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Translational frameshifting mediated by a viral sequence in plant cells

(barley yellow dwarf virus/β-glucuronidase fusion/protoplasts)

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ABSTRACT **It has been proposed that the polymerase gene of barley yellow dwarf virus and related viruses is expressed by a ribosomal frameshift event during translation. The 5′ end of this gene overlaps with the 3′ end of an upstream gene that is in a different reading frame. The region of overlap is similar to sequences in retro- and coronaviruses that are known to express their polymerase genes by frameshifting. This overlap region includes a "shifty" heptanucleotide, followed by a highly structured region that may contain a pseudoknot. Sequences of 115 or 144 base pairs that span this region from barley yellow dwarf virus (PAV serotype) genomic RNA were introduced into a plasmid, so that a reporter gene could be expressed in plant cells only if a minus one (−1) frameshift event occurred. Frameshifting was detected at a rate of ≈1%. This frameshifting was abolished when the stop codon at the 3′ end of the upstream open reading frame was deleted. A sequence expected to form a strong stem-loop immediately upstream of the frameshift site was unnecessary for frameshifting, and initiation at AUG codons within the stem-loop appeared to be inhibited. Like viruses that infect hosts in other kingdoms, plant viruses also can induce frameshifting in translation of their genes.**

A diverse group of viruses and other mobile genetic elements has been shown to translate overlapping open reading frames (ORFs) by inducing ribosomes to change reading frame in the region of overlap during translational elongation (for review, see ref. 1). Minus one (−1) ribosomal frameshifting by eukaryotic RNAs to express an RNA-dependent polymerase has been described for retroviruses (2, 3), coronaviruses (4, 5), retrotransposons (6), and a yeast double-stranded RNA (7). Presumably this frameshifting facilitates controlled low-level synthesis of the polymerase that is needed only in small quantities. The signals responsible for −1 ribosomal frameshifting are encoded in the sequence at and around the frameshift site. These signals include a "slippery site" heptamer at the frameshift site consisting of a run of three adenine, uracil, or guanine residues followed by the tetranucleotide UUUA, UUUU, or AAAC (8); in most cases, this is followed by a sequence predicted to form a pseudoknot (for review, see ref. 9).

The genome organizations of the luteoviruses (refs. 10–12; for review, see ref. 13), dianthoviruses (14), and pea enation mosaic virus (15) suggest, that for each of these viruses, the polymerase gene is expressed via a −1 translational frameshift event. In all cases, the 5′ end of the putative polymerase ORF overlaps with the 3′ end of an upstream ORF, and the region of overlap has structural similarity to the frameshift regions in the above viruses (2–7). Luteoviruses have a single-stranded, positive-sense RNA genome containing six ORFs (13). The first two ORFs (39 kDa and 60 kDa) at the 5′ end of the genome of barley yellow dwarf luteovirus (PAV serotype, BYDV-PAV) overlap by 13 nucleotides (nt) and are in different reading frames (Fig. 1). We proposed that the 60-kDa ORF is expressed by ribosomal frameshifting—i.e., a small proportion of ribosomes slips into the −1 frame, relative to the 39-kDa ORF, before encountering the termination codon and continues to translate the 60-kDa ORF (10). This translation would be expected to result in a low-abundance 99-kDa fusion protein and larger amounts of "correctly" terminated 39-kDa protein. When genomic RNA was translated *in vitro*, products consistent with the above prediction were, indeed, observed (16, 17). In addition, cell-free translations of RNAs from another luteovirus (18) and from red clover necrotic mosaic dianthovirus (RCNMV; ref. 19) provided evidence for translational frameshifting by related viruses. The region of reading frame overlap in BYDV-PAV contains a potential "shifty" heptanucleotide: GGGUUUU followed immediately by a sequence that can form a double pseudoknot or a single large stem-loop (Fig. 2). In addition, a stable hairpin ($\Delta G = -25.6$ kcal/mol) upstream of the shifty heptanucleotide is predicted. Such a structure has not been reported in viruses known to induce frameshifting.

