRF efficiencies in the cell-free system. First, we verified the suitability of *Renilla* luciferase for the intended *in vitro* experiments as described in (30). Transcripts encoding monocistronic *Renilla* luciferase and *Renilla* luciferase fused to firefly luciferase (*Rluc*-*Am*/*Cm*-*luc*) were mixed in different ratios and used to program the *in vitro* translations. Increasing concentrations of transcripts encoding the *Rluc*-*Am*-*luc* fusion resulted in linearly growing firefly luciferase activities. At the same time *Renilla* luciferase activities remained constant, which showed that its enzymatic activity was not sensitive to the C-terminal fusions (Figure 4A). Similar results were obtained with *Rluc*-*Cm*-*luc* mRNA (data not shown). -1 PRF efficiencies were then determined with transcripts that contained CfMV regions A, B and C, and their corresponding inframe controls. In all cases, slightly higher -1 PRF frequencies were obtained than *in vivo*. In nice correlation with the *in vivo* results, enhanced -1 PRF was observed with the region B, although the effect was weaker than *in vivo*. In this context, region C did not differ from the minimal region A in its capacity to program -1 PRF (Figure 4B).

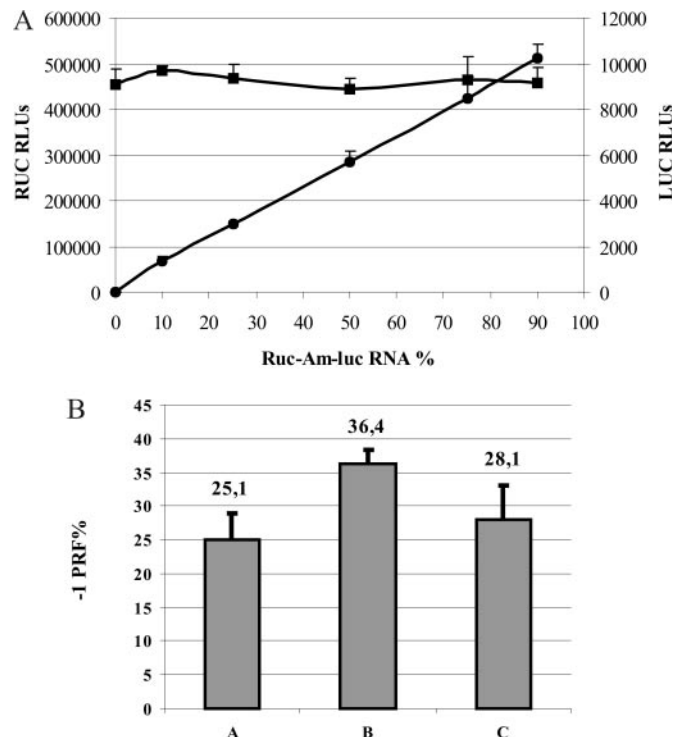


Figure 4. -1 PRF efficiency *in vitro*. (A) The suitability of *Renilla* luciferase for internal control usage was tested by studying changes in its specific activity as a result of C-terminal fusions. *In vitro* translations were programmed with 400 ng of RNA mixture, which contained known proportions of RNAs encoding *Renilla* luciferase without a fusion and *Renilla* luciferase fused to Am inframe control sequence and firefly luciferase gene (*Rluc*-*Am*-*Luc*). Total reaction volume was 8 μ l. *Renilla* luciferase (rectangles) and firefly luciferase (circles) activities were measured as relative light units (RLUs), and plotted against the *Rluc*-*Am*-*Luc* mRNA content in the mixture shown as percentages. Mean activities and the SD values from at least four translations are shown. (B) *In vitro* -1 PRF frequencies were determined with dual luciferase RNAs containing CfMV regions A/Am, B/Bm and C/Cm as the intergenic regions. Translations were performed in triplicate with 64 ng/ μ l of RNA. Results represent an average -1 PRF efficiency calculated from three experiments. Error bars show the SD values calculated from the averages. SD values of the firefly luciferase activities normalized with *Renilla* luciferase were <15% of the average.

