

Chapter 9

Ribosomal Frameshifting in Decoding Plant Viral RNAs

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Abstract Frameshifting provides an elegant mechanism by which viral RNA both encodes overlapping genes and controls expression levels of those genes. As in animal viruses, the -1 ribosomal frameshift site in the viral mRNA consists of a canonical shifty heptanucleotide followed by a highly structured frameshift stimulatory element, and the gene translated as a result of frameshifting usually encodes the RNA-dependent RNA polymerase. In plant viruses, the -1 frameshift stimulatory element consists of either (i) a small pseudoknot stabilized by many triple-stranded regions and a triple base pair containing a protonated cytidine at the helical junction, (ii) an unusual apical loop–internal loop interaction in which a stem-loop in the 3' untranslated region 4 kb downstream base pairs to a bulged stem-loop at the frameshift site, or (iii) a potential simple stem-loop. Other less well-characterized changes in reading frame occur on plant viral RNAs, including a possible $+1$ frameshift, and net -1 reading frame changes that do not utilize canonical frameshift signals. All these studies reveal the remarkable ways in which plant viral RNAs interact with ribosomes to precisely control protein expression at the ratios needed to sustain virus replication.

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9.1 Introduction

9.1.1 *Frameshifting Plant Viruses*

Plant viruses known to employ minus one (-1) programmed ribosomal frameshifting include one genus (*Dianthovirus*) in the Tombusviridae family, all three genera (*Luteovirus*, *Polerovirus*, and *Enamovirus*) of the Luteoviridae, and the *Umbravirus* and *Sobemovirus* genera. The latter two genera bear some resemblance in genome organization and sequence to the *Luteovirus* and *Polerovirus* genera, respectively, but have not been assigned to a family (Hull and Fargette, 2005; Miller et al., 2002; Taliansky et al., 2005). It is likely that translation of the genome of an entirely unrelated genus, *Closterovirus*, undergoes a net $+1$ reading frame change to translate the viral RdRp coding region (Karasev et al., 1995). This would be the first known $+1$ frameshift in any plant viral RNA. A carlavirus may use -1 frameshifting by a novel “P-site only” mechanism (Gramstat et al., 1994). Recently a new ORF was discovered in the large Potyviridae family of plant viruses (Chung et al., 2008). The new ORF, called pipo, overlaps with the major ORF which encodes a large polyproteins. One likely mechanism for expression of the pipo ORF is -1 ribosomal frameshifting.

9.1.2 *Why Frameshift?*

RNA viruses have evolved to compress maximal protein-coding capacity and regulatory signals for RNA replication, localization, encapsidation, and gene expression into minimal sequence space (Belshaw et al., 2007; Holmes, 2003). Programmed ribosomal frameshifting allows the virus to control levels of synthesis of two proteins using one sequence that codes for a portion of both proteins. The result of the frameshift is two proteins that have the same amino terminal sequence up to the frameshift site, at which point the amino acid sequences diverge, with a small portion of the proteins having the -1 frame (frameshifted) sequence, while the majority of the proteins result from conventional unshifted translation of the initial frame (called the zero frame). Thus, only a small percentage of ribosomes change reading frames in plant viral frameshifting. In many viruses, such as those in the *Luteovirus*, *Dianthovirus*, and *Umbravirus* genera, the frameshift occurs immediately upstream of the zero frame stop codon, so the translated proteins consist of a short and long (C-terminally extended) version of the same protein (Fig. 9.1). In contrast, in the

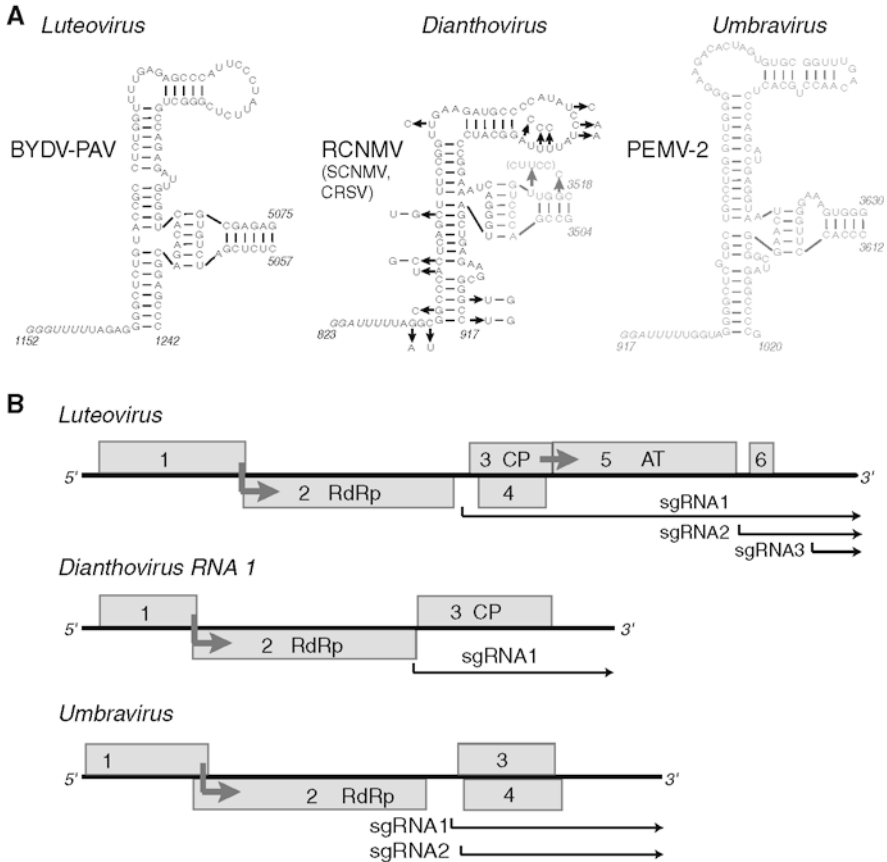


Fig. 9.1 Frameshifting in Luteo-, Diantho-, and Umbraviruses. **(A)** Representative secondary structures required for -1 frameshifting by each of the three genera known or thought to harbor such structures. The shifty heptanucleotide (SH) at which the ribosomes change reading frame is indicated in italics. The apical loop–internal loop frameshift structures comprising the adjacent downstream-bulged stem-loop (ADSL) base-paired to the long-distance frameshift element (LDFE) in the 3' UTR are indicated for each virus. Base numbering indicates positions of the bases in the viral genome. These structures are predicted here for the first time in Diantho- and Umbraviruses. They are conserved in all genus *Luteovirus* (Barry and Miller, 2002; Salem et al., 2008) and *Umbravirus* RNAs but diverge significantly in sequence (not shown). The bases and base pairs that differ from RCNMV in the other two dianthoviruses, SCNMV and CRSV, are shown with *arrows* (as in (Kim and Lommel, 1998)). Known structures are in *black*; our predicted structures are in *gray* text. **(B)** Genome organizations of viruses in each of the three genera. *Gray square arrow* indicates frameshift site. ORF 2 encodes the RdRp and is translated via -1 frameshift from ORF 1. Luteovirus ORFs 3, 4, and 5 encode coat protein (CP), putative movement protein, and a 3' extension of ORF 3 required for aphid transmission (AT), respectively. ORF 5 is translated via ribosomal readthrough of the leaky ORF 3 stop codon (gray straight arrow). Genomes in all three genera lack a 5' cap or VPg and a poly(A) tail. The approximate positions of sgRNAs are indicated by the *black arrows*