To monitor frameshifting and other translational phenomena in a sensitive and quantitative fashion, we used a reporter system in which the putative frameshift-inducing sequence was inserted between the start codon and the remaining portion of the β-glucuronidase (GUS) coding region. The GUS gene was placed in various reading frames relative to the start codon so that frameshifting was required for expression. Plasmids containing these constructs were introduced into plant cells, and the amount of GUS enzyme activity was observed. GUS activity should be proportional to the amount of frameshifting that occurs to allow translation of the GUS gene.

MATERIALS AND METHODS

Protoplast Transformation and β-Glucuronidase Assay. Carrot (*Daucus carota* L.) wild-type cell line 49-1 was provided by E. Wurtele (Botany Department, Iowa State University). The cells were subcultured weekly in MS medium (23) containing 2,4-dichlorophenoxyacetic acid at 1.2 mg/liter in a ratio of 1/5 of suspension/medium. Cultures were grown at room temperature on a rotary shaker (110 rpm) in the light. Protoplasts were isolated and prepared for electroporation 3 days after subculture by the method of Fromm *et al.* (24), except that cellulase treatment was for 20 hr at room temperature. One milliliter of protoplasts (2×10^6 protoplasts per ml) was mixed with 30 μg of either plasmid DNA or carrier DNA (salmon sperm; Sigma). Samples were

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Abbreviations: BYDV, barley yellow dwarf virus; BYDV-PAV, BYDV-PAV serotype; RCNMV, red clover necrotic mosaic virus; ORF, open reading frame; nt, nucleotide(s); GUS, β-glucuronidase.
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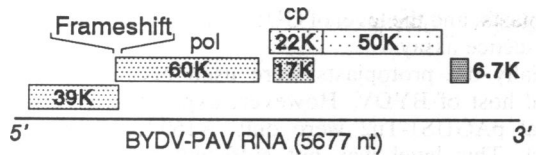


FIG. 1. Major ORFs deduced from the BYDV-PAV RNA sequence (10). pol, Putative RNA-dependent RNA polymerase; cp, coat protein; K, kDa.

kept on ice 15 min before electroporation, which was performed by delivering one pulse at 350 V, 500 μF (Gene Pulser; Bio-Rad). After electroporation, protoplasts were allowed to stand for 15 min in ice and were then diluted with 5 ml of MS medium containing 2% sucrose/0.3 M mannitol and 2,4-dichlorophenoxyacetic acid (24). Electroporated protoplasts were cultured at 26°C in the dark for 40 hr before collection for analysis. Each construct was assayed in triplicate. GUS was assayed as described (25).

Plasmid Construction. The vector used for all constructs was pAGUS1-Tn2 (20) a gift of J. Skuzeski, University of Nebraska. The vector contains a duplicated cauliflower mosaic virus 35S promoter, the Ω 5′ leader of tobacco mosaic virus (26), and a multiple cloning site followed by the GUS gene. The integrity of all constructs was verified by sequencing the entire insert.

The first series of plasmids [pL(0)UAG, pL(−1)UAG pL(+1)UAG, and pL(0)UCG], called set L, contains a 144-base-pair (bp) fragment from nt 1103 to nt 1246 in the BYDV-PAV genome (10) adjacent to the GUS gene. This viral sequence (Fig. 2*A*) encompasses the region of overlap of the 39-kDa and 60-kDa ORFs and contains all the structures thought to be involved in frameshifting. In our plasmid nomenclature, the number in parentheses is the position of the GUS ORF, relative to the initiation codon. The sequence after this indicates whether a stop codon is present at the end of the first (wild-type 39-kDa) ORF.

