Translational Frameshifting by Barley Yellow Dwarf Virus RNA (PAV Serotype) in *Escherichia coli* and in Eukaryotic Cell-Free Extracts

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Received 3 November 1992. Accepted 22 March 1993.

The open reading frame (39K ORF) at the 5′ end of the genome of barley yellow dwarf virus, PAV serotype (BYDV-PAV), overlaps with a 60K ORF by 13 nucleotides. Several approaches were used to show that the 60K ORF (putative polymerase gene) is translated by a low-frequency frameshift event in which some ribosomes shift into the 60K ORF rather than terminate at the 39K ORF stop codon. A sequence encompassing this region of overlap induced minus one (−1) translational frameshifting in heterologous and native contexts. In *Escherichia coli,* with the α subunit of *lacZ* used as a reporter gene, the rate of frameshifting caused by the BYDV-PAV sequence was approximately 3%. Amino acid sequencing of the transframe protein confirmed that ribosomes slip into the −1 frame in the overlapping region which includes a consensus shifty heptanucleotide: GGGUUUU. In a wheat germ translation system, BYDV-PAV genomic RNA from virions frameshifted about twice as efficiently as full-length transcripts from a cDNA clone. Frameshifting in rabbit reticulocyte lysates was much lower for either template. The identity of the 99-kDa wheat germ translation product was verified as the transframe protein by immunoprecipitation with antibody specific for the 60K ORF. These results support our previous observations of frameshifting in protoplasts and illustrate a subtle molecular control mechanism between this pathogen and its host cells.

Additional keywords: expression vectors, *in vitro* translation, protein sequencing, viral gene expression, luteovirus.

Most plant viruses have RNA genomes and replicate in the cytoplasm of their host cells. Thus, they cannot exploit the elaborate host machinery that regulates gene expression at the level of transcription to regulate their own genes. For this reason, translational control is a major step in regulating gene expression of many RNA viruses. Sequences and structures within certain viral RNAs can cause the host translational machinery to decode them via mechanisms not known to occur with host genes (Gesteland *et al.* 1992). One of these unusual events, ribosomal frameshifting, is a means by which the host is induced to translate a viral gene at the controlled, low level required for the proper balance of viral proteins to sustain an infection (Atkins *et al.* 1990; Dinman and Wickner 1992). The polymerase genes of some viruses and retrotransposons are expressed by a minus one (−1) ribosomal frameshift; i.e., before terminating translation of an upstream, overlapping open reading frame (ORF), a proportion of the ribosomes slip back one nucleotide and begin translating the polymerase gene. Control of −1 frameshifting has been studied for retroviruses (reviewed by Hatfield *et al.* 1992), coronaviruses (Brierley *et al.* 1991), and a yeast double-stranded RNA (Dinman and Wickner 1992), as well as members of the luteovirus (Brault and Miller 1992; Prüfer *et al.* 1992) and dianthovirus (Xiong *et al.* 1993) groups. The efficiency ranges from 1 to 50%. Ribosomal frameshifting is induced by signals in the mRNA at and around the frameshift site. These include a shifty heptanucleotide constituting the frameshift site, usually consisting of a run of three adenine, uracil, or guanine residues followed by the tetranucleotide UUUA, UUUU, AAAC (Jacks *et al.* 1988), or AAAU (Prüfer *et al.* 1992). Beginning four to eight bases 3′ of the shifty heptanucleotide is a structured sequence which may form a pseudoknot (Brierley *et al.* 1991; Chamorro *et al.* 1992; Dinman and Wickner 1992; Morikawa and Bishop 1992; ten Dam *et al.* 1990) or a stable stem-loop (Parkin *et al.* 1992; Prüfer *et al.* 1992).

The genome of barley yellow dwarf virus, PAV serotype (BYDV-PAV) consists of a 5,677-nucleotide polycistronic RNA (Miller *et al.* 1988). A number of unusual translational phenomena, including ribosomal frameshifting, initiation at two AUGs of overlapping genes, and in-frame stop codon readthrough are likely to occur during gene expression of BYDV-PAV RNA (Brault and Miller 1992; Dinesh-Kumar *et al.* 1992). The second (60K) ORF in the genome is expected to be translated by low-frequency ribosomal frameshifting at the end of the 39K ORF (Figs. 1,2). It has been shown previously that *in vitro* translation of full-length BYDV-PAV RNA gives products consistent with production of the high molecular weight (99 kDa) polypeptide predicted by translational frameshifting (Young *et al.* 1991), but the peptides were not assigned to ORFs. Previously, we inserted a 115-base sequence that

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MPMI Vol. 6, No. 4, pp. 444-452