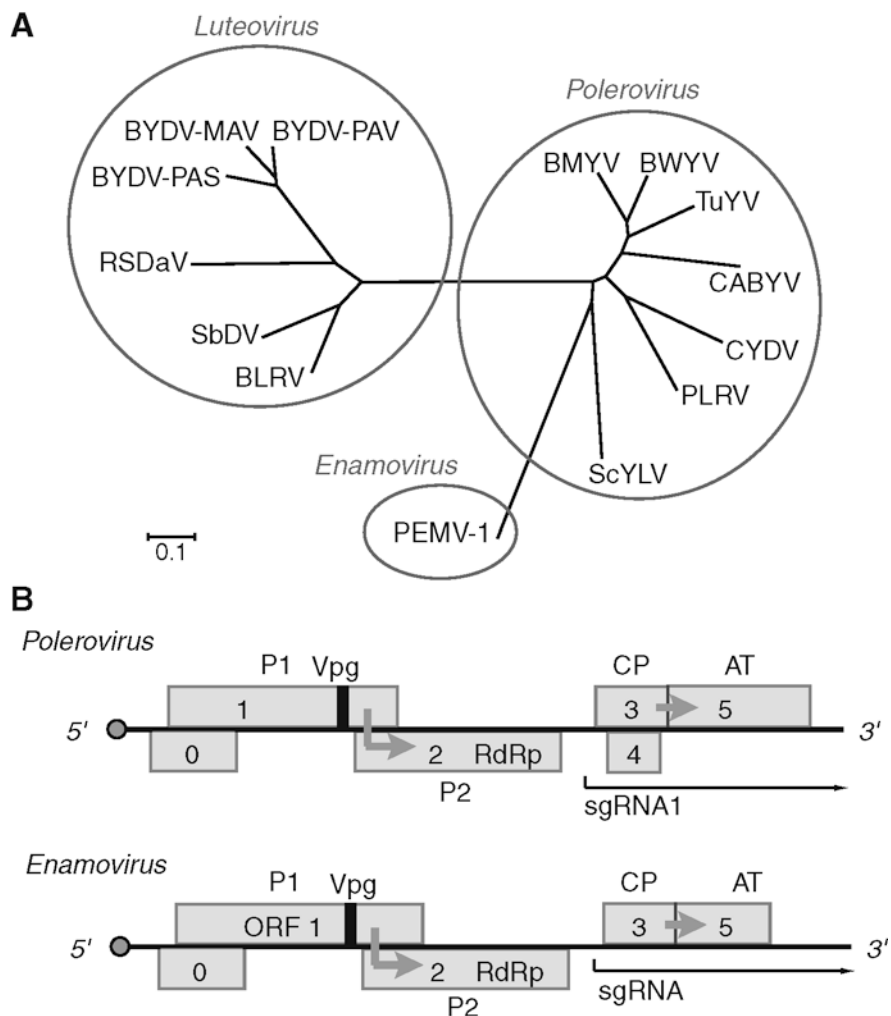


Fig. 9.2 Phylogenetic relationships among the *Luteoviridae* and schematic representations of *Polerovirus* and *Enamovirus* genomic RNAs. (A) Unrooted radial phylogenetic tree of selected *Luteoviridae* sequences. Members of the three genera are circled with the genus indicated in italics. (B) Genome organizations of polero- and enamoviruses, highlighting the P1–P2 frameshift sites (gray square arrow) in each case. ORF designations are from 0 to 5, with ORF 1 and ORF 2 encoding P1 and P2, respectively; CP, coat protein; AT, aphid transmission factor. Gray circle, VPg covalently linked to the 5' end of the genomic RNA; straight gray arrow, termination codon between the CP and AT genes that is readthrough to make a CP–AT fusion protein. The approximate positions of sgRNAs are indicated by the black arrows

polero- and enamoviruses, significant translation continues in the zero frame beyond the frameshift site (Fig. 9.2). In all plant viruses, with the possible exception of the Potyviridae and carlaviruses, the protein translated via ribosomal frameshifting is the RNA-dependent RNA polymerase (RdRp).

The high ratio of a viral protein to a C-terminally extended version of the protein can also be achieved by in-frame readthrough of a stop codon (codon redefinition). In fact, different viruses in the same family (Tombusviridae) use either readthrough or frameshifting to express the RdRp (Lommel et al., 2005). The portion of the RdRp upstream of the leaky (recoded) stop codon in Tomato bushy stunt virus (TBSV, a tobamovirus), called p33, equivalent to the pre-shift portion of the RdRp in frameshifting viruses specifically binds viral RNA (Pogany et al., 2005). The full-length readthrough product (p92^{p01}) includes the p33 and downstream RdRp domains (Panavas et al., 2005). p33 then localizes the viral RNA and p92^{p01} to membrane-bound vesicles derived from the peroxisomal membrane (McCartney et al., 2005). By analogy with Brome mosaic virus (Schwartz et al., 2002), these may serve as replication factories with many more copies of p33 than p92^{p01}. Thus infrequent in-frame readthrough of the stop codon is necessary to ensure a high ratio of p33 to RdRp in TBSV-infected cells. Based on sequence homologies (Koonin and Dolja, 1993) and the observation that the homologous protein (p27) of dianthovirus red clover necrotic mosaic virus (RCNMV) also localizes to membranes (Turner et al., 2004), it is highly likely that the pre-shift ORF of viruses in the *Dianthovirus*, *Luteovirus*, and *Umbravirus* genera play a role similar to that of p33. In these viruses, infrequent frameshifting, rather than readthrough, ensures a low level of RdRp expression. In addition to playing a role in RNA replication, the pre-shift part of ORF 2 of poleroviruses, enamovirus, and sobemoviruses also encodes the viral protease which is needed early in infection and the 5' genome-linked protein (VPg) (van der Wilk et al., 1997) which is absent in the Tombusviridae and in genus *Luteovirus*.