The plasmids were obtained by PCR, starting with pPA142 (10) as template. The upstream primer UP1: 5′-GAAC-CATGGGTCCTGAAGCACGTGCCAG-3′ contained an *Nco* I site (underlined) that introduced an ATG and BYDV-PAV bases 1103–1121. The downstream primer DP1: 3′-GCCAGTGTCTGCCTCGGGCCGAAGTACTTTC-GAATCCCCGGGAAC-5′ contained BYDV-PAV bases 1246–1225 and three restriction sites: *Bsp*HI, *Hin*dIII, and *Apa* I (underlined). The *Hin*dIII and *Apa* I sites are one and two bases, respectively, out-of-frame relative to the same sites in the vector. This facilitated cloning the insert in any of the three reading frames relative to the GUS gene, depending on which of the three restriction sites was used to clone into pAGUS1-Tn2. The amplified fragment was digested with *Nco* I and either (*i*) *Hin*dIII to create pL(−1)UAG, (*ii*) *Apa* I to create pL(+1)UAG, or (*iii*) *Bsp*HI to create pL(0)UAG. These fragments were cloned into pAGUS1-Tn2 that had been cut with the same enzymes, with the exception of the *Nco* I–*Bsp*HI fragment that was cloned into pAGUS1-Tn2 cut with *Nco* I only. After the *Nco* I–*Bsp*HI fragment was cloned, an ATG at the 3′ end of the viral insert, in-frame with the GUS gene, was changed to TAC by using the upstream primer UP1 and a mutagenic primer DP2: 3′-CCTCGGGCCGAAGATG-CATTCGAATCGC-5′ (base changes are underlined). pL(0)UCG, in which the stop codon (TAG) at the end of the 39-kDa ORF in pL(0)UAG was changed to TCG (serine) was constructed by a two-step PCR method (27), using the

