Ribosomal frameshifting in plants: a novel signal directs the −1 frameshift in the synthesis of the putative viral replicase of potato leafroll luteovirus

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The 5.8 kb RNA genome of potato leafroll luteovirus (PLRV) contains two overlapping open reading frames, ORF2a and ORF2b, which are characterized by helicase and RNA polymerase motifs, respectively, and possibly represent the viral replicase. Within the overlap, ORF2b lacks an AUG translational start codon and is therefore presumably translated by −1 ribosomal frameshifting as a transframe protein with ORF2a. This hypothesis was studied by introducing the putative frameshift region into an internal position of the β-glucuronidase (GUS) gene and testing for the occurrence of frameshifting *in vivo* by transient expression of GUS activity in potato protoplasts as well as *in vitro* by translation in the reticulocyte system. Both experimental approaches demonstrate that a −1 frameshift occurs at a frequency of ~1%. Site-directed mutagenesis identified the frameshift region and the involvement of the novel heptanucleotide motif UUUAAAU in conjunction with an adjacent stem−loop structure. Part of this stem−loop encodes a basic region in the ORF2b moiety of the transframe protein which was shown by binding experiments with PLRV RNA to represent a nucleic acid-binding domain. These data support a possible biological significance of the frameshift to occur at this position of the large overlap by including the putative RNA template-binding site of the PLRV replicase in the ORF2a/ORF2b transframe protein.

Key words: −1 frameshift/gene expression/luteovirus/potato leafroll virus/replicase

Introduction

Expression of a single protein from two or more overlapping open reading frames (ORFs) by ribosomal frameshifting is a translational mechanism that has been studied in detail with viruses as model systems (reviewed by Hatfield *et al.*, 1990; Hatfield and Oroszlan, 1990). For example, in animal retroviruses, expression of the viral RNA-dependent DNA polymerase (reverse transcriptase) occurs by −1 ribosomal frameshifting (Jacks and Varmus, 1985; Jacks *et al.*, 1987, 1988a; Moore *et al.*, 1987; Wilson *et al.*, 1988). Frameshifting apparently also operates in the expression of reverse transcriptase for several retrotransposons [*Ty*1 (Mellor *et al.*, 1985; Wilson *et al.*, 1986; Clare *et al.*, 1988), *Ty*912 (Clare and Farabaugh, 1985), 17.6 (Saigo *et al.*, 1984) and *gypsy* (Marlor *et al.*, 1986)]. Other viruses may also depend on frameshifting for the expression of some of their genes as has been documented for infectious bronchitis coronavirus

(IBV) in the translation of the F1 and F2 proteins (Brierly *et al.*, 1987, 1989).

Potato leafroll virus (PLRV), a member of the luteovirus group of plant viruses, is a particularly suitable subject for the study of translation in plants, as several translational mechanisms serve for the expression of various genes. The viral genome consists of a 5.8 kb single-stranded (+) RNA with six major ORFs (Figure 1A; Mayo *et al.*, 1989; van der Wilk *et al.*, 1989; Keese *et al.*, 1990). An intergenic region located in the centre of the RNA genome separates a 5' cluster of genes (ORFs 1, 2a and 2b), which are divergent among the luteoviruses sequenced so far, from a highly conserved gene block (ORFs 3, 4 and 5) in the 3' half. The 3' located genes are translated from a 2.3 kb subgenomic RNA (sgRNA1) in a manner that includes internal translation initiation as well as UAG stop codon suppression for the synthesis of ORF4 and ORF5, respectively (Tacke *et al.*, 1990). In the 5' half of genomic PLRV RNA a small ORF (ORF1) is followed by two large ORFs, ORF2a and ORF2b, which may code for a 70 kDa and a 67 kDa protein, respectively, and contain the motifs characteristic of helicases (ORF2a and ORF2b; Habili and Symons, 1989) and RNA polymerases (ORF2b; Kamer and Argos, 1984). In the German PLRV isolate both ORFs overlap by 582 nucleotides (E.Tacke, unpublished) with ORF2b lacking an AUG translational start codon in this region. As the first ORF2b AUG is located 900 bp downstream of its 5' end which could encode a protein of only 34 kDa, it was assumed that ORF2b would be expressed as an ORF2a/2b transframe protein by −1 ribosomal frameshifting (Mayo *et al.*, 1989).

For a number of retroviruses, the coronavirus IBV and the *gypsy* transposable elements of *Drosophila*, heptanucleotide signals are involved as 'slippery sequences' in the frameshift. These include the sequences AAAAAAC (mouse mammary tumour virus, MMTV), AAAUUUA (Rous sarcoma virus, RSV) and UUUUUUA (human immunodeficiency virus, HIV-1 and HIV-2) which allow for tRNA slippage during translation (Hatfield *et al.*, 1990). For RSV every single base exchange in the heptanucleotide signal except for the 3' terminal base inhibits frameshifting (Jacks *et al.*, 1988b). In addition to the specific sequence signal a second type of information bears relevance to frameshifting (Hatfield and Oroszlan, 1990): stem−loop structures immediately downstream of the 'slippery sequences' have significant influence on the efficiency of the frameshift event. For RSV and IBV (Jacks *et al.*, 1988b; Brierly *et al.*, 1989) it could be shown that stem-destabilizing mutations of the corresponding stem−loop resulted in a decrease in frameshifting, while restoring these base pairings by specific stem-restabilizing mutations rescued the frameshift event. The requirement of a stable stem−loop structure for efficient frameshifting to occur indicates that ribosomes may stall at such RNA secondary structures and thereby allow the change of reading frame at the heptanucleotide signal. In HIV,

however, a stem−loop is not necessary for the frameshift event (Wilson et al., 1988).