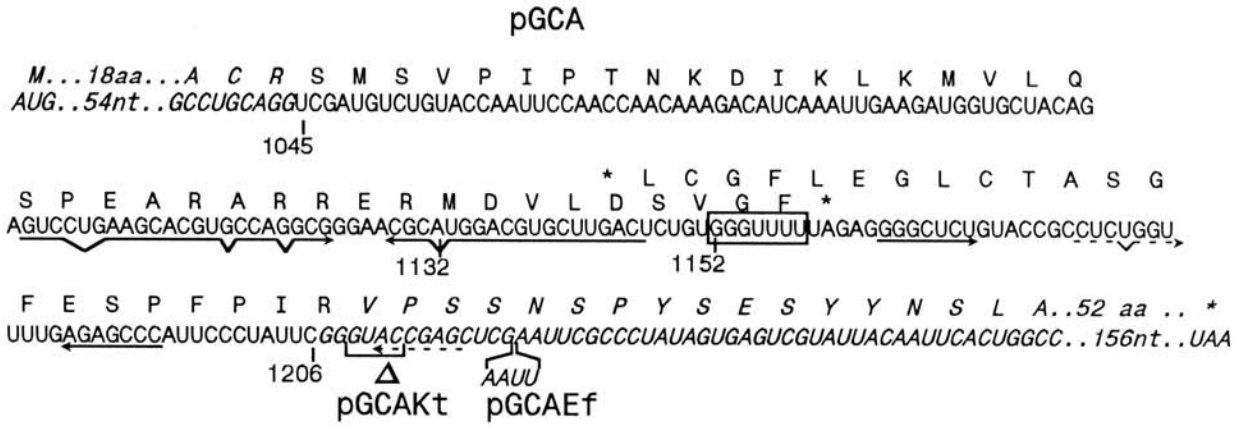


Fig. 1. Sequence of viral insert and flanking bases encoded by pGCA. Vector bases and amino acids (*lacZ* α peptide) are in italics. Amino acid sequence of the C-terminus of 39K ORF and the N-terminus of 60K ORF are shown. Four-base deletion (bracketed above D) and insertion (AAUU) to generate pGCAKt and pGCAEf, respectively, are shown below the sequence. Underlined bases indicate potential base-paired regions with arrowheads indicating proximal ends of each strand. Dashed arrows indicate potential weak pairing between virus (CUCUGGU) and vector (ACCGAG) sequences that may replace viral basepairing to form a pseudoknot. Shifty heptanucleotide is boxed. Viral bases are numbered as in Miller *et al.* (1988).

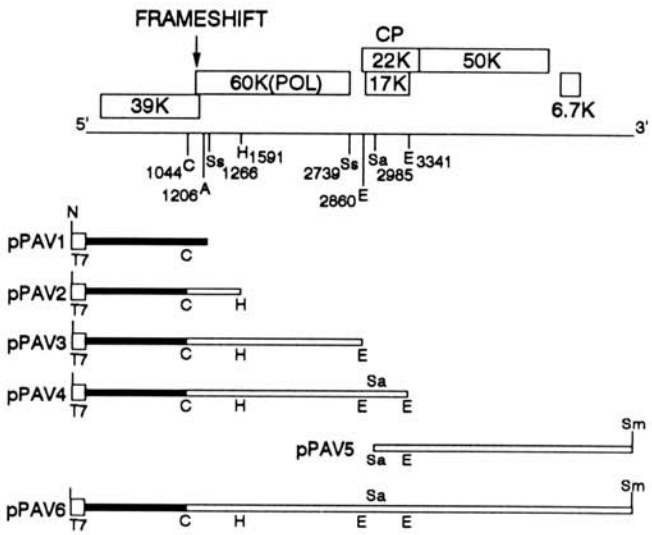


Fig. 2. Genome organization of BYDV-PAV RNA and schematic diagram of full-length clone assembly. Large ORFs are indicated above relevant restriction sites, numbered as in Miller *et al.* (1988). Maps of inserts in intermediate plasmids used to assemble full-length clone (see text) are diagrammed below the genome organization. Boxes at left end of the maps represent the T7 RNA polymerase promoter; solid bars depict the portion from BYDV-PAV-IL, the remainder (open bars) is from the Australian isolate of BYDV-PAV (Miller *et al.* 1988). *Ssp*I fragment spanning bases 1266–2739 was expressed in pETP. Abbreviations: A, *Ava*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Sa, *Sal*I; Sm, *Sma*I; Ss, *Ssp*I.

spans the frameshift signal of BYDV-PAV RNA between the start codon and the remainder of a β-glucuronidase (GUS) gene in a vector so that a −1 frameshift was required for GUS expression (Brault and Miller 1992). In a transient expression assay, in carrot protoplasts, both the size of the GUS enzyme produced and levels of activity were best explained to result from a −1 frameshift event occurring at a frequency of about 1%.

Here, we take advantage of previous observations of accurate frameshifting on eukaryotic viral signals in *Escherichia coli* (Weiss *et al.* 1989), to identify the frameshift site by direct amino acid sequencing of the transframe protein expressed in *E. coli*. Because eukaryotic gene expression *in vivo* is subject to numerous potential RNA modifying activities such as low levels of RNA editing, splicing, or transcriptional stuttering, which may explain the observed expression, we also used eukaryotic cell-free translation systems. Wheat germ and reticulocyte systems were used to confirm observation of translational events only, and to study frameshifting by full-length BYDV-PAV RNA in its native context. Antiserum, prepared against the product of the 60K ORF which had been expressed in *E. coli*, was used to identify the frameshift product produced *in vitro*.

RESULTS

Translational frameshifting in *E.coli*.

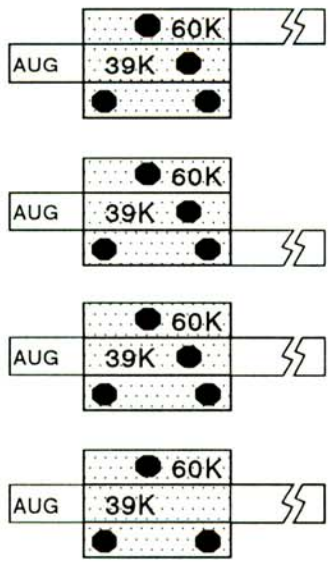
To identify the precise frameshift site, we sought to express the frameshift protein at levels high enough to allow purification of the transframe protein and determination of the amino acid sequence across the frameshift site. We were unable to obtain sufficient quantities of the putative frameshift product from plant protoplasts, so we expressed the protein in *E. coli* using a high level expression vector. A fragment of the BYDV-PAV genome (bases 1045–1206), which included the region of overlap between the 39K and 60K ORFs, was inserted in pGEM3Zf(−) (Fig. 1). For *lacZ* to be translated, ribosomes would have to shift from the initiator AUG reading frame (0 frame) at the 5′ side of the viral sequence to the *lacZ* coding region in the −1 frame at the 3′ side. After induction by IPTG, this plasmid (pGCA) gave about 3% of the β-galactosidase activity of pGCACK which contained the *lacZ* gene in-frame with the AUG (Fig. 3A). A high level (27.8%) of in-frame readthrough of the 39K stop codon was observed in cells containing plasmid pGCAEf. This demonstrated the leaky nature of host strain DH5α which contains a *supE* amber supressor mutation (Hanahan 1983). pGCAKt which contained *lacZ* in the +1 reading frame relative to the AUG, showed a very low level of