Effect of CfMV proteins P27 and replicase on -1 PRF

The ratio between the CfMV P27 and replicase is regulated by -1 PRF during CfMV infection (28). We studied whether these proteins could regulate the -1 PRF process. P27, replicase, or an empty expression vector was co-expressed in yeast together with the dual reporter vectors containing the minimal -1 PRF test and inframe control regions as intergenic sequences (pAC-A and -Am). P27 and replicase expression was verified by a western blot analysis (Figure 5). A faint band having nearly the same mobility as the replicase was detected in cells grown under repressing conditions. However, due to the small size difference, this protein was not regarded as replicase.

Enzymatic activities were measured from yeast lysates prepared from induced cultures. Measurements showed comparable levels of β -galactosidase in all the samples, indicating that P27 or replicase expression did not affect the stability of the dual reporter mRNA or the translatability of the first reporter (Table 2). The effect of P27 or replicase expression

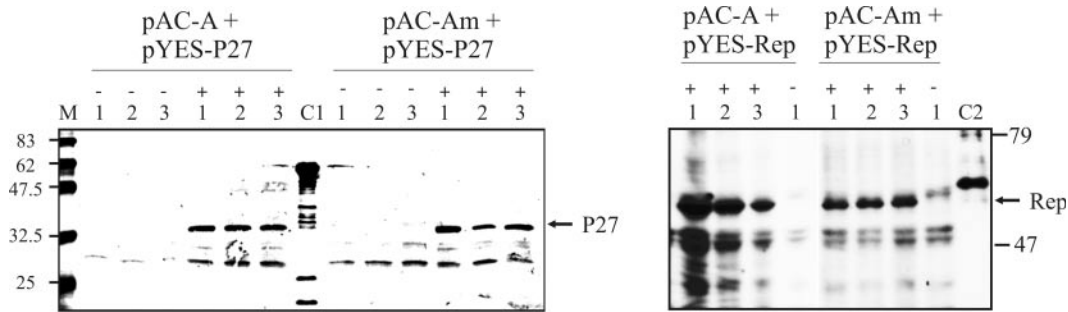


Figure 5. Co-expression of CfMV P27 or replicase simultaneously with the minimal frameshift signal construct pAC-A or the corresponding inframe control pAC-Am in yeast. Yeast total protein samples were separated in 12% SDS-PAGE gels, transferred onto PVDF membranes, and immunocomplexes detected by ECL chemiluminescent system. CfMV P27 expression was verified by western blotting with antisera raised against ORF2A (A), and CfMV replicase expression was detected with antisera raised against ORF2B (B). Abbreviations: -, repressed; +, induced; C1, pMAL-VPg ~53 kDa; and C2, baculovirus expressed CfMV replicase.

Table 2. β -Galactosidase and firefly luciferase/ β -galactosidase activities from co-expression experiments

	LacZ	Luc/LacZ	Number of experiments
-1 PRF constructs			
A + Yes	0.56 \pm 0.09	8.58 \pm 1.63	n = 4
A + P27	0.50 \pm 0.09	6.06 \pm 1.01	n = 4
A + Rep	0.55 \pm 0.11	10.65 \pm 1.92	n = 4
Inframe controls			
Am + Yes	0.52 \pm 0.10	58.56 \pm 9.4	n = 4
Am + P27	0.50 \pm 0.09	26.99 \pm 7.2	n = 4
Am + Rep	0.49 \pm 0.11	56.66 \pm 8.3	n = 4

Enzymatic activities were measured from three parallel yeast lysate samples, where CfMV P27, CfMV replicase, or empty expression vector was co-expressed simultaneously with the dual reporter vectors pAC-A and pAC-Am. Cells were grown under inducing conditions. Results show an average \pm SD from a number of experiments indicated.

was monitored by comparing the reporter activity ratios to those measured from cells harbouring the empty expression plasmids (Table 2). Co-expression of CfMV replicase did not affect the normalized firefly luciferase expression (LUC/LacZ) from the inframe control, whereas slightly increased luciferase expression from the test construct was observed. In contrast, P27 expression reduced firefly luciferase expression both from the test and the inframe constructs. The effect was stronger in the presence of inframe control as normalized firefly luciferase levels reached only 54% of expression measured from the empty vector control.