Unlike in-frame stop codon readthrough, frameshifting requires overlapping genes which allow for compression of coding regions on the genome. Thus, at the slippery site and downstream frameshift-stimulating structure in the mRNA, one sequence tract includes a *cis*-acting frameshift signal and encodes portions of two proteins, exemplifying the remarkable multiple functions that evolution has bestowed on a single tract of viral sequence.

9.1.3 Proposed Mechanisms of -1 Frameshift Stimulation

Ribosomal frameshifting is programmed entirely by the mRNA. For a region of mRNA to promote programmed -1 frameshifting, two sequence elements are required at a minimum. These include a shifty heptanucleotide at which the ribosome pauses and shifts reading frame (below), followed six to eight bases downstream by a highly structured region, such as a pseudoknot, a stable stem-loop, or other structure that greatly stimulates the process (see Chapters 7 and 8). It is currently unknown exactly how a downstream structure, e.g., a pseudoknot, stimulates -1 frameshifting. However, several proposals have been made that attempt to identify the exact step in a translocation cycle where frameshifting occurs on the ribosome (Harger et al., 2002; Jacks et al., 1988; Leger et al., 2007; Plant et al., 2003) (for a review, see Giedroc and Cornish (2009)). For a pseudoknot, what is

clear is that the relatively short spacer between the slip-site and the pseudoknot places the pseudoknot in direct contact with the mRNA entry channel of the translocating ribosome (Namy et al., 2006). In bacteria and probably also in eukaryotes, the mRNA channel is contained totally within the small (30S in bacteria) ribosomal subunit, between the head and body, lined with ribosomal proteins S3, S4, and S5 (Brodersen et al., 2002). Biochemical experiments reveal that the 70S ribosome has helicase activity (Takyar et al., 2005), which may function by passively trapping transiently unfolded secondary structure by dsRNA-binding protein S5; alternatively, the S3/S4/S5 proteins might function as a processivity clamp positioned at the entrance to the mRNA tunnel (Takyar et al., 2005).

Because error-free translation likely requires that the A- and P-site tRNAs maintain hydrogen bonding contact with the mRNA during both 60S and 40S translocation, at some point in an elongation cycle with the paused ribosome positioned over the slip-site, these interactions must be broken for frameshifting to occur. The sequence of the heptanucleotide slip-site in a -1 frameshift signal is such that during re-pairing of the P- and A-site tRNAs in the new -1 reading frame, only the wobble codon–anticodon interaction is changed (“0” frame: X XXY YYZ to “ -1 ” frame: XXX YYY Z) (Brierley et al., 1992). As a result, the new -1 reading frame provides near cognate re-pairing partners for the bound tRNAs. This suggests that the total free energy of codon–anticodon formation of the -1 frame will be comparable to that of the reference frame (frame 0). Since frameshifting does in fact occur over such a sequence, but happens infrequently, a sizable transition state energy barrier to shifting reading frames must be present, for which there is now structural insight (Selmer et al., 2006). A downstream secondary structural element could therefore function by lowering this energy barrier, either by playing an active (mechanical) or passive role in this process.

A mechanical model of frameshift stimulation, using our understanding of prokaryotic ribosomes as a model, hypothesizes that the downstream pseudoknot functions as a kinetic barrier to normal translocation, and in so doing, lowers the energy barrier to tRNA–mRNA re-pairing. It can be imagined that this kinetic barrier would come into play either during spontaneous hybrid A/P P/E states formation (Cornish et al., 2008) (coincident with 50S translocation) and/or during the next step in the elongation cycle, EF-G (eEF2)•GTP-driven translocation (30S translocation) (Namy et al., 2006). In either case, movement of the ribosomal subunits may cause tension to build up in the mRNA strand due to the downstream structural element positioned in the mRNA entry channel. This tension could then be released via -1 frameshifting (Plant et al., 2003).

Recent support for a mechanical model of frameshift stimulation has emerged from cryo-electron microscopy images of mammalian 80S ribosomes (to a resolution of ≈ 16 Å) paused over the infectious bronchitis virus (IBV) pp1a/pp1b pseudoknot frameshift signal (Namy et al., 2006). As expected (Yusupova et al., 2001), the pseudoknot lies at the entrance to the mRNA channel apparently making direct contact with elements of the putative ribosomal helicase, including rps3 (bacterial S3), 16S helix 16, rpS9 (S4), and rpS2 (S5), as well as the

ribosome-regulatory protein RACK1. Furthermore, the clearly defined electron density observed for the pseudoknot strongly suggests that the pseudoknot is largely folded as the ribosome shifts reading frames. This structure is fully compatible with the idea that the ability of the downstream pseudoknot to actively lower the energy barrier for unpairing of the P-site codon–anticodon interaction will be more strongly correlated with frameshift stimulation. By extension, RNA motifs more capable of resisting the force of ribosomal helicase-mediated unwinding and eEF2(EF-G)-catalyzed translocation will thus be more efficient frameshift stimulators (Larsen et al., 1997).

In plant viral RNAs there appear to be three classes of RNA structure downstream of the slippery site that can facilitate -1 ribosomal frameshifting: (i) an “apical loop–internal loop” (ALIL) structure in which a bulged stem-loop, located 5–6 nt downstream of the slippery site, base pairs to a distant loop in the 3′ UTR; (ii) a very small, highly structured hairpin-type pseudoknot; or (iii) a stable, imperfect stem-loop. Other as-yet unidentified structures may facilitate frameshifting in other plant viruses such as the Potyviridae (see below). The following discussion provides an overview of these different structures, with an emphasis on the pseudoknots in the polero and enamoviruses which, due to their small size, are among the best characterized frameshift signals in eukaryotes (Cornish et al., 2005).

9.2 Plant Virus Frameshift Elements

9.2.1 *Luteoviridae*

The *Luteoviridae* are nonenveloped, icosahedral, aphid-transmissible, single-stranded positive(+)-sense RNA viruses. They are grouped in three genera: *Luteovirus*, *Polerovirus*, and *Enamovirus* (D’Arcy and Domier, 2005; Mayo and D’Arcy, 1999; Miller et al., 2002). The genomes of all *Luteoviridae* are 5.6–5.8 kb long and typically have six open reading frames (ORFs) which are divided into two clusters separated by a noncoding intergenic region (Figs. 9.1 and 9.2). ORFs 1 and 2 encode proteins P1 and P2 which form part of the viral replicase. In poleroviruses and enamovirus, P1 is a polyprotein precursor containing a putative helicase, a chymotrypsin-like (3C-like) proteinase, and VPg peptides (van der Wilk et al., 1997), while the exact biochemical function of P1 of genus *Luteovirus* P1 is ill-defined, as viruses in this genus have neither a VPg nor any known proteinase. In all *Luteoviridae*, P1 plays an essential role in RNA replication and P2 contains the RNA-dependent RNA polymerase active site (RdRp) (D’Arcy and Domier, 2005). The RdRp is translated as the C-terminal part of a P1–P2 fusion protein via -1 ribosomal frameshifting (Brault and Miller, 1992; Di et al., 1993; Garcia et al., 1993; Kujawa et al., 1993; Prüfer et al., 1992).