β-galactosidase activity (≤0.6% of wild type; Fig. 3A). Because of the variability between experiments, the results were confirmed visually by plating colonies on X-Gal media (Fig. 3B). The intensity of blue correlated with the β-Gal units observed in the solution assay. To test the effects of sequences downstream of base 1206 that would provide secondary structure that may be needed for maximal frameshifting (Brault and Miller 1992), *lacZ* expression constructs that included all the required downstream sequence (bases 1045–1246) were constructed. However, cells containing these constructs yielded no β-galactosidase activity, even in the positive control construct containing the *lacZ* ORF in-frame with the AUG.

Amino acid sequencing across the frameshift site.

To provide definitive evidence that the β-galactosidase activity was due to frameshifting, and to identify the precise frameshift site, we made a new construct that would bring the AUG close enough to the expected frameshift site to allow direct amino acid sequencing across the frameshift site starting from the amino terminus. A fragment containing bases 1132–1246 of the BYDV-PAV sequence, along with two-thirds of the GUS gene was moved from the plant expression vector, pS(−1)UAG (Brault and Miller 1992), into *E. coli* expression vector pET-11d and expressed in *E. coli*. As predicted by the occurrence of −1 frameshifting, large amounts of a 53.7-kDa protein

A



Plasmid	lacZ reading frame relative to AUG	Expt.	β-gal units	%	Avg. %
pGCA	-1	1	1.10±0.06	2.5	
		2	0.52±0.09	1.4	**3.0**
		3	0.52±0.04	5.1	
pGCAKt	+1	1	0.13±0.02	0.60	
		2	0.09±0.01	0.25	**0.6**
		3	0.11±0.01	0.97	
pGCAEf	0 Readthrough	1	12.85±2.1	29.4	
		2	5.70±0.6	15.6	**27.8**
		3	3.94±0.1	38.4	
pGCACK	0 No Stop	1	43.64±0.17	100	
		2	36.64±2.1	100	**100**
		3	10.26±3.5	100	

B

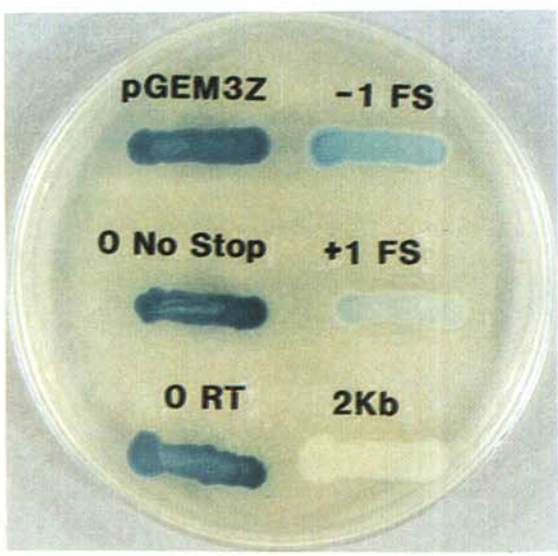


Fig. 3. A, Maps of plasmids and β-galactosidase activity produced by *E. coli* transformed with them. On the left are schematic representations of the *lacZ* a peptide reading frame relative to the viral sequence (not to scale). Sequences of viral inserts are in Figure 1. In the viral sequence portion (shaded), three reading frames are shown, relative to the *lacZ* start codon, with −1 reading frame at top, 0 frame in center, and +1 frame at bottom. Black octagons represent stop codons. Each plasmid was tested in triplicate in three separate experiments (Expt.). β-Galactosidase (β-Gal) units are as in Miller (1972), ± one standard deviation. **B,** Colonies of DH5α cells transformed with pGEM3Z, pGCACK (0 No Stop), pGCAEf (0 RT), pGCA (−1 FS), pGCAKt (+1 FS), and pPA142 (2Kb) on X-Gal-containing media.

were produced within 5 hr after induction with IPTG (data not shown). The amino terminus of this protein was found to be: M-D-V-L-D-S-V-G-F-L-E-G-L (Fig. 4), exactly as predicted by a −1 frameshift at the shifty heptanucleotide.

In vitro translation of full-length BYDV-PAV RNA.

To demonstrate unequivocally that expression of the downstream ORF was a translational event, we used cell-free translation systems. To observe frameshifting in its natural context, full-length BYDV-PAV RNA was translated *in vitro*. Both virion-extracted RNA and *in vitro* transcripts of *Sma*I-linearized pPAV6 (see Materials and Methods) were used. The full-length transcript was a chimera with the 5′-terminal 1,044 nucleotides derived from an Illinois isolate of BYDV-PAV (PAV-IL), while the remainder was from the sequenced Australian isolate (Miller *et al.* 1988). Thus, the frameshift region (bases 1152–1158) was from the Australian isolate. The nucleotide sequence derived from PAV-IL RNA was 93.1% identical to the Australian isolate and 97.7% identical in encoded amino acid sequence (data not shown). Despite the differences, the full-length transcript was highly infectious in oat protoplasts (S.P.D.-K., unpublished).