To verify that the observed differences in firefly luciferase production depended on the studied CfMV proteins, we co-expressed the dual reporter vectors with plasmids having the first translation initiation codons of P27 and replicase deleted (pYES-P27 Δ AUG and pYES-Rep Δ AUG). Western blot analysis with antisera against ORF2A or 2B did not detect any proteins produced from these vectors (data not shown). The obtained LUC/LacZ ratios were compared to those measured from cells expressing the CfMV proteins (pYES-P27 or pYES-Rep). LUC/LacZ ratios measured from cells expressing replicase were slightly lower than the ratios calculated from cells harbouring pYES-Rep Δ AUG plasmids, being ~90% when co-expressed with pAC-A and ~84% when co-expressed with pAC-Am. In the presence of P27, LUC/LacZ ratio of pAC-A reached ~81% of expression measured

from cells transformed with pYES-P27 Δ AUG. Again the effect of P27 expression was more evident with pAC-Am inframe control as P27 expression reduced LUC/LacZ ratio to half (~48%) when compared to the corresponding value measured from the cells harbouring pYES-P27 Δ AUG. This verified that CfMV P27 was able to reduce the downstream reporter expression from dual reporter mRNAs. Since CfMV P27 had a proportionally stronger effect to firefly luciferase production from the inframe control mRNAs in comparison to the test mRNAs (Table 2), the calculated -1 PRF efficiency increased from 14.7 to 22.4%.

DISCUSSION

Since -1 PRF studies are affected by a huge number of different parameters, it is not an easy task to determine the real ratio between the proteins produced via this mechanism *in vivo*. However, in viral systems, the efficiency of -1 PRF is an essential determinant of the stoichiometry of synthesized viral protein products, which must be rigidly maintained for efficient propagation of the virus. For example, frameshifting in retroviruses determines the ratio of structural (Gag) to enzymatic (Gag-Pol) proteins, and plays a critical role in viral particle assembly (5). In this study, the capacity of CfMV frameshift signals to direct efficient -1 PRF was analysed *in vivo* by using dual reporter vectors. The length of the CfMV sequence clearly affected the actual efficiency percent *in vivo*. The PRF efficiency was elevated when longer viral sequences were directing the -1 PRF, but the deletion analysis did not identify any specific region as being solely responsible for the enhancement. Up- and downstream sequences nearby or far away from the *cis*-acting signals have been reported to enhance -1 PRF in other viruses, such as HIV, human T-cell leukaemia virus and BYDV (6,19,20). Also out-of-frame stop codons have been shown to influence -1 PRF frequency *in vitro* in retroviruses (17) and in CfMV (18). A study on the spacer sequences located between the *cis*-acting signals showed that high slippage frequencies were obtained when the first three nucleotides were G/U, G/A and G/A, the first two being the most important (37). In CfMV, the spacer starts with UAC, which partially explains the capacity of the CfMV sequence to promote high slippage levels. In this study, the observed enhancement of -1 PRF was, however, caused by sequences that were not in the immediate vicinity of the

slippery sequence thus indicating that CfMV sequences further away also have an influence on the level of frameshifting *in vivo*. We conclude that the most reliable estimates for -1 PRF and consequently for the amount of replicase versus the 0-frame translation product P27 can be obtained only by using the full-length viral sequences. In reality, such a study would however be hampered by the non-quantitative nature of the western blot analysis, the presence of different polyprotein processing intermediates, and the differences in the stabilities of the end products in the infected cells.