Within the *Luteoviridae*, the RNA structures that facilitate frameshifting differ markedly, depending on the genus. Viruses in genus *Luteovirus* are known

or predicted to have a GGGUUUU shifty site. The structured region (Fig. 9.1A) that begins six to eight bases downstream of the shifty heptanucleotide consists of a large adjacent downstream-bulged stem-loop (ADSL) that forms a complex pseudoknot by base pairing of a bulge loop in the ADSL to a stem-loop in the long-distance frameshift element (LDFE) located four kilobases downstream in the 3' UTR (Barry and Miller, 2002). In contrast, viruses in the other two genera of the Luteoviridae harbor a small, compact, highly structured pseudoknot adjacent to, and downstream of, the shifty site (see below). These striking differences between *Luteovirus* and *Polerovirus/Enamovirus* frameshift signals correlate with highly divergent sequences of the RdRp (ORF 2) and upstream (ORF 1) coding regions (D'Arcy and Domier, 2005). In fact, the RdRp of genus *Luteovirus* is more closely related to those of the Tombusviridae and Umbraviruses than to the other genera of the Luteoviridae (Miller, Liu and Beckett, 2002). In particular it is most closely related to the RdRPs of viruses in the *Dianthovirus* genus (Tombusviridae) which, unlike other Tombusvirids, employ -1 frameshifting via an RNA structure very similar to that of genus *Luteovirus* (Fig. 9.1).

9.2.2 Frameshift Stimulators Involving Long-Distance Base Pairing

The RNA structures for frameshifting by Barley yellow dwarf virus (BYDV, genus *Luteovirus*) and RCNMV (genus *Dianthovirus*) RNAs have been investigated. Both consist of a shifty site (GGGUUUU in BYDV, GGAUUUU in RCNMV) followed by a large bulged stem-loop (the ADSL) (Fig. 9.1A). The BYDV frameshift structure requires an additional stem-loop, the LDFE, located 4 kb downstream in the genome (Paul et al., 2001). The loop of this stem-loop base pairs to a large single-stranded bulge in the ADSL (Barry and Miller, 2002). Based on our predictions, this structure, now called an apical loop-internal loop (ALIL) interaction (Mazauric et al., 2008), is conserved in all members of the *Luteovirus* (Barry and Miller, 2002; Salem et al., 2008) and *Dianthovirus* genera (WAM unpublished, Fig. 9.1). We predict here that members of genus *Umbravirus*, exemplified by pea enation mosaic virus RNA-2 (PEMV2), contain a similar ALIL structure to stimulate -1 frameshifting (Fig. 9.1A).

The requirements for -1 frameshifting on BYDV RNA were demonstrated using a bicistronic reporter system, consisting of a reporter ORF (encoding Renilla luciferase) in the zero frame fused at its 3' end to the 3' end of the viral ORF 1 in the frameshift region, which, in turn, is followed by the 5' end of the -1 ORF (including the region of ORF overlap and the putative frameshift sequence) fused to a firefly luciferase ORF in the -1 frame, so that a -1 frameshift is required for expression of the firefly luciferase (Grentzmann et al., 1998). The ratio of firefly luciferase to Renilla luciferase produced in the frameshifted construct vs. a positive control with both luciferases in the same reading frame provides a measure of frameshift efficiency. Frameshift efficiency is about 1–2% for BYDV frameshift signals (Barry and Miller, 2002; Paul et al., 2001). Using this system Barry and Miller (2002) showed

that mutations that disrupted the ADSL–LDFE base pairing reduced frameshifting to background levels *in vitro* and in plant cells. In the context of the full-length viral RNA genome, the disruptive mutations also blocked frameshifting *in vitro* and prevented virus replication *in vivo* (oat cells). Compensating mutations that restored ADSL–LDFE base pairing also restored frameshifting *in vitro* and allowed virus replication in oat cells (Barry and Miller, 2002).

In contrast to the requirement for an LDFE in BYDV RNA, Kim and Lommel (1998) reported that the shifty heptanucleotide and the adjacent bulged stem-loop (equivalent to the ADSL in BYDV) were necessary and sufficient for significant (~7%) frameshifting by RCNMV RNA and thus did not require downstream sequences including the stem-loop that we predict forms the LDFE (Fig. 9.1A). We suggest that these experiments may not have revealed all of the sequence requirements for frameshifting on the full-length viral RNA because the frameshifting constructs tested contained a very short (10 codon) first (zero frame) ORF from the start codon through the shifty site which is followed immediately by a stop codon. Indeed, in earlier studies of the BYDV frameshift sequences, Brault and Miller (1992) obtained similar results to those of Kim and Lommel (1998) with a similar construct containing a very short (nine codon) ORF upstream of the shifty site, along with the ADSL, but no LDFE. A peculiar finding was that, unlike in other well-studied –1 frameshift elements from retroviruses or coronaviruses, the stop codon immediately adjacent to the shifty site appeared to be required for the –1 frameshift (Brault and Miller, 1992). Thus, these early constructs in BYDV and more recent experiments with RCNMV, both characterized with a very short first (zero frame) ORF, may not behave “naturally.” Certainly, a more native-like context is a full-length ORF encoding a functional protein upstream of the shifty site, as described more recently (Barry and Miller, 2002; Paul et al., 2001), employing either a dual luciferase reporter construct or the natural full-length viral genome. We speculate that ribosomes engaged in decoding a very short first ORF are in an initiation phase of the elongation process that may be “shifter” than elongating ribosomes farther downstream from the start codon. This shiftiness could be aided by the nearby in-frame stop codon (Brault and Miller, 1992). Another possibility is that folding of the nascent protein may influence shiftiness. The nature of the polypeptide in the exit tunnel can influence ribosome elongation rate (Cruz-Vera et al., 2005; Lu and Deutsch, 2008) which, in turn, may influence frameshifting (Ivanov and Atkins, 2007). The amino terminus of the short ORF 1-encoded protein in the constructs (Brault and Miller, 1992; Kim and Lommel, 1998) would not have exited the polypeptide exit tunnel (~30 amino acids) when the ribosome encounters the shifty sequence and thus would be unable to fold. This is in contrast to the point at which the ribosome encounters the shifty site in the context of mRNAs encoding dual luciferase reporter or viral genome proteins. In this case, much or all of ORF1-encoded protein has been synthesized and likely folded outside of the ribosome, but it remains attached to the ribosome via the growing polypeptide chain near the carboxyl terminus. Folding of the nascent peptide after leaving the exit tunnel may affect elongation rate. Moreover, the dual luciferase reporter provides a more accurate measurement of frameshift rate because it allows measurement of the level of

production of the first (zero frame) ORF as well as the post-shift (-1 frame) ORF. Finally, proximity of the frameshift site to the start codon has also been observed to affect $+1$ frameshifting in a misleading way. In this case, frameshifting was abolished when the $+1$ frameshift site was placed three codons downstream of the start codon (Belcourt and Farabaugh, 1990).