When BYDV-PAV virion RNA or full-length transcripts were translated under optimal ionic conditions (below) in a wheat germ extract, an abundant peptide was produced that migrated at an apparent molecular weight of 47 kDa, using BMV translation products as markers (Fig. 5, lanes 2, 3). We believe that this is the product of the 39K ORF. A less abundant product that migrated as a 95 kDa polypeptide was also visible. This is close to the size and abundance expected for the transframe protein (39K + 60K = 99K). To verify this, a deoxycytidilate residue was inserted in the stop codon of the 39K ORF (mutant pFLFSM4) to destroy the stop codon and place the 60K ORF in the same frame as the 39K ORF, resulting in a single 99K ORF. As expected, the translation product of this RNA comigrated with the approximately 95 kDa product from wild-type RNA but was present in much greater abundance (Fig. 5, lane 4). Concomitantly, little or no 47 kDa-migrating translation product was present among translation products of pFLFSM4 RNA, supporting the notion that it is the product of the 39K ORF. Thus, we will refer to the apparent 47 and 95 kDa products as 39 and 99 kDa polypeptides, respectively.

In contrast to the wheat germ translation products, in the reticulocyte extract, using optimal ionic conditions (Dinesh-Kumar *et al.* 1992), much smaller amounts of putative 39K ORF and 99K products were detected (Fig. 5, lanes 7, 8). A faint band migrated at about 99K, but trace amounts of a similar band were present in the extracts to which no RNA was added (Fig. 5, lane 10). Thus any frameshifting that occurs in reticulocyte lysates, is at a much lower efficiency than in wheat germ and is partially obscured by the endogenous band. Translation of the 39K ORF was two- to threefold less efficient in reticulocyte

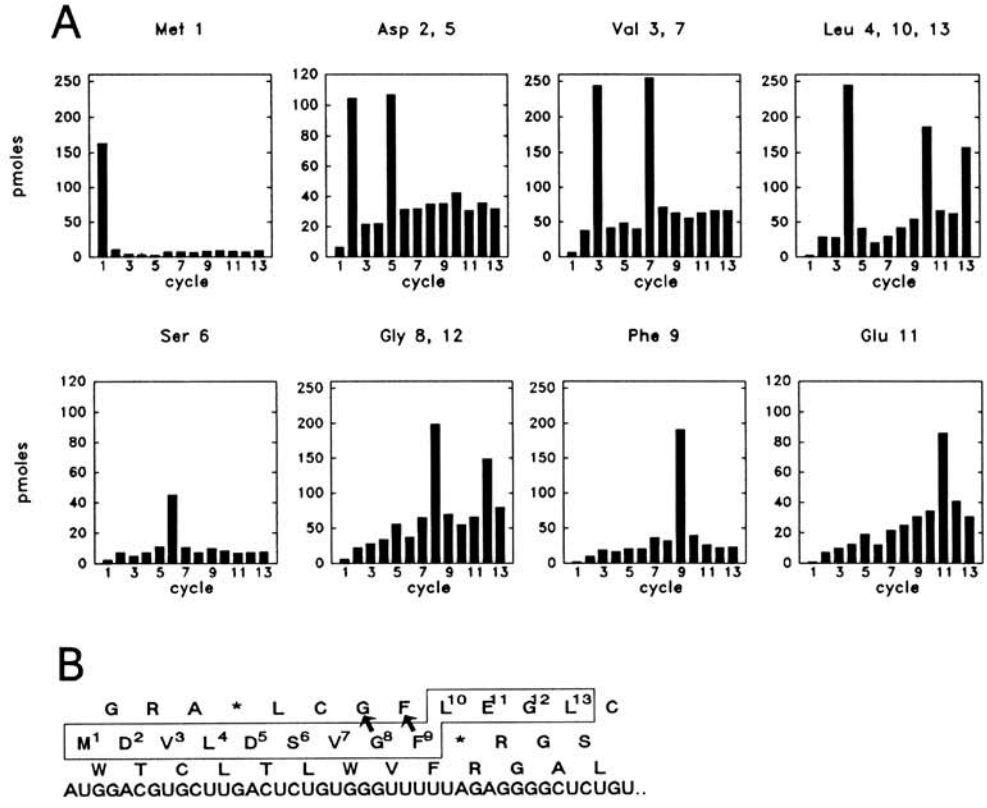


Fig. 4. A, Amino acid sequencing of the N-terminus of the purified 53.7-kDa protein expressed from pETV in BL21(DE3) cells. Histograms of relevant PTH-amino acids through 13 cycles of Edman degradation are shown. The cycles in which the indicated amino acid predominates are indicated beside the amino acid. **B,** BYDV-PAV-derived nucleotide sequence encoding N-terminus of the transframe product sequenced in A, and translation in all three reading frames. Amino acid sequence from A, and predicted by −1 simultaneous slippage frameshift model is outlined and numbered. Arrows indicate possible sites of −1 frameshift by the tRNA in the ribosomal A site.

lysates than in wheat germ, whereas BMV RNA translated more efficiently in reticulocyte lysates. The relative amounts of 39 and 99 kDa polypeptides were measured using ImageQuant software on Phosphorimager scans of the gels. The rate of frameshift of transcripts in wheat germ extracts ranged from 5 (Fig. 5) to 1.6% (Fig. 6), but the virion RNA frameshifted at a rate of 12%. When the background band of approximately 99 kDa in the no RNA lane was subtracted from the reticulocyte lysate translation products, the full-length transcript and virion RNA were found to frameshift at rates of 1.2 and 1.5%, respectively.

Because optimal ionic conditions can vary between mRNAs and between batches of cell-free systems (Kemper and Stolarsky 1977; Kozak 1990), the ionic conditions of the translation systems were varied to see if this would affect the amount of frameshift product. Increasing the magnesium ion concentration in the wheat germ system from 2.1 mM, supplied by the manufacturer, to 2.6, 3.1, 3.5, and 4.1 mM simply reduced overall translation and did not enhance frameshifting (data not shown). In contrast, increasing potassium acetate to 154 mM dramatically improved translation efficiency relative to that at 54 mM supplied by the manufacturer. Wheat germ translations shown in Figures 5 and 6 were performed in 154 and 114 mM potassium acetate, respectively, and 2.1 mM magnesium acetate. A concentration of 8 ng/ml (4 nM) of full-length transcript was used because it gave sufficient amounts of product but would not be expected to saturate translation factors (Kozak 1990). Despite extensive optimization of the magnesium and potassium concentrations in the reticulocyte lysate, the rate of frameshifting remained substantially below that in wheat germ extracts (Fig. 5).