The overall competence of CfMV signals to direct -1 PRF was high, when compared to related plant viruses, such as Potato leaf roll virus and BYDV. -1 PRF values of $\sim 1\%$ have been reported for these viruses when measured with reporter-based assays (6,38). We can hypothesize that one reason for the high efficiency is the slippery tRNA^{Asn} encoding the AAC triplet of the CfMV heptamer. Equal U UUA AAC slippery heptamer has been measured to induce 20–40% of -1 PRF in a diversity of animal viruses [(39); reviewed in (3)]. The low fitness of CfMV -1 PRF signals in bacteria is in agreement with the poor functioning of the eukaryotic slippery heptamers of the order X XXA AAC in prokaryotes (40–42). IBV RNA, having an identical shifty heptamer, has been shown to direct -1 PRF at similar 2–3% level in bacteria (41). A recent study reported that XXXAAAC heptamers dictate -1 PRF to occur via the slippage of two adjacent tRNAs placed over the heptamer, irrespective of whether the host is an eukaryote or a prokaryote (42). Therefore, the inability of prokaryotic translation systems to direct efficient -1 PRF from this heptamer is not an inherited property of prokaryotic tRNA^{Asn}, but results from differences in the ribosomes (42).

Paused ribosomes can pass the -1 PRF site by -1 frameshifting, resumption of 0-frame translation, or termination (43). Transient polypeptide intermediates that result from the pausing of ribosomes in the slippery sequences have been observed during IBV and *S.cerevisiae* L-A virus polyprotein synthesis (12,13,43,44). A pseudoknot structure formed by IBV mRNA causes a translational pause at fixed position upstream the secondary structure regardless of whether the slippery heptamer is present or absent (12). Based on the findings of this study, we propose that also here a certain percent of ribosomes stalled at the secondary structure of the frameshift site in our inframe control and test mRNAs in yeast, and this led to the prematurely terminated products observed with the inframe control constructs pAC-Bm and -Cm. Although not unambiguously proven by this study, high frequency of termination of translation especially at the frameshift site of the pAC-Cm mRNA would nicely explain the extremely high calculated -1 PRF efficiency.

Factors that change the translation fidelity and kinetics have been shown to influence -1 PRF efficiency [(10,15); reviewed in (16)]. Autoregulation of $+1$ frameshifting by mammalian ornithine decarboxylase antizyme has been reported (45). This mechanism allows modulation of frameshifting frequency according to the cellular concentration of polyamines. One could speculate that such a regulation mechanism could also be useful to adjust the amounts of the replication-associated proteins to match the requirements of different phases in viral replication cycle. This hypothesis was studied by expressing CfMV proteins P27 and replicase together with pAC-A and pAC-Am in yeast cells. Since β -galactosidase

production remained constant regardless of the presence or absence of CfMV proteins, they did not interfere with translation initiation from pAC-A/Am mRNAs *per se*. However, P27 expression caused a reduction in the firefly luciferase production especially from the inframe control, whereas replicase production only slightly increased the firefly luciferase production from pAC-A, but not from pAC-Am. Since replicase expression had only a faint effect on the normalized firefly luciferase production via -1 PRF, our conclusion is that CfMV replicase had no pronounced effect on translation at the frameshift site. Co-expression of the non-translatable form of P27 with the dual reporter vectors verified that P27 truly affected firefly luciferase expression on the protein level. Therefore, we propose that CfMV protein P27 may influence translation at the frameshift site. If CfMV P27 indeed interferes with viral protein synthesis during CfMV infection, the mechanism, its specificity and the possible biological role needs to be elucidated in the future.

ACKNOWLEDGEMENTS

We would like to thank Dr Jean-Pierre Rousset of the Université Paris-Sud, France, for the plasmids pAC74, pAC1789 and pAC1790, Dr Deyin Guo for helping with the yeast work, Drs Mikko Frilander and Konstantin Ivanov for critical reading of the manuscript, as well as Jimmy Lucchesi and Prof. Mart Saarma for valuable discussions. This work was supported by the National Technology Agency of Finland (grants 40673/98, 40680/01, 40014/04), the University of Helsinki (grant 2109007) and the Academy of Finland (grants 52265, 206870). Funding to pay the Open Access publication charges for this article was provided by the National Technology Agency of Finland.