Additional strong evidence to support a requirement for the ALIL structure (ADSL–LDFE base pairing) is that it is phylogenetically conserved. A stem-loop in the 3' UTR (putative LDFE, albeit sometimes with a very weak predicted stem) capable of forming five to six base pairs with the 3' bulge in the ADSL is predicted in all members of the *Luteovirus* and *Dianthovirus* genera (Fig. 9.1), despite many differences in sequence. Interestingly, in the dianthoviruses and umbraviruses, the putative LDFE we predict is located upstream of the cap-independent translation element that is also located in the 3' UTR (Mizumoto et al., 2003), whereas in the luteoviruses, the LDFE is downstream of the cap-independent translation element which is located in the 5' end of the 3' UTR (Paul et al., 2001). Phylogenetic conservation of base pairing is strong evidence of a biological function and given that the ADSL–LDFE base pairing is required for BYDV frameshifting, frameshifting is its likely role for the other viruses, including dianthoviruses.

The ALIL structure is not unique to plant viruses. A different type of ALIL in which the small stem-loop precedes the large bulged stem-loop, and base pairs to a bulge in the 5' side rather than the 3' side of the large bulged stem-loop, was shown recently to be required for frameshifting of bacterial transposable elements in the IS3 family (Mazauric et al., 2008). In that study, modeling predicted that the proximal (bottom) helix coaxially stacks with the bulge loop–stem-loop helix (ADSL–LDFE interaction) which forces a sharp bend at the junction of this helix with the upper helix in the ADSL. It is possible that a similar structure forms here and blocks the helicase activity of the ribosome.

The long-distance base pairing spanning up to 4 kb between the ADSL and the LDFE may serve as an RNA traffic control signal, allowing the virus to switch from translation to replication of the viral genome (Barry and Miller, 2002; Miller and White, 2006). All positive-strand RNA viruses must first translate the RdRp (replicase) prior to RNA replication. The replicase initiates complementary RNA synthesis at the 3' end of the viral template. Early in infection this template must be the same genomic RNA that is being translated. However, ribosomes on the viral RNA would interfere with the replicase moving in the opposite direction toward the 5' end (Gamarnik and Andino, 1998). Because frameshifting requires the long-distance interaction, the replicase could disrupt the LDFE in the 3' UTR, and thus block frameshifting, before the replicase reaches the translated ORF. By the time the replicase would reach the RdRp-encoding ORF, that portion of the RNA would be ribosome-free. A similar process is predicted to occur via a cap-independent translation element, located near the LDFE, which must base pair to the 5' UTR to initiate translation of both the pre-shift and post-shift (RdRp) ORFs (Guo et al., 2001). This interaction would also be disrupted by the replicase, shutting off translation initiation, freeing the entire viral genomic RNA of ribosomes, making it available for

full replication (Barry and Miller, 2002). Future experimentation will determine the validity of this model.

9.2.3 *Polerovirus and Enamovirus*

The four viruses studied in these two genera are beet western yellows virus (BWYV), potato leaf roll virus (PLRV), sugarcane yellow leaf virus (ScYLV), and pea enation mosaic virus RNA1 (PEMV-1). BWYV, PLRV, and ScYLV are classified as poleroviruses, while PEMV-1 is the sole member of the *Enamovirus* genus (Fig. 9.2). PEMV is unique in that it harbors a second taxonomically distinct(+)-sense RNA, PEMV RNA2, classified in the *Umbravirus* genus (Demler et al., 1993). RNA1 and RNA2 can replicate independently but both are required for wild-type levels of infectivity (Demler et al., 1994).

The reported frameshifting efficiency of poleroviruses and enamoviruses ranges from 1–15% depending on the frameshift signal itself as well as the biological context within which the measurement was made; indeed, earlier measurements place this efficiency at ≈ 1 –2% using standard in vitro eukaryotic translation systems (Garcia, van Duin and Pleij, 1993; Kujawa et al., 1993). More recent measurements using a dual luciferase assay place this value at 4–6% for BWYV, 9% for PEMV-1, and $\approx 15\%$ for ScYLV -1 frameshifting P1–P2 wild-type signals, as measured by coupled transcription–translation assays with rabbit reticulocyte lysates (Cornish, Hennig and Giedroc, 2005; Kim et al., 2000, 1999; Nixon et al., 2002).

The slippery sequence at which the ribosomes change reading frame is GGGAAAC in PEMV-1 (Nixon et al., 2002), BWYV (Garcia et al., 1993), and ScYLV (Moonan et al., 2000; Smith et al., 2000), and a more efficient sequence UUUAAA(U/C) in PLRV (Garcia et al., 1993; Kujawa et al., 1993) (see Fig. 9.3 below). The second element is a downstream hairpin-type (H-type) RNA pseudoknot positioned 6–8 nt from the 3' edge of the slip-site (see Figs. 9.3 and 9.4). The slip-site alone dramatically increases the intrinsic level of frameshift errors from 0.00005 to ≈ 0.005 per codon depending strongly on the sequence (Stahl et al., 2002), with the pseudoknot further stimulating this process from 5- to 30-fold more.