Here we define by in vitro experiments a new heptanucleotide motif of a frameshift site which is involved in the expression of the putative RNA-dependent RNA polymerase (replicase) of PLRV. Activity of this signal in frameshifting is dependent on a stable stem−loop structure immediately downstream of the heptanucleotide sequence. Furthermore we provide indirect evidence for the biological significance of the frameshift to occur at this particular position within the ORF2a/b 582 nucleotide overlap by demonstrating that the basic amino acid cluster encoded by part of the stem−loop is a nucleic acid-binding domain and possibly represents the site to which PLRV RNA template binds during viral RNA replication by the PLRV replicase.

Results

Localization of the frameshift region

For the analysis of the putative frameshift between ORF2a and 2b the corresponding region was inserted into a heterologous environment, the β-glucuronidase (GUS) gene of Escherichia coli, resulting in clone pSFS-WT (Figure 1; for details see Materials and methods). This strategy was chosen to exclude the possibility that other viral proteins or sequence motifs of PLRV RNA participated in the frameshift. In this construct the coding sequence resulting from the fusion of the N-proximal half of the GUS gene (GUS-N) to the residual ORF2a part would yield a chimeric GUS-N−ORF2a protein of 60 kDa, while a putative frameshift into ORF2b would allow translation to proceed to the GUS carboxy-terminus (GUS-C) and produce a chimeric transframe protein GUS-N−ORF2a−ORF2b−GUS-C of ~100−110 kDa. In vitro transcription/translation experiments with pSFS-WT showed that a frameshift took place as evident by the synthesis of the 103 kDa transframe protein in addition to the 60 kDa GUS-N−ORF2a translation product produced by the ORF2a translational stop signal (Figure 1B). The transframe protein was identified as the only product, when an additional deoxynucleotide (C) was introduced by site-directed mutagenesis in the ORF2a/ORF2b overlap region at position 1771 (Mayo et al., 1989) within the heptanucleotide frameshifting motif (see below) to yield construct pSFS-tf with a continuous open reading frame (Figure 1B). In order to identify the frameshift

region more closely within the 582 nucleotide overlap, several deletion mutants in this region were produced (pSFS-mut1, -mut2 and -mut3; Figure 1A). In all cases ribosomal frameshifting was obtained, as evident from the appearance of translational products of the expected sizes (pSFS-mut1: 103 kDa; pSFS-mut2: 83 kDa; pSFS-mut3: 85 kDa) in addition to the main GUS-N−ORF2a products. Common to all of these deletion mutants is a sequence of 214 nucleotides between the DdeI and PstI restriction sites in the ORF2a/2b overlap (Figure 1A). It was concluded that this sequence carries all types of information required for the frameshift event to take place.

Efficiency of ribosomal frameshifting

To determine the frequency of transframe protein expression in vivo, transient expression experiments were performed in potato protoplasts. For this, ~90% of the overlap region was translationally fused to the amino-terminus of the GUS gene (Jefferson et al., 1986) at the GUS BclI site and flanked by the cauliflower mosaic virus (CaMV) 35S promoter and terminator control sequences. Within this construct (pRFS-WT) the original ORF2a UGA stop codon was removed and replaced by a GUS gene UGA stop codon in the −1 noncoding frame which extends the ORF2a translational product by 12 amino acid residues. The resulting constructs, GUS-N−ORF2a, as well as ORF2b−GUS-C, represents a continuous coding sequence. Ribosomal frameshifting in vivo would result in a GUS−PLRV−GUS transframe protein containing 31 N-terminal GUS amino acids, 217 amino acids of the PLRV overlap region and an additional 572 amino acids of the GUS enzyme. In order to evaluate frameshift efficiencies with respect to control GUS expression of 100%, the identical ORF2a/2b overlap region was cloned into the GUS BclI site such that the introduction of a single additional G nucleotide produced a continuous GUS-N−ORF2a−GUS-C ORF (pRFS-PC). In addition, to account for any putative GUS activity resulting from internal translation initiation, an out-of-frame construct was used as a negative control and transient expression experiments were performed as described previously (Tacke et al., 1990). Table I summarizes the results of transient expression in potato protoplasts. The data demonstrate that −1 ribosomal frameshifting in the expression of the putative polymerase (ORF2b) occurs at a frequency of 0.7−0.9% normalized with respect to the two control constructs.

Table I. Efficiency of ribosomal frameshifting in the expression of ORF2b by in vivo and in vitro experiments

| Expression of ORF | In vivo | | In vitro | |
	GUS activity[a]	% Frameshift	Radioactivity[b]	% Frameshift
2a	13280		2480	
2a/2b	110	0.83	30	1.2
2a	18500		3125	
2a/2b	167	0.90	41	1.3
2a	24580		2635	
2a/2b	233	0.95	32	1.2
2a	17875			
2a/2b	127	0.71		

[a]pmol MU formed/mg protein/min. GUS activity of the pRFS-PC construct was set at 100%. The percentage of frameshifting was determined for pRFS-WT after subtraction of the background value measured for an out-of-frame construct (Tacke et al., 1990). Background activity had a mean value of 72 pmol MU formed/mg protein/min.
[b]Radioactivity measured in the transframe protein ORF2a/2b was corrected for background radioactivity and different methionine content, and frameshift efficiencies were calculated in relation to the 100% control (ORF2a).

