

Differential response to frameshift signals in eukaryotic and prokaryotic translational systems

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ABSTRACT

The genomic RNA of beet western yellows virus (BWYV) contains a potential translational frameshift signal in the overlap region of open reading frames ORF2 and ORF3. The signal, composed of a heptanucleotide slippery sequence and a downstream pseudoknot, is similar in appearance to those identified in retroviral RNAs. We have examined whether the proposed BWYV signal functions in frameshifting in three translational systems, *i.c. in vitro* in a reticulocyte lysate or a wheat germ extract and *in vivo* in *E.coli*. The efficiency of the signal in the eukaryotic system is low but significant, as it responds strongly to changes in either the slip sequence or the pseudoknot. In contrast, in *E.coli* there is hardly any response to the same changes. Replacing the slip sequence to the typical prokaryotic signal AAAAAAG yields more than 5% frameshift in *E.coli*. In this organism the frameshifting is highly sensitive to changes in the slip sequence but only slightly to disruption of the pseudoknot. The eukaryotic assay systems are barely sensitive to changes in either AAAAAAG or in the pseudoknot structure in this construct. We conclude that eukaryotic frameshift signals are not recognized by prokaryotes. On the other hand the typical prokaryotic slip sequence AAAAAAG does not lead to significant frameshifting in the eukaryote. In contrast to recent reports on the closely related potato leafroll virus (PLRV) we show that the frameshifting in BWYV is pseudoknot-dependent.

INTRODUCTION

A number of viruses use ribosomal frameshifting in the -1 direction to produce a single protein from two or more overlapping reading frames. This mechanism is widely encountered in retroviruses where proteins like reverse transcriptase and protease are synthesized as a fusion with the core proteins encoded by the upstream *gag* gene (1).

Two different signals in the mRNA have been found to play a major role in the frameshifting event. First, there is a

heptanucleotide consensus sequence where the frameshifting actually takes place (2). This heptanucleotide or slippery sequence is composed of a stretch of 3 U, G or A residues, followed by either AAAC, UUUU or UUUA. From a mutational analysis of the sequence in and around the heptanucleotide of the RSV *gag-pol* overlap, Jacks *et al.* (2) proposed that frameshifting involves the simultaneous slippage of the aminoacyl-tRNA and the peptidyl-tRNA. Such a shift in the -1 direction would maintain stable interaction with the codon for at least two out of the three nucleotides of each of the two anticodons.

The second signal is a structural element just downstream of the heptanucleotide sequence (Figure 1). Initially, a single hairpin was proposed to be involved (2–5) but Brierley *et al.* (6) later demonstrated that in the case of the coronavirus IBV the folding of this hairpin into a pseudoknot structure was necessary to obtain efficient frameshifting. The latter authors also observed that potential pseudoknots can be folded downstream of the slippery sequence of some other eukaryotic viral RNAs. This observation was supported by an extensive analysis of all kind of viral RNAs, including plant and yeast viruses, having translational overlap regions and potential or demonstrated slippery sequences (7). The essential role of these pseudoknots in the frameshifting process has now been experimentally verified for IBV (6,8), yeast viruses (9,10), MMTV (11), FIV (12) and SRV-1 (Ten Dam, personal communication). The requirement for a pseudoknot structure, however, seems not to be absolute for all eukaryotic viruses, e.g. HIV (13).

Frameshifting in the -1 direction has also been reported to occur in prokaryotes. There too, it is caused by a slippery sequence belonging to the consensus one mentioned above (14–17). Weiss *et al.* (18) studied frameshifting in *E.coli* using sequences from the MMTV *gag-pro* and HIV-1 *gag-pol* overlaps. It turned out that the efficiency of frameshifting was mainly due to the presence of AAAAAAG, a slippery sequence which is in fact used in natural prokaryotic messengers to induce frameshifting (14,15).