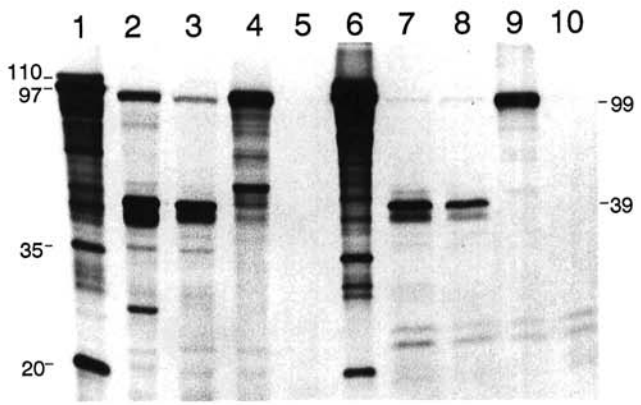


Fig. 5. Fluorograph of *in vitro* translation products of BMV RNA (lanes 1, 6), BYDV-PAV virion RNA (lanes 2, 7), transcripts of *Sma*I-linearized pPAV6 (lanes 3, 8) and pFLFSM4 (lanes 4, 9), and no added RNA (lanes 5, 10), following SDS-PAGE. Lanes 1–5: wheat germ translation products (2.5-ml aliquot of BMV RNA reaction, 5-ml aliquots of BYDV-PAV RNA reactions). Lanes 6–10: rabbit reticulocyte lysate products (2.5-ml aliquot of BMV RNA reaction, 10-ml aliquots of BYDV-PAV RNA reactions). All reactions contained 0.2 mg of RNA template in 25-ml reaction mixture with 1,071 Ci/mmole ^{35}S-methionine. Details are in the text. Mobilities (in kilodaltons) of BMV products are shown on the left: expected sizes of BYDV-PAV products are on the right.

The 99-kDa peptide was precipitated by antiserum against the protein encoded by the *Ssp*I fragment (C-terminal 92%, Fig. 2) of the 60K ORF, but not by preimmune serum (Fig. 6, lanes 4 and 5). A small fraction of the abundant 39K product was precipated equally by both anti-60K and preimmune sera, apparently due to nonspecific or fortuitous binding by antibodies in both sera. Its presence in vast excess compared to other proteins prior to immunoprecipitation probably exacerbated its nonspecific precipitation. A 57-kDa protein that was present in varying amounts in wheat germ translation products was also immunoprecipitated.

DISCUSSION

BYDV-PAV sequence mediates frameshifting in *E. coli*. In the previous work, in which GUS activity was monitored in protoplasts (Brault and Miller 1992), the remote possibility remained that the GUS activity was due to an unanticipated event such as a low-frequency, RNA-modifying event. Here we show that the BYDV-PAV RNA frameshift sequence can also mediate −1 frameshifting in *E. coli* as with some retroviruses (Weiss *et al.* 1989) beet western yellows virus (BWYV) (Garcia *et al.* 1993) and yeast L-A virus (Dinman and Wickner 1992). This demonstrates the universality of frameshift signals for both 70S and 80S ribosomes.

The higher frameshifting rate in *E. coli* (approximately 3%) cannot be compared directly with the rate in proto-

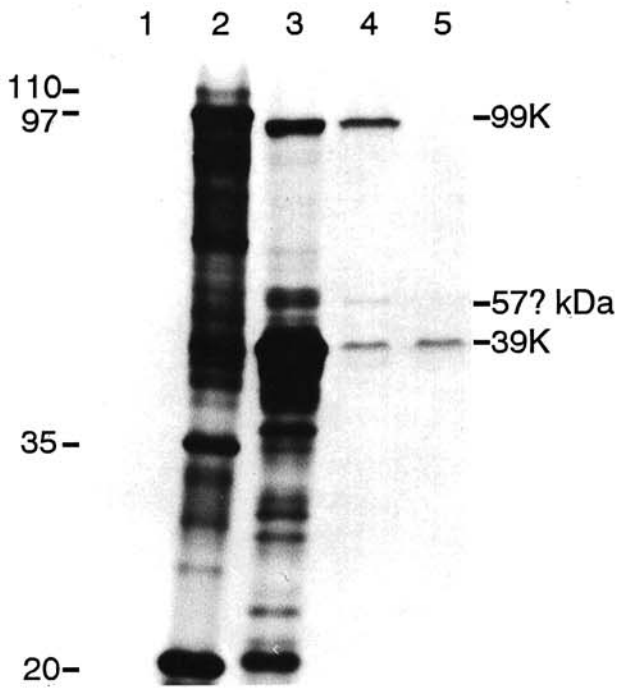


Fig. 6. Immunoprecipitation of wheat germ translation products of BYDV-PAV transcripts. No RNA (lane 1); BMV RNA (lane 2). Full-length transcript of *Sma*I-linearized pPAV6 (lane 3) and immunoprecipitation of these translation products by 60K-specific antibody (lane 4) and preimmune serum (lane 5). Mobilities of BMV markers are on the left. The expected (39 and 99) and calculated (57?) sizes of BYDV-PAV translation products are on the right.