Conflict of interest statement. None declared.

REFERENCES

- Farabaugh, P.J. (1996) Programmed translational frameshifting. *Microbiol. Rev.*, **60**, 103–134.
- Gesteland, R.F. and Atkins, J.F. (1996) Recoding: dynamic reprogramming of translation. *Annu. Rev. Biochem.*, **65**, 741–768.
- Brierley, I. (1995) Ribosomal frameshifting viral RNAs. *J. Gen. Virol.*, **76**, 1885–1892.
- Dinman, J.D. and Wickner, R.B. (1992) Ribosomal frameshifting efficiency and *gag/gag-pol* ratio are critical for yeast M1 double-stranded RNA virus propagation. *J. Virol.*, **66**, 3669–3676.
- Hung, M., Patel, P., Davis, S. and Green, S.R. (1998) Importance of ribosomal frameshifting for human immunodeficiency virus type 1 particle assembly and replication. *J. Virol.*, **72**, 4819–4824.
- Barry, J.K. and Miller, W.A. (2002) A -1 ribosomal frameshift element that requires base pairing across four kilobases suggests a mechanism of regulating ribosome and replicase traffic on a viral RNA. *Proc. Natl Acad. Sci. USA*, **99**, 1133–1138.
- Dinman, J.D., Ruiz-Echevarria, M.J. and Peltz, S.W. (1998) Translating old drugs into new treatments: ribosomal frameshifting as a target for antiviral drugs. *Trends Biotechnol.*, **16**, 190–196.
- Hammell, A.B., Taylor, R.C., Peltz, S.W. and Dinman, J.D. (1999) Identification of putative programmed -1 ribosomal frameshift signals in large DNA databases. *Genome Res.*, **9**, 417–427.
- Jacks, T., Madhani, H.D., Masiarz, F.R. and Varmus, H.E. (1988) Signals for ribosomal frameshifting in the Rous sarcoma virus *gag-pol* region. *Cell*, **55**, 447–458.
- Dinman, J.D., Ruiz-Echevarria, M.J., Czaplinski, K. and Peltz, S.W. (1997) Peptidyl-transferase inhibitors have antiviral properties by altering programmed -1 ribosomal frameshifting