9.2.4 *Frameshift Stimulators with Compact Pseudoknots*

Although a diverse array of RNA motifs are capable of stimulating -1 frameshifting when placed ≈ 6 –8 nt downstream of the slip-site (Giedroc and Cornish, 2009), the atomic (1.3–1.6 Å) resolution crystallographic structure of the 28-nt P1–P2 pseudoknot from BWYV clearly established how an RNA pseudoknot with a very short 3-bp pseudoknot-forming stem S2 could fold and stimulate -1 frameshifting (Egli et al., 2002; Su et al., 1999) (Fig. 9.3A). This RNA is compact and is largely triple-stranded RNA with the majority of the L1 and L2 loop nucleotides making

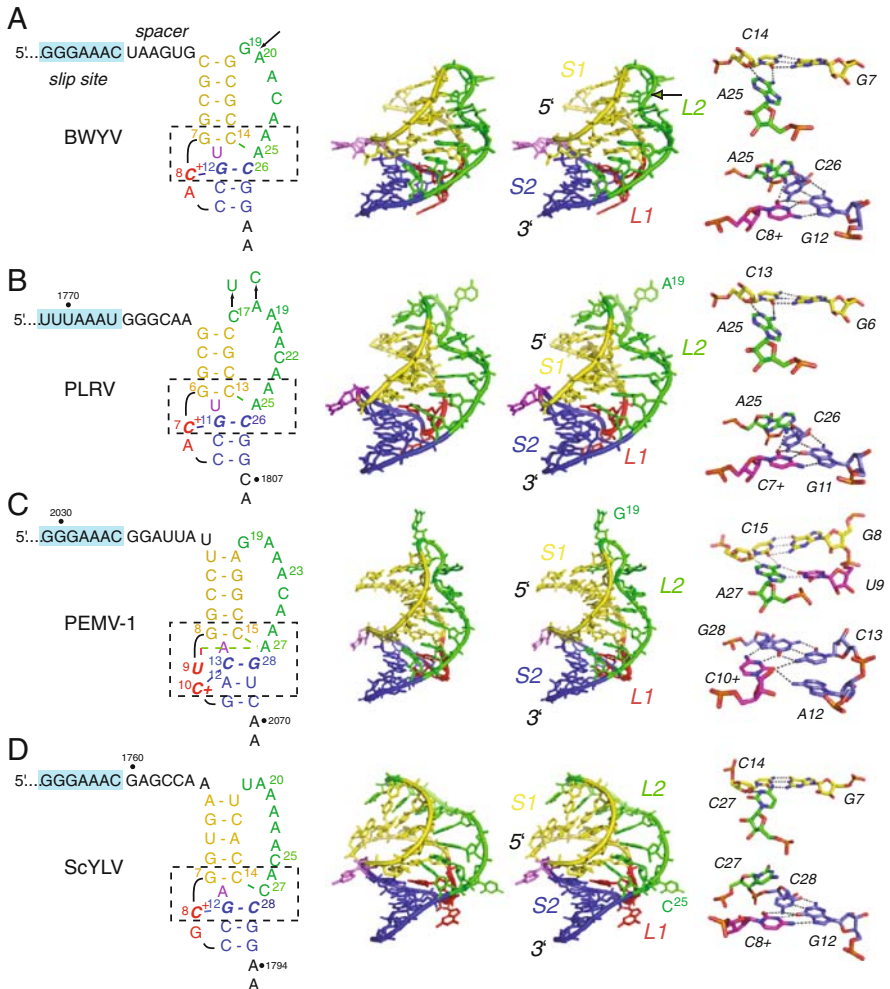


Fig. 9.3 Stereo views of the structures of four related luteoviral P1–P2 pseudoknots. (A) BWYV (PDB 1L2X solved to 1.25 Å resolution) (Egli et al., 2002); (B) PLRV (PDB 2A43 solved to 1.34 Å resolution) (Pallan et al., 2005); (C) PEMV-1 (PDB 2RP1) (Giedroc and Cornish, 2009); (D) ScYLV (PDB 1Y4) (Cornish, Hennig and Giedroc, 2005). Secondary structure models of the four frameshift signals are shown on the *far left*, with nucleotides in S1 (yellow), L1 (red), S2 (blue) and L2 (green) color-coded as in the stereo views of the structures (*middle*). Genomic RNA nucleotide numbers are also shown for the PLRV, PEMV-1, and ScYLV RNAs. Close-ups of the helical junction regions of all four RNAs are shown to the *far right* (these correspond to the regions *inside* the dashed box on the secondary structure diagrams, *far left*) with the L2–S1 minor groove base triple highlighted at the *top*, and the L1–S2 major groove base quadruple shown at the *bottom*. Stereo images and helical junctions are reprinted from Virus Research 139, Giedroc DP, Cornish PV, Frameshifting RNA pseudoknots: Structure and mechanism, pp 193–208, with permission from Elsevier

noncanonical base–base and base–sugar edge hydrogen bonds with base pairs of the two stems. A protonated C8+ from loop L1 forms a standard Hoogsteen-type base pair with an accepting G12 as part of a C8+•(G12–C26) base triple at the helical junction (Fig. 9.3A). The two helical stems are strongly overrotated relative to one another, with the S1 G7–C14 base pair actually stacked on the S2 C8+•G12 Hoogsteen pair; this overrotation is partly mediated by an unpaired nucleotide (U12 in BWYV) that is flipped out of the stack and may function as a “spacer” at the helical junction. Thermodynamic and structural studies of the three other luteoviral RNA pseudoknots confirm that the C+•(C–G) L1–S2 major groove triple base pair is a common feature of all P1–P2 RNA pseudoknots from polioviruses and the enamovirus (Fig. 9.3) (Nixon et al., 2002; Nixon and Giedroc, 2000). Although protonation of this cytidine is strongly stabilizing, the degree to which protonation affects frameshifting efficiency or mechanical stability (Tinoco et al., 2006) remains unknown.

The other striking structural feature of the BWYV pseudoknot is a minor groove triplex, in which a run of three consecutive adenosines at the 3' end of the loop L2 (A23–A24–A25) forms a series of Watson–Crick–sugar edge hydrogen bonding interactions with base pairs of the upper stem S1 closest to the helical junction (see Fig. 9.3A) (Egli et al., 2002; Su et al., 1999). While reminiscent of classical A–minor interactions found in other complex RNAs and the large ribosomal subunit (Doherty et al., 2001; Nissen et al., 2001), these interactions are characterized by direct adenosine N1–2'OH hydrogen bonding interactions, many of which could be detected directly in solution using NMR methods (Cornish et al., 2005, 2006; Giedroc et al., 2003; Nixon et al., 2002) (see below). Other distinct L2–S1 hydrogen bonding interactions are found further from the helical junction in all luteoviral RNAs but these tend to be unique to individual RNAs.