Efficiency of frameshifting was also determined by *in vitro* transcription/translation of pSFS-mut3 (Figure 1A and B) and estimation of the radioactivity present in the transframe protein as opposed to the protein produced by translational stop at the ORF2a carboxy-terminus. The data were corrected for the different methionine contents of the products. By these *in vitro* experiments in the heterologous translation system (rabbit reticulocytes), a comparable efficiency of frameshifting was observed (Table I).

Analysis of the frameshift site

The heptanucleotide signals required for frameshifting have been classified into three classes on the basis of the consensus sequences xxxAAAC, xxxUUUA and xxxUUUU within their 3' termini (Hatfield *et al.*, 1990). Computer analysis of the PLRV sequence represented by the *Dde*I−*Pst*I fragment and identified to be involved in frameshifting (see above; Figure 1A) revealed a similar sequence motif UUUAAAU (coordinates 1768−1774) that could possibly serve as the signal for frameshifting. In order to prove this,

pSFS-mut3 (Figure 1A) was used for the generation of further deletion mutants (Figure 2A). Deletion of most of the PLRV sequences upstream of the presumptive signal (pSFS-mut4) did not change frameshifting efficiency from that of pSFS-mut3 (Figure 2B). However, in the mutant pSFS-mut5, which lacks the signal UUUAAAU, frameshifting was totally abolished.

Additional mutations were based on single base substitutions within the signal. In pSFS-mut6 the motif UUUAAAU was changed to UUUACAU. In this mutant the heptanucleotide codes for Leu−His in the zero frame (UUA CAU) and Phe−Thr in the −1 frame (UUU ACA) as opposed to Leu−Asn (UUA AAU) and Phe−Lys (UUU AAA) in the wild-type. In the mutant tRNA^{His} is obviously unable to participate in frameshifting, because frameshifting was not observed (Figure 2B). Another point mutation that changed the heptanucleotide signal from UUUAAAU to CUUAAAU (pSFS-mut7) was meant to analyse the UUUA part of the heptanucleotide for its possible involvement in frameshifting. As with pSFS-mut6, frameshifting was totally
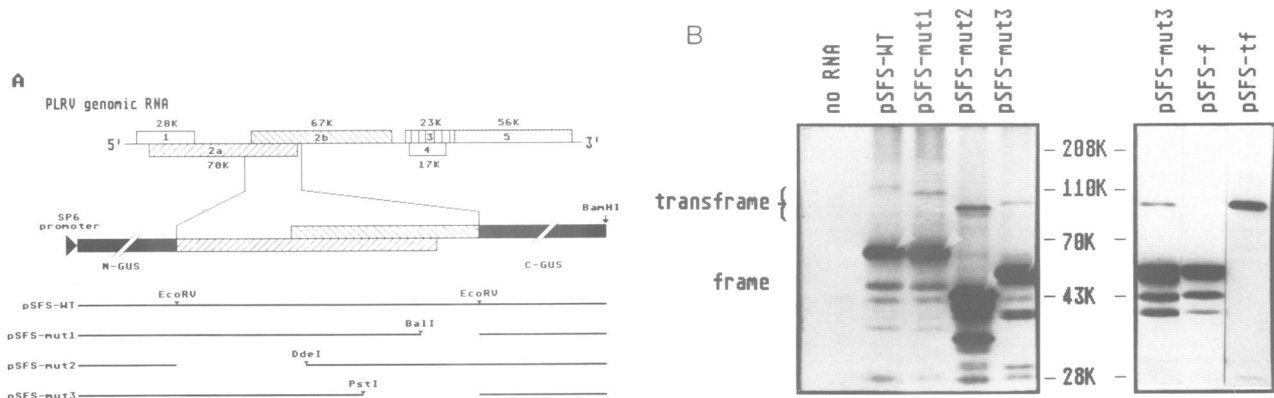