On the basis of similarities of the -1 frameshifting process in *E.coli* and that in eukaryotes, it is of interest to compare the response of both translational machineries to a single frameshift

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signal. To this end we choose to study the frameshift signal occurring in the ORF2-ORF3 overlap of beet western yellows virus (BWYV) (19) for a number of reasons. First, we would learn whether pseudoknots regulate frameshifting in plant viral RNAs (see also ref. 20). Secondly, BWYV contains a potential pseudoknot which is relatively simple in having two stem regions of 5 and 4 basepairs, respectively (7) (Figure 2; A1) while the heptanucleotide sequence consists of GGGAAAC, an efficient slippery sequence in the retroviral SRV-1 RNA (Ten Dam,

personal communication). We have studied this frameshift signal in three different translational systems: *in vitro* in wheat germ extract and reticulocyte lysate and *in vivo* in *E. coli*.

We here present evidence that the BWYV signal induces frameshifting in the eukaryotic translational systems used but not in prokaryotes.

MATERIALS AND METHODS

Strains and plasmids

In all experiments, *E. coli* K-12 strain M5219 was used (21). Cells were grown in LC medium containing (per liter) 10 g tryptone, 5 g yeast extract (Difco), 8 g NaCl, 5 ml 1 M Tris.HCl pH 7.3, and 100 µg/ml ampicillin. This strain carries the genes for the thermosensitive λ repressor and the λ-derived transcription antitermination protein N. Enzymes used for the construction of plasmids were purchased from Pharmacia LKB. All plasmids are derived from pIF.TA (22) containing the 3' portion of the *lac Z* gene and the pL promoter of phage λ. The original EcoRI restriction site in the polylinker of pIF.TA was removed by opening the site, filling in the extremities and religation. Then, the XbaI-BamHI fragment of pIF.TA was replaced by the XbaI-BamHI fragment containing nucleotides 1304–2057 of phage MS2 and harboring the gene for MS2 coat protein (MS2 cp). The fragment of the MS2 genome from the EcoRI site (1628) to the BamHI site (2057) was then replaced by the BWYV slippery site synthesized as an EcoRI-BamHI fragment, in such a way that the *lac Z* gene is in the -1 frame with respect to the MS2 cp gene and to the UAA stop codon present just past the BWYV slippery sequence (Figure 3). To allow *in vitro* transcription, the T7 RNA polymerase promoter derived from plasmid pT7-2 (Promega) was cut out (fragment PvuII/XbaI), and inserted between the filled HindIII and XbaI restriction sites of the polylinker (Figure 3). The BWYV frameshift signal in its wild type or mutated form (clones A1, A2, A3 and A4) were constructed by phosphorylation and annealing of two

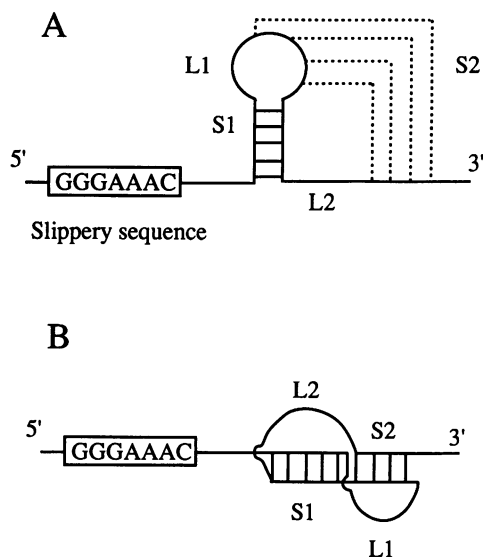


Figure 1. General structure of a pseudoknot-dependent frameshift signal. (A) Indication of the base-paired regions. (B) Folding into a pseudoknot structure. The slippery sequence of BWYV is shown. S1 and S2 represent stem 1 and stem 2, and L1 and L2 are loop 1 and loop 2 of the pseudoknot.