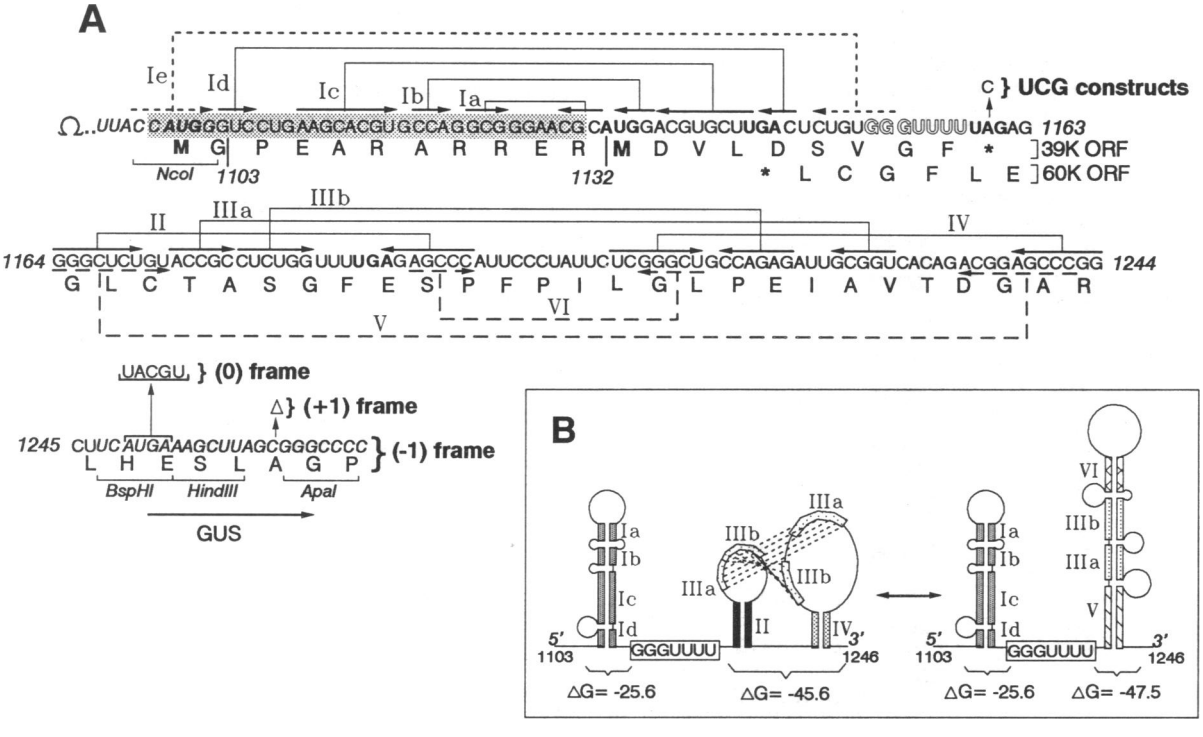


FIG. 2. (*A*) Linear representation of the viral sequence cloned into pAGUS1-Tn2 (20). Sequence of pL(−1)UAG, beginning at the 3′-terminal three bases of the Ω sequence is shown. Base numbering is as in BYDV-PAV genomic RNA (10). Vector-derived bases are italicized, and the shifty heptanucleotide is in outlined text. Amino acid sequences of the 39-kDa ORF, 60-kDa ORF, and beginning of the GUS ORF are shown. Constructs containing GUS in the 0 frame and +1 frame relative to the 39-kDa ORF have the indicated substitutions or deletion (Δ). Plasmids with UCG in their name have the indicated base change in the 39-kDa ORF stop codon. The shaded sequence is absent from S series plasmids that are otherwise identical to those in series L. This recreates the *Nco* I site around the AUG at nt 1132 in the S series. Sequences that have the potential to base-pair are indicated by connected arrows. Helix Ie (dotted lines) requires the presence of vector sequences. Helices V and VI (dashed lines) are alternatives to helices II and IV. (*B*) Schematic diagram of alternative structures (in the absence of vector sequences) that can result from the base pairing in *A*. Free energies for stem-loops (in kcal/mol) were calculated with the RNASE computer program (21), using the parameters in ref. 22. Dotted lines connecting helices IIIa and IIIb illustrate the potential pseudoknot.

mutagenic primer DP3: 3′-CACCCAAAAAGCTC-CCCGAGA-5′ and flanking primers UP1 and DP2. The final amplified fragment was digested with *Nco* I and *Hin*dIII and cloned into pAGUS1-Tn2 cut with the same enzymes.

Plasmid series S was derived from the previous set of plasmids by deleting 28 viral bases and the first AUG from the 5′ extremity of the viral sequence in set L (underlined in Fig. 2*A*). The constructs were obtained by PCR using upstream primer UP2: 5′-ACCCATGGACGTGCTTGACTCT-GTGG-3′ with each of the above L series plasmids as templates. UP2 includes an *Nco* I site (underlined) and the viral nt 1131–1153. When pL(0)UCG or pL(0)UAG were templates, the downstream primer was DP2, and the amplified fragments were cloned into the *Nco* I and *Hin*dIII sites of pAGUS1-Tn2, to give pS(0)UCG and pS(0)UAG, respectively. When pL(−1)UAG and pL(+1)UAG were templates, the downstream primer was DP1. The fragments were cloned into *Nco*I–*Hin*dIII and *Nco* I–*Apa* I-cut pAGUS1-Tn2 to generate pS(−1)UAG and pS(+1)UAG, respectively. pS(−1)UCG was derived from pS(−1)UAG by the method described for pL(0)UCG, except that UP2 was the upstream primer.

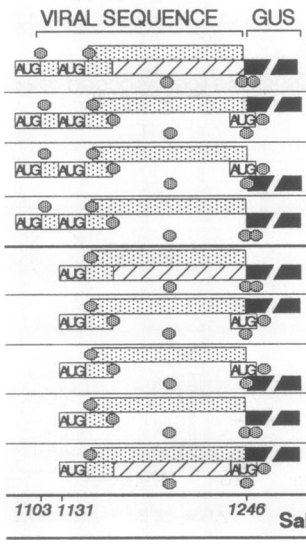
RESULTS

To determine whether ribosomal frameshifting could be induced *in vivo*, plasmid series L was constructed in which the BYDV-PAV sequence from nt 1103 to 1246 was inserted between the start codon of the GUS gene and the remainder of the coding region so that the start codon was in a different reading frame in each construct relative to the GUS coding region (Fig. 3). Bases 1103–1246 were selected because they contain the entire region of overlap, including the putative shifty heptanucleotide site and a sequence that can fold in either of the two secondary structures in Fig. 2*B*. This plasmid series also contains an upstream stem-loop not found in other frameshifting RNAs, the significance of which we wanted to test. The above plasmids were electroporated into protoplasts, and the level of GUS activity was determined by fluorescence assay.

Initially oat protoplasts were used because oats are a natural host of BYDV. However, expression levels of the original pAGUS1-Tn2 were only ≈160-fold above background. This level was not sufficient to allow accurate quantitation of frameshifting, which was ≈1%, giving GUS activities ≈1.5- to 2-fold greater than background (data not shown). This result was likely due to the relatively low expression from both the 35S promoter (24) and the Ω sequence (28) in monocots compared to dicots. Thus, all subsequent experiments were performed using (dicotyledonous) carrot protoplasts, in which GUS activity 48 hr after electroporation with pAGUS1-Tn2 was 1000-fold greater than background (data not shown).