Fig. 1. Localization of the ORF2a/ORF2b frameshift site. **A.** Chimeric constructs used for *in vitro* transcription/translation. The overlap region of ORF2a and ORF2b was translationally fused to the GUS gene (Jefferson *et al.*, 1986) in the pSP65 transcription vector to yield pSFS-WT. Deletion mutants were obtained by digestion with the restriction enzymes indicated. Solid lines represent sequences retained in the constructs. The *Dde*I site is at PLRV coordinates 1735−1739 (corresponding to ORF2b coordinates 91−95). **B.** *In vitro* translation of pSFS-WT and mutant mRNAs. RNA was synthesized from *Bam*HI-linearized pSFS-WT and mutant template DNAs with SP6 RNA polymerase and translated in the rabbit reticulocyte system in the presence of [^{35}S]methionine. The products were separated on a 10% SDS−polyacrylamide gel and detected by fluorography. White arrowheads (left panel) indicate the main GUS-N−ORF2a translational products. The 40 kDa protein represents the product of endogenous mRNA activity in the translation system. Lane pSFS-f: translational products from a mutant in which the heptanucleotide signal for frameshifting was deleted. Lane pSFS-tf: translational product from a mutant in which by insertion of a single nucleotide at the heptanucleotide signal a continuous ORF2a/2b was produced.
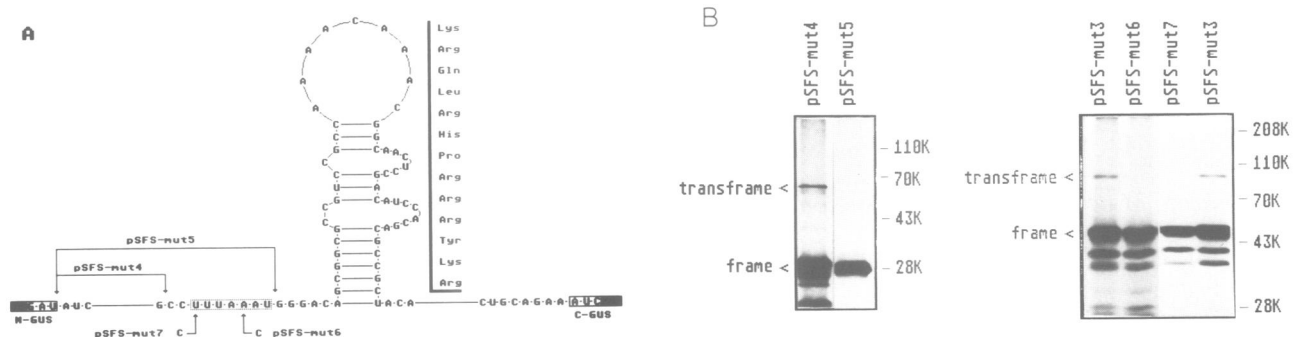

Fig. 2. Analysis of the frameshift site. **A.** The position of specific deletions (mut4 and mut5) and point mutations (mut6 and mut7) created within the ORF2a/2b overlap region of plasmid pSFS-mut3 are indicated by arrows. The diagram shows the suspected 'slippery sequence', UUUAAAU (boxed) and a stem−loop structure immediately downstream of the signal. The heptanucleotide sequence is located at PLRV coordinates 1768−1774 (ORF2b coordinates 123−130). GUS coding sequences (N-GUS and C-GUS) are represented by black areas. The boxed sequences GUA and AUC represent the 5' and 3' terminal halves of the GUS internal *Eco*RV sites. The black bar indicates the basic amino acid cluster identified as the nucleic acid-binding domain in the ORF2b protein. **B.** Translation of mRNAS transcribed from the mutants described in A. Analysis of products was done as described in the legend to Figure 1B.
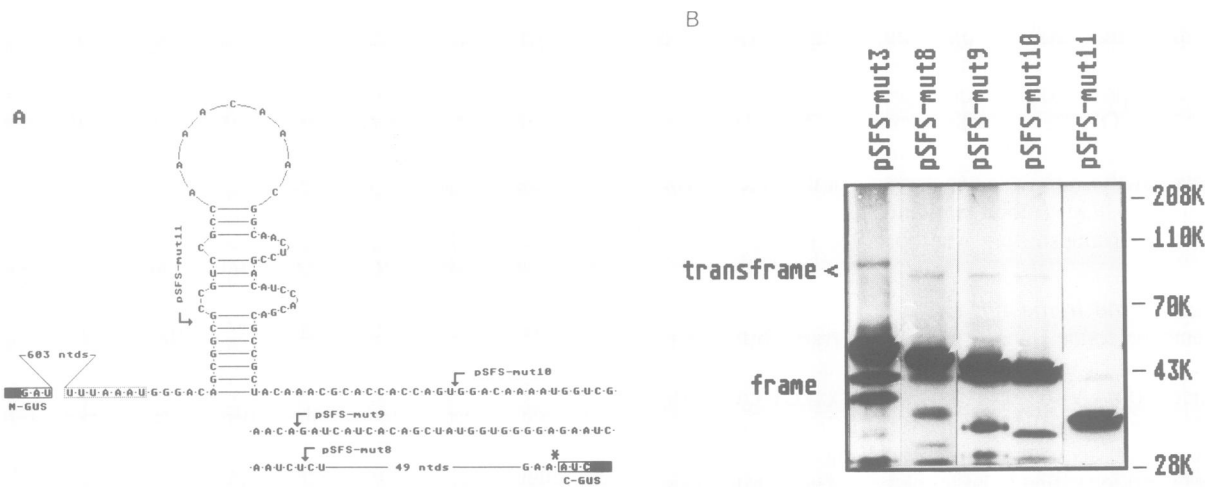
Fig. 3. Requirements of downstream sequences for efficient ribosomal frameshifting. **A.** The specific deletions produced in pSFS-mut3 start at the positions indicated by arrows and extend to the same position in the C proximal part of the GUS gene as indicated by an asterisk. **B.** Translational products derived from RNAs of the mutant templates described in panel A. Analysis was performed as described in the legend to Figure 1B.
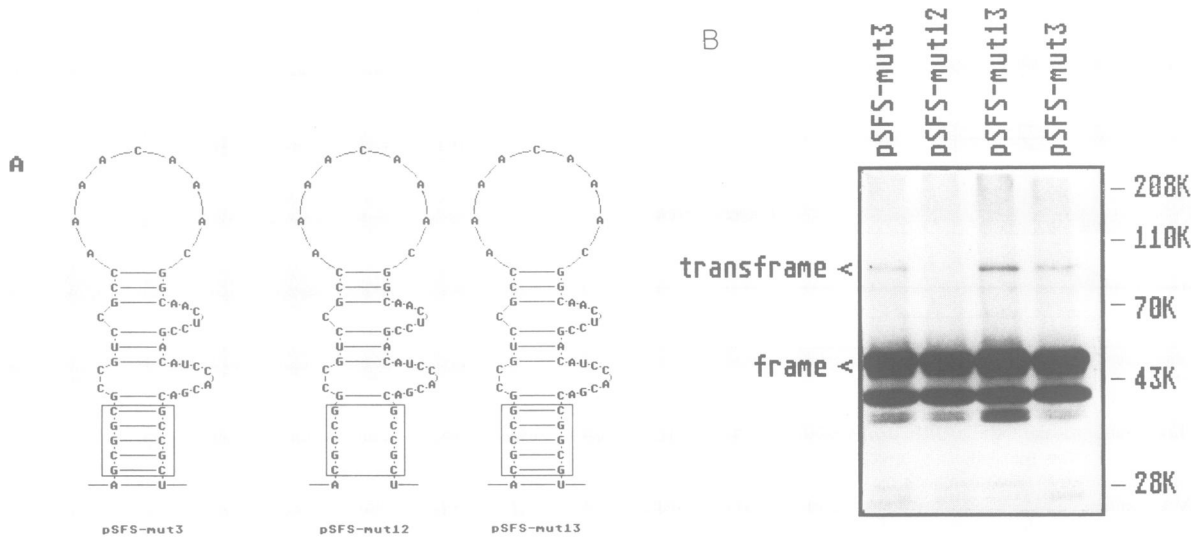