An extensive mutational analysis of BWYV pseudoknot revealed that the hydrogen bonding interactions that stabilize the distinct helical junction region and the minor groove triplex are critical for supporting high levels of frameshift stimulation in vitro (Kim et al., 1999). However, a remarkable finding not predicted by the structure was that a single nucleotide insertion between G19 (or U19) and A20 at the tip of loop L2 dramatically increased frameshift stimulation, by upward of 300% (Kim et al., 1999) (see Fig. 9.3A, *arrow*). The origin of this stimulation is unknown and it is unclear the degree to which these mutations alter hydrogen bonding interactions of A20 with the G4–C17 base pair (Su et al., 1999). It is interesting to note, however, that this region of the pseudoknot is likely in closest proximity, or in direct contact, with the ribosome during frameshifting. These findings seem to parallel those found for a single nucleotide *deletion* mutant in loop L2 in the ScYLV P1–P2 frameshifting pseudoknot, which stimulates frameshifting by 200% relative to the wild-type RNA (Δ C25; see Fig. 9.4D, below) (Cornish, Hennig and Giedroc, 2005), via an as-yet unknown mechanism. Along similar lines, substitution of the extrahelical “spacer” nucleotide at the helical junction (U13 in the BWYV RNA; U12 in PLRV; see purple base in Figs. 9.3A–B) with A or G gives rise to a detectable *decrease* in frameshift stimulation (ranging from 35 to 90% of WT levels in vitro) (Kim et al., 1999, 2000). Since the analogous spacer adenosine in the PEMV-1 and ScYLV pseudoknots is highly dynamic in solution (Nixon et al., 2002;

Cornish et al., 2005), the structural origin of this small effect is unknown; one possibility of course is that this nucleotide also makes physical contact with the ribosome mRNA entry tunnel given its close proximity to the “top” of stem S1.

Figure 9.3B–D compares the structures of three other luteoviral P1–P2 frameshifting pseudoknots to that from BWYV (Fig. 9.3A) (Egli et al., 2002; Su et al., 1999). These include a crystallographic structure of the PLRV pseudoknot (Pallan et al., 2005), an NMR solution structure of the P1–P2 signal from PEMV-1 (Giedroc and Cornish, 2009; Nixon et al., 2002), and a solution structure of the frameshifting motif from ScYLV (Cornish et al., 2005). The PEMV-1 RNA structure clearly reveals that the five most 3′ residues of loop L2 (5′-A23-C24-A25-A26-A27) adopt a conformation that is essentially identical to that found in the BWYV (Egli et al., 2002) and PLRV (Pallan et al., 2005) pseudoknots, each of which contains the same 5′-ACAAA 3′ L2 sequence (Fig. 9.3). This L2 structure is achieved by anchoring the Watson–Crick edges of C24 through A27 into the S1 minor groove where they are engaged in numerous hydrogen bonding interactions.

A critical and unique aspect of the PEMV-1 RNA structure is that both Watson–Crick and Hoogsteen edges of the 3′ nucleotide of loop L2, A27 are tied up in hydrogen bonding (Giedroc et al., 2003; Nixon et al., 2002). In addition to A27 (and A25), the N1/N6 face of A26 forms two hydrogen bonds with the N3–N2 edge of G7. The C24 N4 amino group is also close to the 2′ OH of G17. A23 is stacked on C24, with the Watson–Crick edge rotated out of the stack, leaving the N7 and N6 nitrogens within hydrogen bonding distance of the 2′-OH of G17. At this point the PEMV-1 structure diverges from the BWYV/PLRV structures, with A22 stacked on A23, and essentially extruded from the triple helix, with A21 inserted back into the S1 minor groove near the top two S1 base pairs. G20 is extrahelical. In fact, if one considers A22 an extrahelical insertion in the PEMV-1 loop L2, the entire 5′-A(A)ACAAA loop sequence adopts essentially identical conformations in all three pseudoknots (Fig. 9.4). As was found for the BWYV RNA, strong support for the functional importance of the key structural features was also obtained for the PEMV-1 RNA; for example, efforts to replace the unique PEMV-1 helical junction region with that of the BWYV RNA failed (Nixon et al., 2002).

The solution structure of the ScYLV P1–P2 pseudoknot is characterized by several unique features relative to the PEMV-1 and BWYV/PLRV RNAs (see Fig. 9.3D) (Cornish et al., 2005). Notably, all of loop L2 is very well ordered



Fig. 9.4 Structures of the BWYV (A), PLRV (B), PEMV-1 (C), and ScYLV (D). P1–P2 pseudoknots emphasizing the structure of the minor groove spanning L2 loop (Giedroc and Cornish, 2009). The complete nucleotide sequence (5′–3′) of loop L2 is indicated for each RNA, with extrahelical nucleotides in parentheses. Lower case nucleotides in the PLRV RNA represent substitutions of the wild-type RNA sequence (C17u; A18c) incorporated to facilitate crystal packing (Pallan et al., 2005). u17 in the PLRV RNA (B) is grayed out since it is not colored in the structure shown. Bound Mg²⁺ ions are also indicated as *stippled spheres* in the BWYV and PLRV pseudoknot structures. Reprinted from Virus Research 139, Giedroc DP, Cornish PV, Frameshifting RNA pseudoknots: Structure and mechanism, pp 193–208, with permission from Elsevier