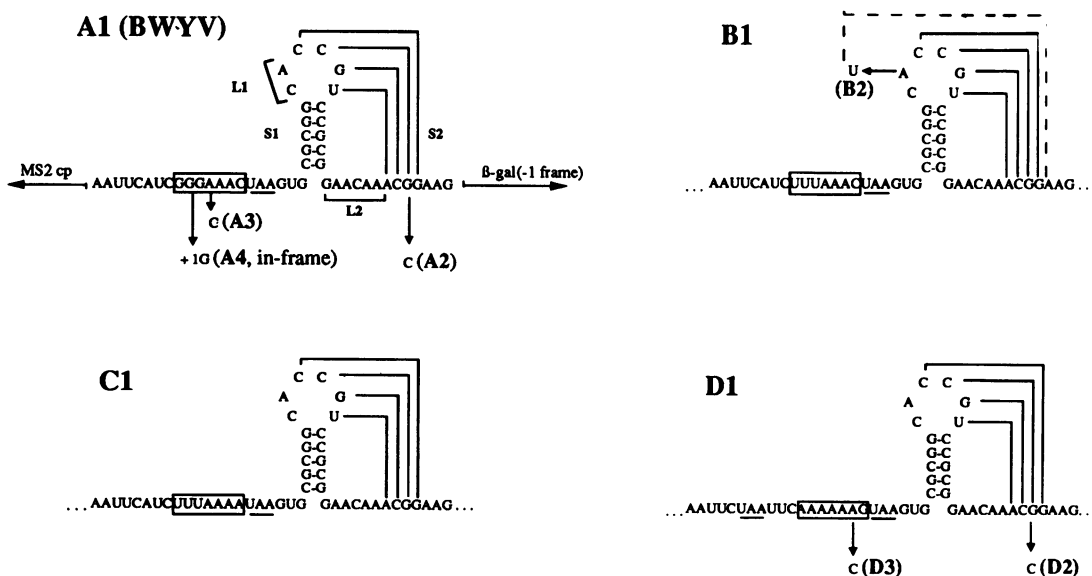


Figure 2. Outline of the various frameshift signals used in this study. Each panel represents a construct with its derived mutants (indicated as bold letters between brackets). Each mutation is indicated by an arrow. Slippery sequences are boxed and stop codons are underlined. A1 corresponds to the wild type BWYV sequence (19).

complementary oligonucleotides (synthesized on a Pharmacia Gene Assembler and desalted over a Sephadex G-25 column). The clones B1, B2, C1, D1, D2 and D3 were constructed taking advantage of the internal SacII site located in the BWYV sequence, and thus the EcoRI-SacII fragment was replaced by the corresponding oligonucleotides. As a control and for technical reasons, a UAA stop codon in the -1 frame was inserted upstream of the slippery sequence in clones D1, D2 and D3. The clone SRV-1 was a generous gift from E. ten Dam. All sequences were checked by double-stranded DNA sequencing using the deaza T7 sequencing kit from Pharmacia LKB.

In vitro transcription

Plasmids were prepared by alkaline-SDS extraction and purified on CsCl gradients as described by Sambrook *et al.* (23). T7 RNA polymerase was prepared following the procedure of King *et al.* (24). For T7 transcription, plasmids were cut by ClaI, except the plasmid SRV-1 which was digested by BamHI and transcribed with SP6 RNA polymerase. The T7 transcription mixture contained: 4 μ g of digested plasmid, 40 mM Tris.HCl pH 8, 1.5 mM MgCl₂, 5 mM DTE, 20 U of RNasin (Boehringer-Mannheim), 0.5 mM m7GpppG, 2 mM ATP, 2 mM UTP, 2 mM CTP, 12.5 μ M GTP and 4 μ l (70 U) of T7 RNA polymerase. To allow the transcripts to begin with the cap, the mixture was incubated for 15 min at 37°C, then GTP was added to a final concentration of 2 mM and the reaction was continued for another 45 min. *In vitro* SP6 RNA polymerase transcription on 4 μ g of digested plasmid was performed in 40 mM Tris.HCl pH 7.5 containing 10 mM NaCl, 6 mM MgCl₂, 2 mM spermidine, 10 mM DTE, 20 U RNasin, 0.5 mM m7GpppG, 1 mM ATP, 1 mM UTP, 1 mM CTP, 12.5 μ M GTP and 90 U of SP6 RNA polymerase (Pharmacia). An incubation of 30 min at 37 °C was performed, before adding GTP at the final concentration of 1 mM. The reaction was continued for 30 more min. The transcripts were subsequently submitted to phenol extraction followed by ether treatment, and precipitated with ethanol, dried, and the pellet dissolved in H₂O. The integrity of mRNA was checked by electrophoresis on a 1% agarose gel: a single mRNA band was observed.