Clone pL(−1)UAG, which contained the GUS coding region in the −1 reading frame relative to the AUG, expressed GUS at a level of 0.6–1.8% of that induced by the positive control plasmid pL(0)UCG containing GUS in the same frame as the AUG with no intervening stop codons (Fig. 3, experiments 1 and 3). In both experiments, the level of GUS activity from pL(−1)UAG was ≈4-fold greater than that measured in protoplasts inoculated with salmon sperm DNA alone. When the GUS gene was in the +1 frame and separated from the AUG by two additional stop codons [pL(+1)UAG], GUS activity was indistinguishable from background. Plasmid pL(0)UAG, in which the GUS gene is in-frame with the AUG, but which retains the 39-kDa ORF stop codon, gave 0.6% of the GUS activity of the positive control in experiment 1. This activity was ≈1.8 times background and 36% of the level induced by pL(−1)UAG after subtracting background.

Fortuitously, the 5′ nonviral sequence in the L series of constructs slightly extended the predicted stem-loop I, incorporating the first AUG (Fig. 2*A*, helix Ie). To test the effect of stem-loop I on GUS expression, this stem-loop was disrupted by deletion of 28 bases from the 5′ end of the viral sequence of the above series of vectors to create plasmid



Plasmid	Expt. 1 nmol MU/ug/min	%	Expt. 2 nmol MU/ug/min	%	Expt. 3 nmol MU/ug/min	%
pL(0)UCG	347.3±20.4	100			609±23*	100
pL(-1)UAG	8.7±0.7	1.8			4.9±0.6	0.6
pL(+1)UAG	2.3±0.3	0			1.2±0.1	0.01
pL(0)UAG	4.8±0.6	0.6				
pS(0)UCG			3397±494*	100	2453±80	100
pS(-1)UAG			32.0±3	0.8	26.9±4.3	1.1
pS(+1)UAG			4.2±0.3	0.01	1.3±0.1	0.01
pS(0)UAG			7.5±2	0.1		
pS(-1)UCG					1.4±0.2	0.01
Salmon sperm DNA	2.6±0.5	0	4.0±1.0	0	1.1±0	0

FIG. 3. Maps of plasmids and levels of GUS activity produced by protoplasts transformed with them. At left are schematic representations of relevant portions (shown in Fig. 2) of plasmids containing viral sequence fused to the GUS gene in pAGUS1-Tn2. Positions of ORFs and all AUGs and stop signals (shaded octagons) are shown with −1 reading frame at top, 0 frame in center, and +1 frame at bottom for each construct. Position of the GUS ORF (solid interrupted box) relative to first AUG is indicated in parentheses in the plasmid name. Portions of the 39-kDa and 60-kDa ORFs (nt 1103–1246) are represented by stippled boxes. Diagonally filled box is the extension of the 39-kDa ORF that results from deletion of the stop codon. The results of three transient expression experiments in carrot protoplasts are shown to the right of maps. GUS activity is expressed as nmol of methylumbelliferone (MU) synthesized per μg of total protein per min. Each point represents the average of three replicates ± SD, except those marked with an asterisk, which are averages of two replicates. Percentage of GUS activity is calculated relative to in-frame constructs pL(0)UCG or pS(0)UCG, after subtraction of background GUS activity in cells electroporated with salmon sperm DNA. Expt., experiment.

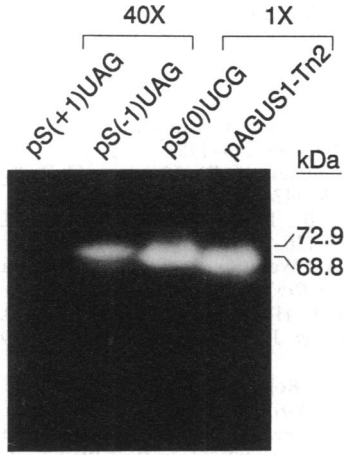


FIG. 4. Gel electrophoresis of GUS proteins. Extracts from protoplasts transformed with indicated plasmids were prepared as described (25). GUS proteins were visualized by fluorescence assay in a 7.5% polyacrylamide gel after electrophoresis by the method of Scott *et al.* (29). Extracts from protoplasts electroporated with pS(−1)UAG and pS(+1)UAG were concentrated 40-fold using microconcentrators (Centricon-30; Amicon), before loading. Known molecular mass of GUS from pAGUS-Tn2 vector (68.8 kDa) and expected molecular mass of virus–GUS fusion protein (72.9 kDa) are indicated at right.