plasts. Firstly, the *cis*-acting sequences that control frameshifting may have different quantitative effects in *E. coli* (Weiss *et al.* 1989, Garcia *et al.* 1993). The tRNA populations and ribosomal RNA sequences in *E. coli* are different from those in plants. Secondly, the viral sequences and structures that were fused to the reporter genes (GUS in the protoplast system, *lacZ* in the *E. coli* system) were different. In the protoplast assay, the viral structure, extending to base 1244, included two potential downstream stem-loops or pseudoknots (Brault and Miller 1992) which may contribute to frameshifting (Jacks *et al.* 1988; Weiss *et al.* 1989; Brierley *et al.* 1991; Morikawa and Bishop 1992; Prüfer *et al.* 1992; Chamorro *et al.* 1992; Parkin *et al.* 1992). However, because the viral sequence in the β-galactosidase reporter constructs ended at base 1206, only the first stem-loop of one of the possible pseudoknots could still form, although fortuitous base-pairing by the vector sequence may be sufficient to allow formation of a weak pseudoknot (Fig. 1). A small stem-loop, predicted earlier (Miller *et al.* 1988) also may form. None of the *lacZ* expression constructs that included these additional viral bases (1045–1246) yielded β-galactosidase activity. Presumably the short (86-amino acid) *lacZ* α peptide was inactivated by fusion of such a long (67-amino acid) sequence to its N-terminus.

Amino acid sequencing of the transframe protein.

Direct amino acid sequencing of the transframe protein was employed to determine the exact position at which ribosomes slip in translation (Jacks *et al.* 1988; Weiss *et al.* 1989). We were unable to observe the frameshift rate of pETV due to the short (nine-amino acid) pre-frameshift ORF. The high level of transframe protein expressed in *E. coli* from pETV, which contained the same viral sequences as the vectors used in plant cells, suggested efficient frameshifting. This is consistent with the above hypothesis that additional downstream sequences absent from the β-galactosidase reporter constructs but present in the protoplast constructs and pETV, may be required for maximal frameshifting.

Because the 0 and −1 reading frames both encode a glycine-phenylalanine doublet at the frameshift site determined by amino acid sequencing, there is ambiguity as to the precise site of frameshift. According to the simultaneous slippage model, the $tRNA^{gly}$ in the 0 frame peptidyl (P) site, and the $tRNA^{phe}$ in the aminoacyl (A) site both slip one base in the 5′ direction either before (Jacks *et al.* 1988) or during (Weiss *et al.* 1989) translocation. Thus, the phenylalanine in the amino acid sequence obtained from the pETV product is predicted to be encoded by the 0 frame (F^9) codon, followed by L^{10} from the −1 frame (right arrow, Fig. 4B). Alternatively, the shift could occur one codon upstream (left arrow, Fig. 4B), with $tRNA^{val}$ and $tRNA^{gly}$ simultaneously slipping at the heptanucleotide UGUGGGU. Because this does not fit the consensus for shifty heptanucleotides (Jacks *et al.* 1988; Hatfield *et al.* 1992), we predict that the shift occurs at GGGUUUU. Introducing mutations that do not inactivate the shifty heptanucleotide, but do change the amino acids in each of the frames should resolve this ambiguity.

Frameshifting by full-length BYDV-PAV RNA *in vitro*.

The *in vitro* results allowed observation of frameshifting in the full-length BYDV-PAV RNA context and assured observation of only *translational* events controlling expression in an extract from a natural host of BYDV (wheat). The biological relevance of these results is established by the observations that the same pattern was generated by virion RNA and an infectious transcript (Fig. 5 and Young *et al.* 1991) and that the transcript used here is highly infectious. Virion RNA may frameshift more efficiently due to sequence differences between the isolates, although the frameshift region is very highly conserved between them. No differences in shifty heptanucleotide or sequences involved in any predicted 3′ secondary structures exist. The transcript and virion may differ in the nature of their 5′ ends. The transcript contained a 5′ triphosphate. BYDV-RPV contains a genome-linked protein (Murphy *et al.* 1989), but this may not be the case with the distantly related BYDV-PAV. A 5′ m^7GpppG cap on the transcript did not increase frameshift efficiency (data not shown).

Although frameshift rates varied between experiments and batches of translation mixes, frameshifting in reticulocyte lysates was consistently below that in wheat germ extracts, and rarely detectable. The reason for this is unknown. In *E. coli*, the short frameshift domain was inserted in a gene that is well expressed in that host. In contrast, the entire viral genome being translated *in vitro* may reveal sequences distant from the frameshift site that affect initiation or frameshift efficiency in wheat germ but not reticulocyte extracts (R. Di, unpublished observations). Our observations are consistent with those of Xiong *et al.* (1993) who observed an apparently very low frameshift rate by red clover necrotic mottle virus (RCNMV) RNA in reticulocyte lysates. Although the frameshift rate was not calculated, a very high amount of upstream ORF product had to be loaded on the gel to allow visualization of the frameshift product. RCNMV RNA frameshifting in wheat germ extracts was not reported, but the BWYV frameshift signal functions equally well in either *in vitro* system (Veidt *et al.* 1992; Garcia *et al.* 1993).

The immunoprecipitation results demonstrated clearly that the 99-kDa polypeptide shares epitopes with the 60K ORF gene product. This, combined with the facts that the 99-kDa product comigrated with the fused 39K + 60K ORF product and that no other ORFs in the genome are big enough to produce a 99-kDa product, provides convincing evidence that it results from a −1 frameshift. The trace amount of 57-kDa product appears to contain all or part of the 60K ORF product, based on its immunoprecipitation with anti-60K antibody. This product may have arisen from internal initation at the first AUG (amino acid 95) of the 60K ORF which would give a protein with a predicted molecular weight of 50 kDa, or it could be due to proteolytic cleavage of the 99K polypeptide. Whether this is an artifact or is biologically significant is unknown. A similar event appeared to occur in the postshift ORF of RCNMV RNA (Xiong *et al.* 1993).

Comparison with other plant viruses.