In vitro translation

The capped mRNAs were translated using wheat germ extract or rabbit reticulocyte lysate, both from Promega, and following the supplier's manual. The incubations were carried out at 30°C for 1 hr in the presence of 0.8 μ Ci of ³⁵S-methionine (Amersham), together with 0.3 to 1 μ g of mRNA in 50 μ l. Translation products were analysed on 3% stacking-17.5% polyacrylamide (acryl/bis 30 : 0.8)-SDS gel (25). After electrophoresis, the gel was fixed, soaked in amplifier solution ('Amplify', Amersham) and dried. The labeled proteins were detected by autoradiography and the amount of ³⁵S from each band was quantified using a Betascope (Betagen, Inc.). It is assumed that, analogous to the *in vivo* situation, the N-terminal methionine of the MS2 coat protein is cleaved off in the extract. The ratio of methionines in trans-frame and MS2 cp gene is then 6:1. If cleavage does not take place it is 7:2.

β -galactosidase activity assays

β -gal activity was measured as described by Miller (26) and corresponded well to the amount of fusion protein estimated from western-blots developed with antibodies against MS2 coat protein

(results not shown). Four different time points were measured for each construct and synthesis rates were determined. Results are presented as the ratio between β -gal activities in trans-frame and in-frame constructs.

RESULTS

Frameshifting in *E. coli* was assayed *in vivo* using plasmids containing the wild type and modified versions of the putative BWYV shift signal placed between the coat protein gene of phage MS2 and the *lacZ* gene (Figure 3). If the BWYV sequence induces a -1 frameshift this will result in the formation of a MS2 cp- β -gal fusion protein. Translation without frameshifting starting at the MS2 coat protein will terminate at a stop codon present in the BWYV-derived insert between the slip sequence and the first stem of the pseudoknot (Figure 2; A1). *In vivo* transcription from this plasmid is driven by the temperature-inducible λ promoter p_L. Frameshift is expressed as the ratio of β -gal activities measured in the trans-frame and in-frame constructs (Figure 2; A4).

Frameshifting *in vitro* in eukaryotic translation systems was performed with T7 transcripts translated in the presence of ³⁵S-methionine in either a reticulocyte lysate or a wheat germ extract. Labeled translational products were fractionated on SDS-polyacrylamide gels and the percentage of frameshifting calculated as the radioactivity ratio between trans-frame and MS2 cp products (see Materials and Methods).

The wild type frameshift stimulator of BWYV, shown in Figure 2 as A1, was tested in the two *in vitro* eukaryotic systems and found to give around 1% frameshifting (Figure 4, Table 1). This number is low, but of the same order as that recently reported for the frameshift signal in the related plant virus PLRV (20). To ascertain that the low efficiency was not due to any trivial cause we tested the shift signal of the retroviral SRV-1 RNA, which is known to be quite efficient, in the same assay systems.

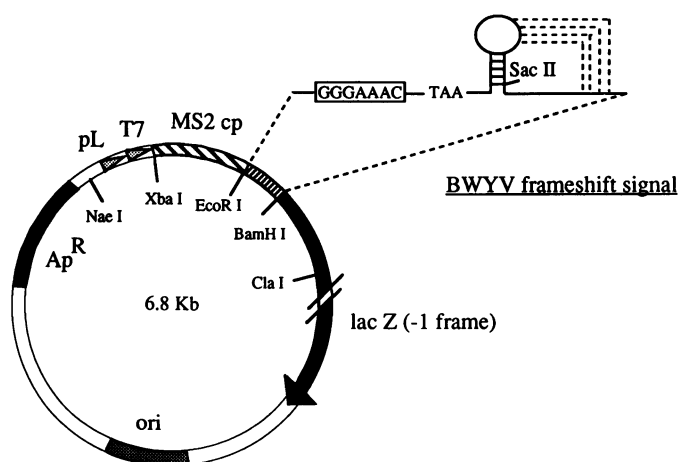


Figure 3. Plasmid used to test efficiency of the BWYV frameshift signal and its derivatives. Translation starts at the coat protein gene of phage MS2 (MS2 cp) and proceeds into the *lacZ* gene when a -1 shift is made in the BWYV sequence. Translation *in vivo* in *E. coli* takes place on transcripts started at the p_L promoter. Translation *in vitro* is performed on transcripts started at the T7 promoter. The BWYV frameshift signal is inserted between the MS2 cp and *lacZ* reading frames.