series S. This series was designed to initiate translation at a viral AUG (nt 1132), which normally encodes an internal methionine in the 39-kDa ORF, 27 bases upstream of the stop codon. This deletion reduced the predicted stability of the helix containing the first AUG from $\Delta G = -34.7$ to -8.7 kcal/mol. The relative level of GUS activity in cells electroporated with pS(−1)UAG was 0.8–1.1% of the positive control in two separate experiments (Fig. 3). These levels are 8- and 24-fold higher than background. When the L and S sets were compared in the same experiment, expression of GUS by the plasmids in set S was 4- to 6-fold greater than their counterparts in set L (Fig. 3, experiment 3). The only exceptions were the +1 constructs that yielded no significant GUS activity in either case. Thus, although total expression was increased in set S, the amount of frameshifting was not greatly affected by deletion of the upstream bases.

Unlike most other known shifty heptanucleotides (8, 9), that of BYDV-PAV is immediately 5′ of the stop codon of the first reading frame. To test any role the UAG codon at the end of the 39-kDa ORF may play in frameshifting, this stop codon in pS(−1)UAG was changed to UCG, extending the length of the overlap by 99 bases to obtain plasmid pS(−1)UCG (Fig. 3, experiment 3). GUS activity in cells electroporated with this plasmid was indistinguishable from background, indicating that the 39-kDa stop codon is required for GUS to be expressed from the −1 frame.

Were the activity from pS(−1)UAG, indeed, from frameshifting, the GUS fusion gene product should be 45 amino acids larger than the wild-type GUS protein. To test this hypothesis, size of the functional GUS gene product was determined by gel electrophoresis of proteins from electroporated protoplasts. As expected, the fluorescent protein GUS from cells electroporated with pS(0)UCG or pS(−1)UAG migrated slightly more slowly than that from cells transformed with pAGUS1-Tn2 (Fig. 4).

DISCUSSION

Evidence for Translational Frameshifting. As predicted, frameshifting by the ribosomes into the −1 frame best explains the results. Although the level was quite low—0.6–1.8%—the values are clearly significant. Two features of the system were essential to allow detection of this event. (*i*) The system is so sensitive that the activity of the pS(−1)UAG construct, for example, was as high as 24 times background level (Fig. 3, experiment 3), even though it was only 1.1% as active as the positive control [pS(0)UCG]. The presence of virus-derived amino acids at the amino terminus of the GUS protein did not reduce enzyme activity (data not shown, and Fig. 4). (*ii*) There was little variation among the samples, verifying the significance of small differences within an experiment.

Explanations other than frameshifting for the GUS activity induced by the −1 constructs include the following: (*i*) internal initiation within the GUS ORF, (*ii*) RNA splicing that aligns the start codon with the GUS ORF and eliminates intervening stop codons, (*iii*) initiation at the intended AUG followed by ribosomal hopping (30) over the viral sequence into the GUS ORF, and (*iv*) some type of RNA editing. The first possibility is ruled out because the first AUG within the GUS ORF is not until the 110th amino acid. Initiation at this site would delete 18% of the protein; yet the GUS product in cells transformed with pS(−1)UAG is slightly larger than wild-type GUS. Because of this observation, any RNA splicing or ribosomal hopping would have to involve very short sequences. The likelihood of the latter possibilities as well as RNA editing occurring is further reduced by the fact that a single base change in the 39-kDa stop codon (UAG to UCG) eliminated GUS activity in the −1 frame. This change would have had to fortuitously eliminate the signals required for splicing, hopping, or editing. Editing would be most unlikely because it is known to occur only in mitochondria (31). Finally, *in vitro* translation of BYDV-PAV genomic RNA (17) revealed a high abundance 39-kDa polypeptide and low levels of a 99-kDa product, as predicted by −1 frameshifting. Although the net change in reading frame is −1, direct amino acid sequencing of the polypeptide product will be required to distinguish this from −4, +2, and other larger shifts that can occur within the 13-nt overlap.