Fig. 4. Involvement of the stem−loop in frameshifting. **A.** Specific mutations were introduced into the stem (boxed) of the stem−loop structure base of pSFS-mut3 by site-directed mutagenesis and (B) analysed as described in the legend to Figure 1B.

abolished and indicated that tRNA at this site (tRNA^{Leu}) also participates in the frameshifting. These results imply that ribosomal frameshifting requires a concerted slippage of tRNA^{Leu} and tRNA^{Asn} at the ribosomal P and A sites for the expression of ORF2b via ribosomal frameshifting. Thus the heptanucleotide signal UUUAAAU represents a new class of frameshift signals xxxAAAU.

Requirements of downstream sequences for efficient frameshifting

Further deletion mutants (pSFS-mut8, -mut9, -mut10 and -mut11; Figure 3A) were produced from pSFS-mut3 in order to identify information located downstream of the heptanucleotide signal and necessary for efficient frameshifting. With mutants 8, 9 and 10, frameshifting occurs with wild-type efficiency (Figure 3B). However, in mutant mut11, in which a stable stem−loop structure (−15.4 kcal/mol) five nucleotides downstream of the heptanucleotide sequence was destroyed, frameshifting was totally abolished.

To document further the significance of this stem−loop

in frameshifting efficiency, further mutations were produced in the stem region of the RNA secondary structure. In pSFS-mut12 (Figure 4A) the stem was destabilized by replacing in pSFS-mut3 the nucleotide sequence GCGGC at the 5' end of the stem by its complementary sequence CGCCG. This mutation leads to a strong reduction in ribosomal frameshifting (Figure 4B). Restoration of a stable stem structure was done in the double mutant pSFS-mut13 in which both strands of the stem were exchanged (Figure 4A) and led to a frameshifting efficiency comparable with that of the wild-type. This result demonstrated that, in addition to the heptanucleotide signal, the adjacent stem−loop plays an important role in the frameshift event irrespective of the sequence that forms the stable stem.