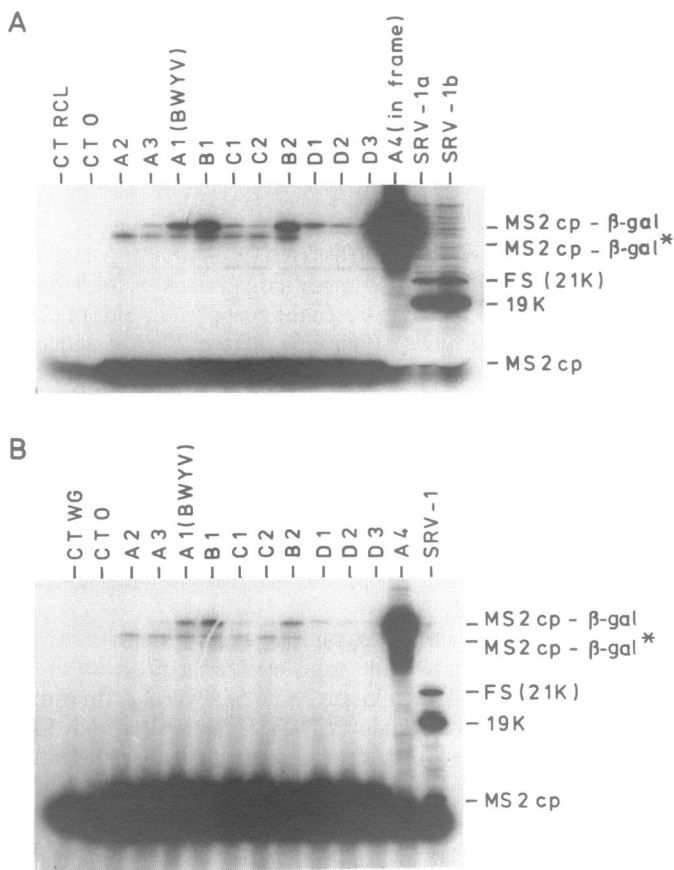


Figure 4. Autoradiography of ^{35}S -labeled translation products after fractionation over SDS-polyacrylamide gels. A—mRNA translated in a reticulocyte lysate. B—mRNA translated in a wheat germ extract. (C_T RCL): control for endogenous mRNA translation from reticulocyte lysate; (C_T WG): control for endogenous mRNA translation from wheat germ extract; (C_T 0): control for unspecific mRNA translation products brought with the plasmid preparation (1 μg of plasmid is added); (SRV-1a) and (SRV-1b) correspond to the same sample but a double amount is loaded in (SRV-1b); (MS2 cp- β -gal): corresponds to the fusion protein (40 K) obtained by frameshifting; (MS2 cp): corresponds to the truncated MS2 coat protein (11K) expressed from the of MS2 cDNA; (FS (21K)) is the fusion protein obtained by -1 frameshifting mediated by the SRV-1 slippery site (ten Dam, in preparation) and (19 K) is the protein read in 0 frame. A band migrating just below the fusion protein MS2 cp- β -gal and indicated as MS2 cp- β -gal* is observed in all constructs except for D1, D2 and D3. The D-series has one important difference with the others: it contains a stop codon in the -1 frame before the slippery sequence. The absence of the band in the D-series demonstrates that this lower molecular weight product is not produced by frameshifting, but rather by an initiation at a -1 AUG codon before the slippery sequence. An AUG in the -1 frame is located downstream of the start of the MS2 cp gene. Translation from this point gives a protein of near 31 K. C2 is a clone not discussed in this paper.

Indeed, this RNA yielded some 25–30% shift (Table 1). As further evidence that the 1% frameshift was significant and occurred in response to the presence of the shift stimulator, we made an A to C substitution at position 4 of the slip sequence. In this construct (Figure 2, A3) frameshifting is reduced tenfold to 0.15%. Similarly, destabilization of stem S2 of the predicted pseudoknot (A2) decreases the shift more than 5 times in the reticulocyte lysate. Also in *E. coli* all three constructs give a low amount of shifting (Table 1), but here the phenomenon is hardly responsive to changes in either the shifty sequence or the pseudoknot structure. Apparently, in *E. coli* the BWYV shift signal is poorly recognized.