In addition to RCNMV and the luteoviruses, luteovirus-

like pea enation mosaic virus also is likely to express both of its polymerases by frameshifting (Demler and de Zoeten 1991; Demler *et al.* 1993). The satellitelike ST9-associated RNA of BWYV contains truncated polymerase ORFs with homology to those of BYDV-PAV and RCNMV which may be expressed by readthrough and frameshifting (Chin *et al.* 1993). A downstream stem-loop, like one possible for BYDV-PAV RNA, was reported to be required for frameshifting *in vitro* by the PLRV overlap sequence inserted in a GUS gene (Prüfer *et al.* 1992). However, another group reported that a pseudoknot rather than a stem-loop is required for frameshifting by PLRV (Kujawa *et al.* 1993). This seems to apply to the related BMYV as well (Garcia *et al.*, 1993). The frameshift signal of PLRV also differs from that of BYDV-PAV in that the shifty heptanucleotide is UUUAAAU, and it occurs in the middle of a 582-base overlap. In contrast, the putative frameshift signal of RCNMV (GGAUUUU in a seven-base ORF overlap followed by a stem-loop, Xiong *et al.* 1993) more closely resembles that of BYDV-PAV and the closely related BYDV-MAV (Ueng *et al.* 1992) than any of the other published luteoviruses. Likewise, the polymerase of BYDV-PAV (and BYDV-MAV) is much more closely related to that of RCNMV (38.4% amino acid sequence identity; Xiong and Lommel 1989) than it is to the polymerases of the other luteoviruses such as PLRV, BWYV, and even BYDV-RPV (Vincent *et al.* 1991).

Why frameshifting?

It is likely that frameshifting and in-frame stop codon readthrough are alternative means by which viruses frugally maximize the functional capacity of their RNA. Indeed, retroviruses can use either strategy to control the ratio of *gag* to *pol* products in the cell (Hatfield *et al.* 1992). The sequences that facilitate frameshifting or readthrough simultaneously encode amino acid sequences and regulate gene expression. This results in low-level expression of the catalytic domain of the RNA-dependent RNA polymerase fused to the upstream ORF, a protein that is needed only in small amounts relative to the upstream ORF alone. In many cases the upstream ORF contains helicase motifs, including an NTP-binding domain (Habili and Symons 1989), but its role and why it is needed in large quantities are unknown.

The frameshift rates observed *in vivo* were mediated by small portions of the viral genome. It will be valuable to observe the frameshift rate in the full-length context *in vivo*. In addition, the role of any virus-encoded or viral induced proteins in frameshifting are unknown. Thus, in the future we will investigate the frameshift rate in virus-infected cells.

MATERIALS AND METHODS

Construction of *lacZ* frameshifts and β-galactosidase assay.

A region spanning the frameshift site, corresponding to BYDV-PAV nucleotides 1045–1206 in clone pPA142 (Miller *et al.* 1988) was digested with *Ava*I (base 1206), made blunt-ended with mung bean nuclease (Sambrook *et al.* 1989), then digested with *Cla*I (base 1045). This fragment was inserted into the *Acc*I and *Sma*I sites of pGEM3Zf(−) (Promega, Madison, WI), to create pGCA in which most of the *lacZ* α peptide coding region was in the −1 frame relative to the start codon (Fig. 1). pGCAEf was derived from pGCA by cutting with *Eco*RI, filling in the ends with Klenow polymerase (Sambrook *et al.* 1989) and religating. pGCAKt was derived by linearizing pGCA with *Kpn*I, removing the overhangs with T4 DNA polymerase, and religating. The *lacZ* gene was in-frame with the start codon (0 frame) in pGCAEf, and in the +1 frame in pGCAKt. Plasmid pGCACK was constructed from pGCAEf by two-step PCR mutagenesis (Herlitze and Koenen 1990) to change the 39K ORF stop codon TAG into TCG. The primers used were VB4: 5′-AGAGCCCCTCGAAAAACCCAC-3′ (base change underlined), and the M13 forward and reverse sequencing primers (Promega). β-Galactosidase (β-Gal) activity was measured by the assay of Miller (1972) using *o*-nitrophenyl-β-D-galactoside (ONPG) substrate. Each construct was assayed in triplicate, in three different experiments. The frameshifting level was calculated by dividing β-gal activity of each construct by that of pGCACK.

Sequencing of amino acids around frameshifting region.

The viral frameshift sequence and two-thirds of the GUS gene from pS(−1)UAG (Brault and Miller 1992) was cloned into *Nco*I and *Bam*HI-digested expression vector pET-11d (Novagen, Madison, WI) to create pETV. This places the frameshift construct under control of a T7 RNA polymerase promoter. A 53.7-kDa fusion protein would be expressed if −1 frameshifting occurred during expression of this construct in *E. coli*. After transformation of *E. coli* strain BL21(DE3), large quantities of expressed protein were purified according to Sambrook *et al.* (1989). The protein was separated by polyacrylamide gel electrophoresis (PAGE) and electroblotted onto a polyvinylidene diflouride (PVDF) membrane (ProBlott membrane, Applied Biosystems) in 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer. The membrane was stained with Coomassie blue. The desired peptide was cut out and sequenced by Edman degradation (Matsudaira 1987) on an Applied Biosystems Protein Sequencer model 4778.

Construction of a full-length cDNA clone of BYDV-PAV RNA.