Nucleic acid-binding activity of the PLRV ORF2b protein

The experiments described above showed that ORF2b was expressed as an ORF2a−ORF2b transframe protein by −1 ribosomal frameshifting involving a new class of frameshift
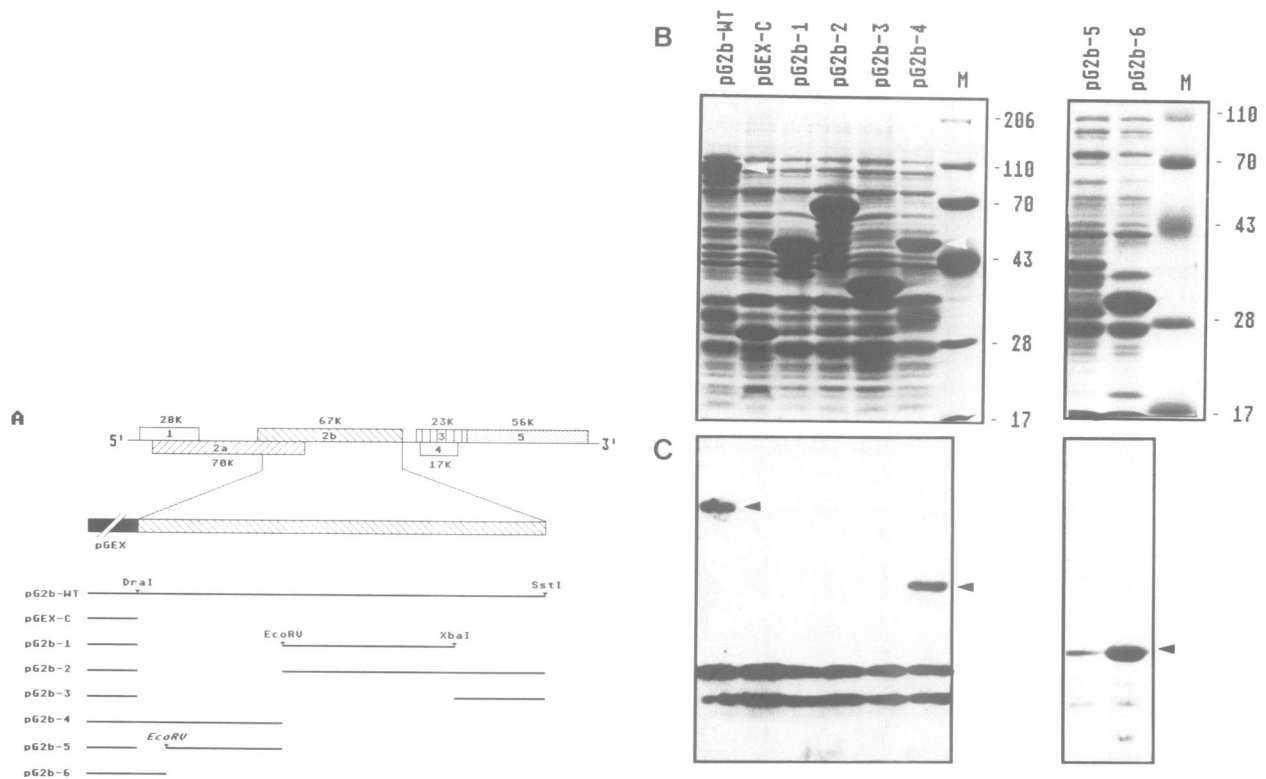
Fig. 5. Nucleic acid-binding activity of the ORF2b protein. **A.** Expression of ORF2b and deletion mutants in *E.coli*. ORF2b was subcloned into the *Sma*I site of the bacterial expression vector pGEX3 (Smith and Johnson, 1988) to yield pG2b-WT. Deletion mutants were produced as indicated by the restriction sites. The *Eco*RV restriction site (italic letters, pG2b-5) was introduced by site-directed mutagenesis. Solid lines represent expressed sequences with the solid line in pGEX-C (control pGEX expression vector without ORF2b sequences) representing the 28 kDa glutathione-*S*-transferase. **B.** PAGE analysis of fusion proteins. Total bacterial extracts were separated on a 10% SDS−polyacrylamide gel and proteins were visualized by staining with Coomassie blue. White arrowheads indicate fusion proteins with RNA-binding capacity. **C.** Autoradiogram of B after blotting of separated proteins onto nitrocellulose membranes and incubation with ^{32}P-labelled single-stranded (+) PLRV RNA. Arrowheads indicate RNA-binding fusion proteins.

sites and an adjacent stem−loop structure. Moreover, we were able to demonstrate that this frameshift event occurred at the 5′ end of the 582 nucleotide overlap between ORF2a and ORF2b (the heptanucleotide motif starts at position 124 of ORF2b). Since a GDD motif, which is typical of viral RNA polymerases (Kamer and Argos, 1984), is found in the C-terminal part of ORF2b, we assumed that an additional important function(s) of the putative PLRV ORF2a/ORF2b replicase resided in the N-terminal part of ORF2b which was included in the transframe protein.

Inspection of the corresponding ORF2b amino acid sequence revealed the presence of a cluster of basic amino acids encoded by the 3′ half of the stem−loop (Figure 3A). As the basic region of another PLRV protein (ORF4, coding for a 17 kDa protein; Figure 1A) has recently been shown to display nucleic acid-binding properties (Tacke *et al.*, 1991), we examined the possibility that the ORF2b basic amino acid cluster would exhibit a similar activity. For this the close to full-length ORF2b protein as well as several deletion mutants (Figure 5A) were expressed in *E.coli* as fusion proteins with glutathione-*S*-transferase (GST) in the pGEX expression vector system (Smith and Johnson, 1988). Total bacterial extracts were subjected to PAGE (Figure 5B), the separated proteins were electroblotted to nitrocellulose membranes and RNA-binding experiments with radiolabelled single-stranded (+) PLRV RNA were performed as described previously (Gramstat *et al.*, 1990; Tacke *et al.*, 1991). Nonspecific binding to *E.coli* proteins of low molecular weight was observed in all lanes. Only those

fusion proteins that contained the basic amino-terminus of ORF2b exhibited the capacity to bind to RNA (pG2b-WT, pG2b-4 and pG2b-6; Figure 5C). Thus the basic amino acid cluster KRQLRHPRRRYKR could possibly represent part of the RNA template-binding site of the PLRV ORF2a/ORF2b replicase. Based on this assumption, ribosomal frameshifting would have to occur to the N-terminal side of the basic cluster (as shown in this paper) in order to preserve this important functional domain in the viral replicase.