Table 1. Frameshift efficiency of various BWYV constructs

| CONSTRUCTS | Frameshifting in <i>E. coli in vivo</i> (%) | Frameshifting in reticulocyte lysate (%) | Frameshifting in wheat germ extract (%) |
|---------------|---------------------------------------------|------------------------------------------|-----------------------------------------|
| in frame (A4) | 100 | — | — |
| BWYV (A1) | 0.85 | 1.40 | 0.80 |
| A2 | 0.70 | 0.25 | 0.25 |
| A3 | 0.45 | 0.15 | 0.20 |
| B1 | 0.95 | 3.95 | 1.70 |
| B2 | 0.75 | 2.75 | 1.25 |
| C1 | 0.65 | 0.75 | 0.50 |
| D1 | 5.40 | 1.50 | 0.55 |
| D2 | 4.00 | 1.10 | 0.30 |
| D3 | 1.60 | 1.00 | 0.30 |
| SRV-1 | — | 27.5 | 28 |

Frameshifting efficiency obtained (i) *in vivo* in *E. coli* by measurements of relative β -gal activities (in comparison to the in-frame construct) and (ii) *in vitro* by quantification of ^{35}S -labeled products incorporated in the fusion protein and in the corresponding MS2 cp band produced for each assay. For *in vitro* experiments, the results are given as a percentage of the radioactivity incorporated in the fusion protein band and corrected for the number of ^{35}S -methionine residues present in each protein MS2 cp band.

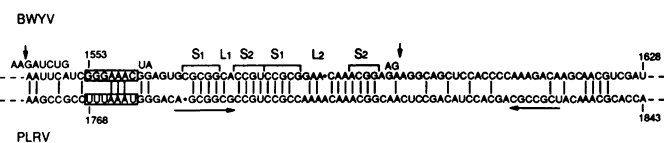


Figure 5. Sequence alignment of BWYV and PLRV RNA around the frameshift site. The sequence of BWYV is from ref. 19 and that of PLRV RNA as reported by Prüfer *et al.* (20). The shifty heptanucleotide is boxed. S₁ and S₂ indicate the two stem regions and L₁ and L₂ the two loop regions of the pseudoknot (see also Fig. 2 (A₁)). The horizontal arrows represent the stem of the hairpin proposed by Prüfer *et al.* (20). The vertical arrows delineate the BWYV RNA fragment examined in this study. Changes in the BWYV RNA sequence introduced for reasons of cloning are shown in the upper line.

To extend our analysis we changed the slip site from GGG-AAAC to UUUAAAAG (Figure 2; B1). This change increased frameshifting threefold in the reticulocyte system, but the response in *E. coli* was again insignificant (from 0.85% to 0.95%) (Table 2). We tried to increase the efficiency of construct B1 by allowing an extra basepair in stem S2 (B2), but as frameshifting did not go up we presume that the pair did not form.

It has been suggested that AAC at positions 5–7 in the slip site is so efficient because the anticodon of the cognate tRNA^{Asn} in reticulocytes has queuosine in the wobble position, which would interact weakly with the C residue (11,27). In *E. coli* the tRNA^{Asn} for AAC has a G at the wobble position and the presence of a strong G.C pair could be a reason for the absence of shifts here. To increase chances to get pseudoknot-induced frameshifting in *E. coli* we changed the slip sequence from UUUAAAAG to UUUAAAAA (Figure 3: C1). In agreement with Brierley *et al.* (27), we find for the eukaryotic system that this change (B1 to C1) diminishes frameshifting. However, there is hardly a response in *E. coli* suggesting again that eukaryotic frameshift signals are not recognized in this bacterium in the same way.

In the last experiment we turned things around and started out with the heptanucleotide AAAAAAG, a sequence known to cause substantial frameshifting in *E. coli*. It occurs at the slip site in

the *dnaX* gene (14,15,28) and was also tested extensively in artificial constructs by Weiss *et al.* (18). Indeed, construct D1 containing this sequence in front of the BWYV pseudoknot (Figure 2) yields 5.4% frameshifting in *E. coli* (Table 1). As expected, shifts are sensitive to mutations in the A₆G sequence. There is a more than threefold drop when the last A is changed into a C (Figure 2; D3). The reaction of *E. coli* to the disruption of stem 2 is very small as if the ribosomes do not 'feel' the pseudoknot. The eukaryotic ribosomes do not respond very much to changes in construct D1. The substitution in the slip site only results in about 30% decrease in frameshifting and the same holds for the destabilizing mutation in the pseudoknot. It is not understood why frameshifting remains so high in eukaryotes with the D2 and D3 constructs.