A full-length clone of BYDV-PAV RNA was constructed for *in vitro* transcription (Fig. 2). The protocol of Frohman *et al.* (1988) was used to amplify the 5′ end of PAV-IL RNA. The oligonucleotide: 3′-*CCAGTGTCTGCCTCGGGCCGA*AGTACTTTCGAATCCCCGGGAAC-5′, containing sequence complementary to bases 1225-1246 (italics) and added *Bsp*HI, *Hin*dIII and *Apa*I restriction sites (underlined) was used to prime first-strand cDNA synthesis using M-MLV RNase H⁻ reverse transcriptase (Superscript, BRL) (Sambrook *et al.* 1989). The upstream primer, 5′-ACGCGGCCGC*TAATACGACTCACTATA*-GAGTGAAGATTGACCATCTTCACA-3′, was similar to that used by Young *et al.* (1991). It contained (5′ to 3′) a *Not*I site (underlined), the T7 promoter sequence (italics), followed by a G residue and the first 23 nucleotides of the 5′ end of the virion RNA. The amplified product was

gel-purified and cloned into *Not*I-*Apa*I-cut pGEM5Zf(+) (Promega), resulting in plasmid pPAV1 (Fig. 2). To obtain pPAV2, the *Cla*I-*Hin*dIII fragment of pSP15 (bases 1044–1591, Dinesh-Kumar *et al.* 1992) was cloned into pPAV1 that had been digested with the same enzymes. To generate pPAV3, the *Hin*dIII-*Apa*I fragment of pSP15 comprising bases 1591–2860 (*Apa*I site is in the vector) was cloned into *Hin*dIII-*Apa*I-cut pPAV2. Plasmid pPAV4 was constructed by cloning the 481 base pair (bp) *Eco*RI fragment (bases 2860–2985) of pSP17 (Dinesh-Kumar *et al.* 1992) into *Eco*RI-cut pPAV3.

Intermediate cloning vector pUC118–1180-H3 was constructed by cloning the small *Sac*I-*Sal*I fragment of pSL1180 (Pharmacia, Milwaukee, WI) into pUC118 from which the *Hin*dIII site had been deleted. Then the *Sal*I-*Sma*I fragment of pSP18 (Dinesh-Kumar *et al.* 1992) was cloned into pUC118-1180-H3, resulting in pPAV5. Finally, the *Not*I-*Sal*I fragment of pPAV4 was cloned into *Not*I-*Sal*I-cut pPAV5 to obtain pPAV6, which contains a full-length cDNA copy of BYDV-PAV (Fig. 2). All the cloning junctions were confirmed by double-stranded DNA sequencing (TaqTrack sequencing kit, Promega).

Mutations in the frameshift site.

The BYDV-PAV sequence from nucleotide 1044 (*Cla*I site) to 1591 (*Hin*dIII site) was cloned into the multiple cloning site of pSL1180. Mutations were made by the method of Herlitze and Koenen (1990), using M13 forward and reverse primers and mutagenic primer DIFS1: 5′-AGAGCCCCTCTGAAAAACCCAC-3′ (inserted base underlined). Primer DIFS1 changed the 39K stop codon from UAG to UCAG, creating an in-frame 99K fusion of the 39K and 60K ORFs. After mutagenesis, the *Cla*I-*Hin*dIII fragment was subcloned into pPAV2, then the *Not*I-*Hin*dIII fragment was cloned back into the full-length clone to create plasmid pFLFSM4.

Templates for *in vitro* transcription and translation.

BYDV-PAV virions were extracted from oat plants and genomic RNA purified from virions as described by Waterhouse *et al.* (1986). Full-length RNA was also obtained by *in vitro* transcription of *Sma*I-linearized pPAV6 with T7 RNA polymerase (New England Biolabs, Beverly, MA) as described by Melton *et al.* (1984). *In vitro* translation was carried out with 200 ng of uncapped transcripts or virion RNA in a wheat germ extract in the presence of ^{35}S-methionine as described by the manufacturer (Promega) or in a rabbit reticulocyte lysate (Promega) as described by Dinesh-Kumar *et al.* (1992). One-fifth of the *in vitro* translation reaction mix was electrophoresed through 5% stacking and 10% resolving polyacrylamide-SDS gels using a discontinuous buffer system (Laemmli 1970). Gels were fixed and fluorographed (Hames 1990). The relative radioactivity in each gel band was measured using Imagequant 3.0 software on a Phosphorimager 400E (Molecular Dynamics, Sunnyvale, CA). Frameshift rate was calculated as: (relative radioactivity in 99K band/28) × 100/[(relative radioactivity in 39K band/10) + (relative radioactivity in 99K band/28)]. This normalized the radioactivity for the 28 methionine residues in the (39K + 60K) = 99K transframe ORF and the 10 methionines in the 39K ORF alone.

Preparation of antibody and immunoprecipitation.

An *Ssp*I fragment of pPA142, representing 92% of the PAV 60K ORF (nucleotides 1266–2739, Fig. 2), was cloned into expression vector pET-11d which had been digested with *Nco*I and *Bam*HI and in-filled with Klenow polymerase (Sambrook *et al.* 1989) to obtain plasmid pETP. Induction of this gene and partial purification of the resulting 53.9-kDa protein were as described for pETV. Following SDS-PAGE, the acrylamide piece containing approximately 100 μg of the 53.9 kDa protein was excised and lyophylized overnight, ground in liquid nitrogen, resuspended in 0.5 ml 0.01 M phosphate buffered saline (PBS, pH 7.2) and mixed with 0.5 ml complete Freund's adjuvant. After emulsifying, the mixture was injected into a rabbit, both intramuscularly and subcutaneously. A boosting injection with 100 μg of protein was made 3 wk later with Freund's incomplete adjuvant and twice more at fortnightly intervals. Antisera were tested for their titers by indirect ELISA (Koenig and Paul 1982). *In vitro* translation products were immunoprecipitated as described by Ball (1990).

ACKNOWLEDGMENTS

This work was funded by USDA NRICGP grant number 91-37303-6424 and a McKnight Foundation Individual Investigator Award (to WAM). We thank A. Hewings for providing BYDV-PAV-IL, H. Sipes and R. Beckett for technical assistance, C. Roberts and Ju-Whan Eom for advice on use of pET plasmids, and the Iowa State University Protein Facility and Hybridoma Service. This is paper number J-15131 of the Iowa Agriculture and Home Economics Experiment Station. Project no. 2936.

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