- efficiencies: development of model systems. *Proc. Natl Acad. Sci. USA*, **94**, 6606–6611.
11. Giedroc, D.P., Theimer, C.A. and Nixon, P.L. (2000) Structure, stability and function of RNA pseudoknots involved in stimulating ribosomal frameshifting. *J. Mol. Biol.*, **298**, 167–185.
 12. Kontos, H., Naphthine, S. and Brierley, I. (2001) Ribosomal pausing at a frameshifter RNA pseudoknot is sensitive to reading phase but shows little correlation with frameshift efficiency. *Mol. Cell. Biol.*, **21**, 8657–8670.
 13. Tu, C., Tzeng, T.-H. and Bruenn, J.A. (1992) Ribosomal movement impeded at a pseudoknot required for frameshifting. *Proc. Natl Acad. Sci. USA*, **89**, 8636–8640.
 14. Plant, E.P., Muldoon Jacobs, K.L., Harger, J.W., Meskauskas, A., Jacobs, J.L., Baxter, J.L., Petrov, A.N. and Dinman, J.D. (2003) The 9-Å solution: how mRNA pseudoknots promote efficient programmed –1 ribosomal frameshifting. *RNA*, **9**, 168–174.
 15. Meskauskas, A., Harger, J.W., Muldoon Jacobs, K.L. and Dinman, J.D. (2003) Decreased peptidyltransferase activity correlates with increased programmed –1 ribosomal frameshifting and viral maintenance defects in the yeast *Saccharomyces cerevisiae*. *RNA*, **9**, 982–992.
 16. Harger, J.W., Meskauskas, A. and Dinman, J.D. (2002) An ‘integrated model’ of programmed ribosomal frameshifting. *Trends Biochem. Sci.*, **27**, 448–454.
 17. Honda, A. and Nishimura, S. (1996) Suppression of translation frameshift by upstream termination codon. *Biochem. Biophys. Res. Commun.*, **221**, 602–608.
 18. Lucchesi, J., Mäkeläinen, K., Merits, A., Tamm, T. and Mäkinen, K. (2000) Regulation of –1 ribosomal frameshifting directed by Cocksfoot mottle sobemovirus genome. *Eur. J. Biochem.*, **267**, 3523–3529.
 19. Paul, C.P., Barry, J.K., Dinesh-Kumar, S.P., Brault, V. and Miller, W.A. (2001) A sequence required for –1 ribosomal frameshifting located four kilobases downstream of the frameshift site. *J. Mol. Biol.*, **310**, 987–999.
 20. Kim, Y.G., Maas, S. and Rich, A. (2001) Comparative mutational analysis of *cis*-acting RNA signals for translational frameshifting in HIV-1 and HTLV-2. *Nucleic Acids Res.*, **29**, 1125–1131.
 21. Dinman, J.D., Richter, S., Plant, E.P., Taylor, R.C., Hammell, A.B. and Rana, T.M. (2002) The frameshift signal of HIV-1 involves a potential intramolecular triplex RNA structure. *Proc. Natl Acad. Sci. USA*, **99**, 5331–5336.
 22. Dulude, D., Baril, M. and Brakier-Gingras, L. (2002) Characterization of the frameshift stimulatory signal controlling a programmed –1 ribosomal frameshift in the human immunodeficiency virus type 1. *Nucleic Acids Res.*, **30**, 5094–5102.
 23. Mäkinen, K., Tamm, T., Näss, V., Truve, E., Puurand, Ü, Munthe, T. and Saarma, M. (1995) Characterization of cocksfoot mottle sobemovirus genomic RNA and sequence comparison with related viruses. *J. Gen. Virol.*, **76**, 2817–2825.
 24. Ryabov, E.V., Krutov, A.A., Novikov, V.K., Zhelenzikova, O.V., Morozov, S.Y. and Zavriev, S.K. (1996) Nucleotide sequence of RNA from the sobemovirus found in infected cocksfoot shows a luteovirus-like arrangement of the putative replicase and protease genes. *Phytopathology*, **86**, 391–397.
 25. Mäkinen, K., Näss, V., Tamm, T., Truve, E., Aaspõllu, A. and Saarma, M. (1995) The putative replicase of the cocksfoot mottle sobemovirus is translated as a part of the polyprotein by –1 ribosomal frameshift. *Virology*, **207**, 566–571.
 26. Stahl, G., Bidou, L., Rousset, J.-P. and Cassan, M. (1995) Versatile vectors to study recoding: conservation of rules between yeast and mammalian cells. *Nucleic Acids Res.*, **23**, 1557–1560.
 27. Gietz, D., St Jean, A., Woods, R.A. and Scheistl, R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, **25**, 1425.