DISCUSSION

Pseudoknot-dependent frameshifting has recently been described for a number of mammalian and yeast viruses (6,9–12). In this paper we have shown that such a frameshift signal is likewise used for the expression of the ORF3 gene of beet western yellows virus (BWYV). This signal was predicted by Ten Dam *et al.* (7) to consist of the slippery sequence GGAAAC and a pseudoknot having two stems of 5 and 4 basepairs, respectively, located 6 nucleotides downstream of the slippery heptanucleotide. Our results demonstrate that this signal functions in eukaryotic translational systems, albeit with the rather low efficiency of 0.8 and 1.4% in wheat germ extract and reticulocyte lysate, respectively. This low level is comparable to the 1% frameshifting found at the same position in the related potato leaf roll virus (PLRV) RNA as measured in potato protoplasts and reticulocyte lysate (20). We find a two- to threefold higher frameshifting efficiency in the reticulocyte lysate system than in the wheat germ extract. A similar difference was reported for IBV when tested in both systems (5). On the other hand we find identical efficiencies in the case of the retroviral SRV-1 RNA. The reasons for these differences are unclear.

The mutational analysis of the BWYV frameshift signal shows that it acts differently in prokaryotic and eukaryotic systems. Combination of four different slippery heptanucleotides with the BWYV pseudoknot structure (A–D, Figure 3) showed that in *E. coli* only the heptanucleotide AAAAAAG is active. This result is in agreement with the findings of Weiss *et al.* (18) who studied mutants of the MMTV *gag-pro* frameshift site. The heptameric sequence AAAAAAG followed by a stem of 4 to 5 basepairs led to a frameshifting efficiency of 5.9 to 4.0%. This corresponds closely to the value of 4% obtained for construct D2 (Table 1). A possible explanation for the efficiency of this AAAAAAG sequence in *E. coli* is that the unique tRNA^{Lys} isoacceptor recognizes both AAA and AAG, but that it has a better interaction with AAA, due to the modified U residue at the wobble position, thus favouring the –1 frameshift (16). The sequence UUUAAA–C (construct B1) gives the best frameshifting in the eukaryotic systems. It is identical to the one found in the coronaviral IBV and MHV-A59 where 25 and 40% frameshifting have been reported (6,31). Replacement of the terminal C by an A residue (construct C1) gives a 5-fold decrease in frameshifting efficiency as compared to B1.

The pseudoknot predicted for the BWYV case (7) belongs to the more simple and probably less stable ones and may therefore be a weak frameshifting stimulator, ensuring a functionally relevant high ratio between the ORF2 products and the fusion protein (see also ref. 9).

It is interesting to note that Prüfer *et al.* (20) recently reported that PLRV, another luteovirus, is not dependent on a downstream pseudoknot for frameshifting. This was concluded from studies in which a stem region in an alternative potential stem-loop structure was disrupted and restored. This stem region in PLRV RNA is indicated in Fig. 5. From the sequence alignment of BWYV and PLRV RNA around the frameshift site it is clear that it is not possible to fold an identical or even similar hairpin in the case of BWYV RNA. PLRV RNA, however, can be folded in almost the same pseudoknot structure as BWYV RNA (Fig. 2; A1) due to the strong sequence homology in the region of the pseudoknot structure. It is striking that the homology between PLRV and BWYV RNA only extends over the frameshift signal as envisaged in our model. Moreover, our BWYV construct misses the downstream sequence needed to form the hairpin proposed by Prüfer *et al.* (20), but still gives rise to the same amount of frameshifting as reported for PLRV. It is conceivable that the double mutant in PLRV, which eliminates the pseudoknot structure, forces PLRV RNA to fold in an alternative stem-loop structure which is able to restore frameshifting to that of the wild type.

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