A low level of in-frame readthrough may have occurred. GUS activity induced by pL(0)UAG and pS(0)UAG was 36% and 12%, respectively, of that induced by their (−1) counterparts (Fig. 3, experiments 1 and 2). However, in both cases the activity was only 1.8-fold greater than background. Thus its significance may be questionable.

Frameshift Mechanism. The sequence sufficient to promote −1 frameshifting has primary and secondary structural similarities with other sequences that have been demonstrated to promote frameshifting in animal cells (32, 33), in yeast (6, 7), and in *Escherichia coli* (34, 35). Thus, the BYDV-PAV sequence likely uses the same mechanism. Jacks *et al.* (8) proposed a simultaneous slippage mechanism in which the tRNAs in the peptidyl and aminoacyl sites of the ribosome slip 1 nt in the 5′ direction on the mRNA, after which translation proceeds in the new reading frame. For BYDV-PAV, the slippery site (GGGUUUU) would cause the slippage of a $tRNA^{Gly}$ (ACC) in the peptidyl site and a $tRNA^{Phe}$ (AAA) in the aminoacyl site. After the −1 slippage only the wobble base (adenine) of the $tRNA^{Gly}$ would not be base-paired to the BYDV RNA.

The rate of frameshifting induced by the BYDV-PAV sequence is lower than that reported for coronaviruses (25%; ref. 32) but is more similar to that of human immunodeficiency virus (1.5%–4%; ref. 33). Because the viral sequence can induce frameshifting in an uninfected cell and in the absence of viral proteins, like all other known examples, no trans-acting viral or virus-induced factors are necessary for the level of frameshifting seen. However, the rate of frameshifting may be affected by the infection process. For example, Hatfield *et al.* (36) suggested that an increase in frameshift efficiency in human immunodeficiency virus-infected cells may be brought about by an increase in the population of unmodified, "shifty" tRNAs. The effect of infection on

frameshifting of a reporter gene has not been reported for any virus.

Role of the 39-kDa ORF Stop Codon. The crucial role in frameshifting played by the 39-kDa ORF stop codon located immediately 3′ to the shifty heptanucleotide was unexpected. An identical situation is found in Rous sarcoma virus where the stop codon of the *gag* gene borders the shifty site. However, in that case, the mutation of this stop codon to a sense codon did not affect the efficiency of frameshifting *in vitro* (8). In contrast, Weiss *et al.* (35) demonstrated that deletion of a stop codon immediately adjacent to a synthetic −1 frameshift site dramatically reduced frameshift efficiency in *E. coli*. For BYDV-PAV, we suspect the stop codon may act by causing a pause in decoding, enhancing slippage by the ribosome at the shifty site. A downstream pseudoknot proposed to serve this ribosomal pausing function is required for coronavirus frameshifting (32) but not for human immunodeficiency virus (34, 37). Two alternative structures downstream of the BYDV-PAV shifty heptanucleotide are possible (Fig. 2). One is an unusual double pseudoknot involving two stem-loops with base-pairing between the loops (Fig. 2, IIIa and IIIb). The other structure is a large stem-loop, where only the IIIa and IIIb helices are maintained when compared with the former representation. This latter structure is similar to that proposed 3′ of the probable frameshift site of RCNMV (19). The existence of these structures and their role (if any) in mediating frameshifting remain to be investigated.

Role of the Upstream Putative Stem-Loop. Reduced GUS expression in the L series relative to the S series of plasmids may have been due to confinement of the two in-frame AUGs in a stem-loop structure. Inhibition of initiation by confinement of a start codon within a stem-loop has been reported (38–40). Stem-loop I is likely to be even more stable than its predicted free energy, due to the sequence of the loop connecting the two strands of the helix: GGAA. This fits the consensus for a "tetraloop": GNRA, a sequence that has been shown to confer anomalously high stability to stem-loops (41). This inhibition of initiation is unlikely to play a role in viral gene expression, because no natural initiator AUGs exist within it. This sequence appears unnecessary for frameshifting.

Gene Expression Strategy. In summary, the fact that a plant viral sequence can induce ribosomal frameshifting indicates that plant viruses, like various vertebrate and yeast viruses, can use this unusual translational event to produce a protein needed only in small quantities relative to the upstream gene product. This frameshifting provides an elegant regulatory mechanism requiring minimal genetic information from the virus.

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