 28. Mäkinen, K., Mäkeläinen, K., Arshava, N., Tamm, T., Merits, A., Truve, E., Zavriev, S. and Saarma, M. (2000) Characterization of VPg and the polyprotein processing of Cocksfoot mottle virus (genus *Sobemovirus*). *J. Gen. Virol.*, **81**, 2783–2789.
 29. Bidou, L., Stahl, G., Grima, B., Liu, H., Cassan, M. and Rousset, J.-P. (1997) *In vivo* HIV-1 frameshifting efficiency is directly related to the stability of the stem–loop stimulatory signal. *RNA*, **3**, 1153–1158.
 30. Grentzmann, G., Ingram, J.A., Kelly, P.J., Gesteland, R.F. and Atkins, J.F. (1998) A dual-luciferase reporter system for studying recoding signals. *RNA*, **4**, 479–486.
 31. Bidou, L., Stahl, G., Hatin, I., Namy, O., Rousset, J.-P. and Farabaugh, P.J. (2000) Nonsense-mediated decay mutants do not affect programmed –1 frameshifting. *RNA*, **6**, 952–961.
 32. Harger, J.W. and Dinman, J.D. (2003) An *in vivo* dual-luciferase assay system for studying translational recoding in the yeast *Saccharomyces cerevisiae*. *RNA*, **9**, 1019–1024.
 33. Weiss, R.B., Dunn, D.M., Shuh, M., Atkins, J.F. and Gesteland, R.F. (1989) *E. coli* ribosomes re-phase on retroviral frameshift signals at rates ranging from 2 to 50 percent. *New Biol.*, **1**, 159–169.
 34. Yelverton, E., Lindsley, D., Yamauchi, P. and Gallant, J.A. (1994) The function of a ribosomal frameshifting signal from human immunodeficiency virus –1 in *Escherichia coli*. *Mol. Microbiol.*, **11**, 303–313.
 35. Corchero, J.L. and Villaverde, A. (1999) Tolerance of *Escherichia coli* beta-galactosidase C-terminus to different-sized fusions. *Biotechnol. Bioeng.*, **64**, 644–649.
 36. Corchero, J.L., Viaplana, E., Benito, A. and Villaverde, A. (1996) The position of the heterologous domain can influence the solubility and proteolysis of β -galactosidase fusion proteins in *E. coli*. *J. Biotechnol.*, **48**, 191–200.
 37. Bekaert, M., Bidou, L., Denise, A., Duchateau-Nguyen, G., Forest, J.P., Froidevaux, C., Hatin, I., Rousset, J.-P. and Termier, M. (2003) Towards a computational model for –1 eukaryotic frameshifting sites. *Bioinformatics*, **19**, 327–335.
 38. Prüfer, D., Tacke, E., Schmitz, J., Kull, B., Kaufmann, A. and Rohde, W. (1992) Ribosomal frameshifting in plants: a novel signal directs the –1 frameshift in the synthesis of the putative viral replicase of potato leafroll luteovirus. *EMBO J.*, **11**, 1111–1117.
 39. Brierley, I., Bourns, M.E., Binns, M.M., Bilimoria, B., Blok, V.C., Brown, T.D. and Inglis, S.C. (1987) An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV. *EMBO J.*, **6**, 3779–3785.
 40. Garcia, A., van Duin, J. and Pleij, C.W. (1993) Differential response to frameshift signals in eukaryotic and prokaryotic translational systems. *Nucleic Acids Res.*, **21**, 401–406.
 41. Brierley, I., Meredith, M.R., Bloys, A.J. and Hagervall, T.G. (1997) Expression of coronavirus ribosomal frameshift signal in *Escherichia coli*: influence of tRNA anticodon modification on frameshifting. *J. Mol. Biol.*, **270**, 360–373.
 42. Naphthine, S., Vidakovic, M., Girnary, R., Namy, O. and Brierley, I. (2003) Prokaryotic-style frameshifting in a plant translation system: conservation of an unusual single-tRNA slippage event. *EMBO J.*, **22**, 3941–3950.
 43. Lopinski, J.D., Dinman, J.D. and Bruenn, J.A. (2000) Kinetics of ribosomal pausing during programmed –1 translational frameshifting. *Mol. Cell. Biol.*, **20**, 1095–1103.
 44. Somogyi, P.A., Jenner, A.J., Brierley, I. and Inglis, S.C. (1993) Ribosomal pausing during translation of an RNA pseudoknot. *Mol. Cell. Biol.*, **13**, 6931–6940.
 45. Matsufuji, S., Matsufuji, T., Miyazaki, Y., Murakami, Y., Atkins, J.F., Gestland, R.F. and Hayashi, S.-I. (1995) Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. *Cell*, **80**, 51–60.