Discussion

Here we present the first detailed analysis of −1 ribosomal frameshifting in plants using potato leafroll luteovirus (PLRV) as a model system. The 5′ half of the viral RNA genome contains three major open reading frames, ORF1, ORF2a and ORF2b, with a 582 nucleotide overlap between ORF2a and 2b. The first AUG in ORF2b is located at coordinate 2440 of the PLRV sequence (van der Wilk *et al.*, 1989) some 900 nucleotides downstream of the 5′ end of the overlap with ORF2a. A subgenomic RNA that could serve to translate ORF2b has not been detected in PLRV-infected plants (Tacke *et al.*, 1990) and it had been suggested that ORF2b is expressed as a transframe protein with ORF2a by −1 ribosomal frameshifting (Mayo *et al.*, 1989). We have verified this suggestion by *in vivo* and *in vitro* experiments. Transient expression of chimeric PLRV−GUS constructs in protoplasts of the host plant potato indicated

that the frameshift occurred at an efficiency of ~1%. Similar results were obtained by *in vitro* transcription/translation experiments suggesting that the plant viral signals for ribosomal frameshifting were equally efficient in animal (reticulocytes) and plant (potato protoplasts) systems.

The site of ribosomal frameshifting represents a new class of frameshift signals

A sequence of ~80 nucleotides that mediates frameshifting was identified at the 5' end of the PLRV ORF2a/2b overlap. Two types of information were necessary for the frameshift to occur in this region, (i) a heptanucleotide sequence and (ii) a stem−loop structure located five nucleotides downstream of this sequence. The frameshift sites for a large number of retroviruses, retrovirus-related systems and other vertebrate viruses can be classified into three groups by the presence of consensus sequences with the 3' terminal sequences AAAC, UUUA or UUUU (Hatfield *et al.*, 1990). The PLRV ribosomal frameshift site UUUAAAU, the first plant frameshift sequence analysed in detail, represents another class of sites ending in AAAU. Determination of the transframe protein sequence at the frameshift site has been done for several retroviruses and established that the shift occurred at the 3' codon in the 'slippery' sequence [UUU<u>UUUA</u> (HIV-1, Jacks *et al.*, 1988a), AAA<u>AAAC</u> (MMTV, Hizi *et al.*, 1987) and AAA<u>UUUA</u> (RSV, Jacks *et al.*, 1988b)]. By analogy, ribosomal frameshifting would take place at the sequence AAAU within the PLRV slippery sequence leading to the protein sequence Leu−Asn at the frameshift site. Sequencing of this part of the transframe protein will be needed to confirm this.

Alterations of single nucleotides within the frameshift signal indicated that for PLRV ORF2a/2b frameshifting, UUUA is necessary as well as AAU. This observation is consistent with a model for ribosomal frameshifting which involves simultaneous slippage of the aminoacyl-tRNA at the ribosomal A site and the peptidyl-tRNA at the P site as proposed by Jacks *et al.* (1988b) for RSV and supported by results obtained for IBV. For IBV Brierly *et al.* (1989) reported that single base substitutions within the IBV frameshift site UUUAAAC to UUUACAC or UUUAUAC greatly reduced or even abolished frameshifting, whereas the mutation CUUAAAC retained some activity (~2%). In contrast, with PLRV such differences in frameshift efficiencies were not noted with comparable mutations. As the IBV transframe protein is produced at much higher efficiency (25−30%) than that of PLRV ORF2a/2b (0.8−1.0%), the absence of such differences could be explained by a lack of sensitivity.

A stem−loop structure participates in ribosomal frameshifting

The RNA secondary structure (stem−loop) necessary for efficient expression of ORF2b by ribosomal frameshifting is located only five nucleotides downstream of the heptanucleotide signal. For RSV and IBV the introduction of stem-destabilizing mutations resulted in a decrease of frameshifting (Jacks *et al.*, 1988b; Brierley *et al.*, 1989). Restoration of base pairing and stem formation by specific stem-restabilizing mutations rescued the frameshift event. Also in PLRV destabilizing and restabilizing mutations in the stem region drastically influenced the expression of ORF2b by ribosomal frameshifting. In contrast to PLRV and

other viral systems, frameshifting in HIV only requires the heptanucleotide signal (Wilson *et al.*, 1988). The mechanism by which a stem−loop could influence ribosomal frameshifting is still unknown. Jacks *et al.* (1988b) suggested that ribosomes may slow down or stall at such RNA secondary structures and thereby allow tRNA slippage. Additionally the single-stranded loops may participate in the formation of pseudoknot structures as noted for IBV (Brierley *et al.*, 1989). Mutational analysis of the two largest loops in the PLRV stem−loop (Figure 5A) showed only little, if any change in the frameshifting efficiency (data not shown) and would not favour an involvement of the stem−loop in tertiary structure formation (formation of pseudoknots) as a prerequisite for ribosomal frameshifting. This conclusion is supported by the experiments with pSFS-mut10 (Figure 3) in which the sequences downstream of the stem−loop have been deleted except for 17 nucleotides and which shows wild-type ribosomal frameshifting.

An additional feature of the stem−loop involved in the ribosomal frameshifting event is its capacity to code for a cluster of basic amino acids. This strongly basic region KRxxRHxRRRxKR defines a nucleic acid-binding domain as revealed by binding experiments with bacterially expressed proteins, and may well represent the RNA template-binding site of the viral ORF2a/2b replicase. Similar sequences of eight to ten amino acids high in lysine and arginine are also part of other nucleic acid-binding proteins, where they serve as signals for nuclear localization [for a recent review see Garcia-Bostos *et al.* (1991)]. One example for a nucleus-targeted plant virus RNA polymerase is the tobacco etch potyviral replicase NIb which contains a bipartite signal sequence that directs chimeric GUS−NIb fusion proteins into the nucleus (Restrepo *et al.*, 1990; Carrington *et al.*, 1991).

In addition to this basic region in the ORF2a/2b transframe protein, three other motifs have been assigned to certain functions on the basis of amino acid sequence homologies (Habili and Symons, 1989): one helicase motif each in ORF2a and ORF2b and the putative catalytically active amino acids of the RNA polymerase function in ORF2b. PLRV cDNA encoding ORF2b (modified at the amino-terminus by introduction of an AUG translational start codon) has been stably transformed into potato (D.Prüfer, unpublished data). Such and other transgenic potato lines expressing the ORF2a/ORF2b protein as an in-frame protein may serve as a source to test for the various catalytic activities described. These studies may also provide clues as to whether other viral or cellular proteins have to cooperate with the transframe protein ORF2a/2b in order to form an active PLRV replication complex.

Materials and methods

ORF2a/ORF2b overlap region was inserted into pSP65GUS-*Eco*RV such that both ORF2a and ORF2b were in frame with the flanking GUS sequences (pSFS-WT). Deletion mutants were produced as depicted in Figure 1A and contained the frameshift region from coordinates 1165−2190 (pSFS-mut1), 1735−2397 (pSFS-mut2) and 1165−1947 (pSFS-mut3). The integrity of all constructs obtained was verified by sequence analysis. Plasmid DNAs were linearized with *Bam*HI before *in vitro* RNA transcription.

For *in vivo* frameshift studies a *Sal*I−*Ava*I fragment (coordinates 1505−2160) was isolated from clone pCPL3 by *Sal*I digestion, mung bean nuclease treatment, digestion with *Ava*I and treatment with Klenow DNA polymerase and dCTP followed by mung bean nuclease digestion. The resulting 650 bp fragment containing ~90% of the frameshift region was translationally fused to the GUS gene by insertion into the *Bcl*I-digested, flush ended vector pRT103GUS (Töpfer *et al.*, 1988). This construct (pRFS-WT) served as the wild-type construct for ORF2a/ORF2b frameshifting. As a positive control (100% expression) the same fragment was used after *Sal*I and mung bean nuclease digestion, *Ava*I digestion, repair by Klenow DNA polymerase in the presence of dCTP and dGTP followed by a final mung bean nuclease treatment, and then translationally fused to the GUS gene in the *Bcl*I site of the pRT103GUS vector to yield construct pRFS-PC. An out-of-frame construct (Tacke *et al.*, 1990) served as a control for internal translation initiation in the GUS gene.

Site-directed mutagenesis of pSFS-mut3

Restriction of pSFS-mut3 with *Hind*III released a 1400 bp fragment containing the 5′ half of the ORF2a/ORF2b overlap region including the heptanucleotide signal and stem−loop and the GUS gene carboxy-terminus. This fragment was inserted into the *Hind*III site of the pSELECT vector (Promega) and transformed into the *E.coli* strain JM109 (Hanahan, 1985). After infection with the helper phage RK408, single-stranded template DNA for mutagenesis was isolated and used for site-directed mutagenesis according to the protocol. Mutations were verified by sequence analysis, appropriate plasmid DNAs were cut with *Hind*III and the mutant fragments were reinserted into the *Hind*III-digested pSFS-mut3 vector to reconstitute the mutant derivatives described in Figures 2, 3 and 4.

In vitro transcription/translation experiments

Single-stranded RNAs were obtained by *in vitro* transcription of *Bam*HI-linearized pSP65 constructs by SP6 RNA polymerase in the presence of the cap analogue m^{7}GpppG according to established protocols (Melton *et al.*, 1984). RNAs were translated in the rabbit reticulocyte system (Amersham, Brauschweig) in the presence of ^{35}S-labelled methionine, and translational products were analysed on 10% SDS−polyacrylamide gels.

Transient expression experiments

Protoplasts were isolated from *Solanum tuberosum* (cv. Desiree), and Ca(NO$_3$)$_2$/polyethylene glycol-mediated DNA transfer was performed as described by Negrutiu *et al.* (1987) using 3.3 × 10^5 protoplasts and 10 μg of plasmid DNA per transfection experiment. GUS activity in protein extracts was determined by a fluorometric assay (Jefferson, 1987).

Bacterial expression of PLRV proteins and nucleic acid-binding studies

ORF2b fusion proteins with glutathione-*S*-transferase (GST) were expressed in the pGEX vector system (Smith and Johnson, 1988). An ORF2b cDNA fragment (*Dra*I−*Sac*I fragment, coordinates 1770−3500) was treated with mung bean nuclease and inserted into the pGEX3 *Sma*I site. Deletion derivatives were produced as described in Figure 5A. Filter-binding assays with ^{32}P-labelled PLRV RNA were performed as described by Gramstat *et al.* (1990).

Acknowledgements

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