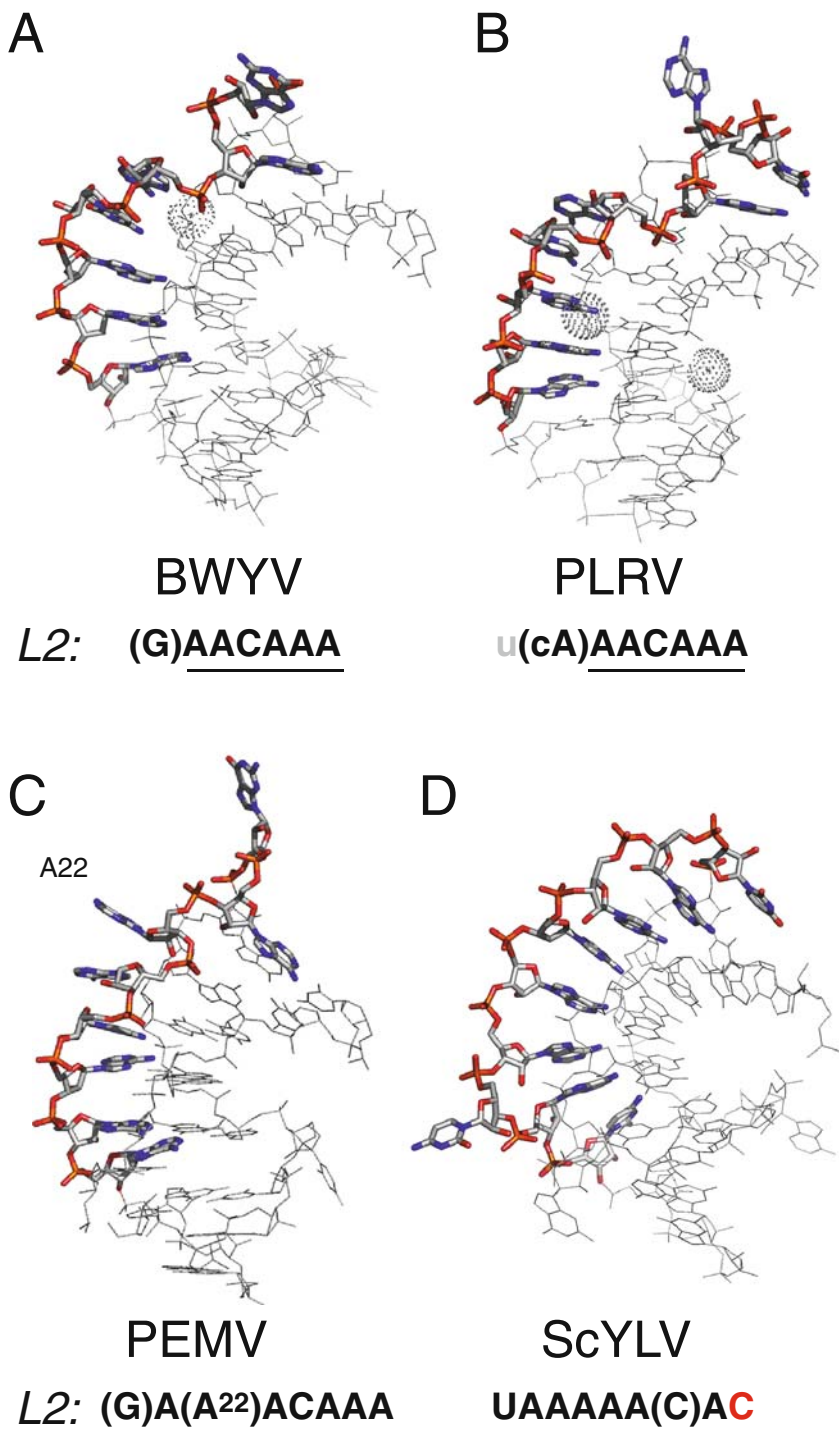


Fig. 9.4

and exhibits continuous stacking of A20 through C27 into the minor groove of S1, with C25 flipped out of the triple-stranded stack. Five consecutive triple base pairs flank the helical junction where the 3' nucleotide of L2, C27, adopts a cytidine 27 N3–cytidine 14 2'-OH hydrogen bonding interaction with the C14–G7 base pair (Cornish et al., 2006). This interaction is isosteric with the adenosine N1–2'-OH interaction found in the BWYV (Su et al., 1999), PLRV (Pallan et al., 2005), and PEMV-1 RNAs (cf. Fig. 9.3, *right-most column*); however, the ScYLV and BWYV mRNA structures differ in their detailed L2–S1 hydrogen bonding and L2 stacking interactions.

Given the isosteric nature of the C27 N3●●●C14 2' OH (ScYLV) and A25 N1●●●C14 2' OH (BWYV) hydrogen bonds in these two RNAs by NMR (Cornish et al., 2006; Giedroc et al., 2003), the extent to which a BWYV-like adenosine in the ScYLV context could functionally substitute for the 3' L2 cytidine (C27) was investigated using an in vitro coupled transcription–translation assay with rabbit reticulocyte lysates. The unexpected finding from these experiments is that the C27A ScYLV RNA is a very poor frameshift stimulator in vitro (Cornish et al., 2005). This surprising finding, in turn, made the prediction that substitution of A25 in the BWYV RNA with cytidine (A25C) would *increase* frameshifting in this context; this is exactly what was found. Thus, a 3' L2 cytidine is a positive determinant for frameshift stimulation by luteoviral RNAs (Cornish et al., 2006). It would be of interest to determine the degree to which other *Luteoviridae* frameshifting pseudoknots with a cytidine in this position, e.g., cereal yellow dwarf virus (CYDV) and beet chlorosis virus (BCV) are also superior frameshift stimulators (Smith et al., 2000). The degree to which the C27A substitution (C1790A in the full-length genome) influences replication of ScYLV in plants is unknown; indeed, such an experiment is complicated by the fact that this substitution would result in a Thr-to-Asn substitution in the P1–P2 fusion protein (Moonan et al., 2000).

Strikingly, the global structure of the poorly functional C27A ScYLV RNA is nearly indistinguishable from the wild-type counterpart, despite the fact that the helical junction region is altered and incorporates the anticipated isostructural A27·(G7–C14) minor groove base triple (Cornish et al., 2006) in adopting a helical junction region that is basically indistinguishable from the BWYV and PLRV pseudoknots (Su et al., 1999), as expected. These results suggest that the lowest energy “ground-state” structure is *not* strongly correlated with frameshift stimulation; instead, these findings point to a reduced stability that derives from an altered helical junction architecture in the C27A ScYLV RNA as of critical importance, for which there is some evidence (Cornish and Giedroc, 2006; Cornish et al., 2005).

Studies in other frameshifting systems have uncovered a correlation between thermodynamic stability of the downstream element and frameshifting efficiency (Bidou et al., 1997; Larsen et al., 1997; ten Dam et al., 1995), although it is not obvious why this has to be the case (Cao and Chen, 2008; Theimer and Giedroc, 2000). In fact, as discussed above, insertion or deletion of an unpaired and extrahelical residue(s) in loop L2 can strongly *increase* frameshifting efficiency, a finding often attributed to a direct interaction with the ribosome (Kim et al., 1999, 2000). We have argued that distinct unfolding thermodynamics measured for closely related

RNAs, e.g., WT vs. C27A ScYLV RNAs, may in fact be reporting on different kinetics of pseudoknot unfolding, with increased rates of unfolding, i.e., unfolding at lower applied forces, negatively correlated with frameshift stimulation (Giedroc et al., 2000; Theimer and Giedroc, 2000). In other words, these studies suggest that the helical junction in luteoviral RNAs is mechanically stable and functions as a classic kinetic barrier to force-induced unfolding (Onoa et al., 2003), essentially placing the unfolding of the entire pseudoknot under kinetic control. This barrier is predicted to be altered in functionally compromised RNAs. Mechanical force-induced unfolding/refolding experiments will be required to obtain direct evidence for this proposal (Green et al., 2008; Hansen et al., 2007).

9.2.5 *Sobemoviruses*

Sobemoviruses are similar to the poleroviruses and enamovirus based on sequence homology of the RdRp and the presence of a VPg (Makinen et al., 2000; Skaf et al., 2000; van der Wilk et al., 1998). Despite homology at the genome level, sobemoviruses are not classified in the Luteoviridae because, unlike Luteovirids, sobemoviruses (i) have a significantly smaller genome (just over 4 kb), (ii) encode a larger coat protein that lacks the readthrough domain found in the Luteoviridae, (iii) are mechanically transmissible, (iv) are not persistently transmitted by aphids, and (v) are not limited to the phloem (Miller et al., 2002; Tamm and Truve, 2000). All sobemoviruses employ -1 frameshifting, but recent resequencing results have challenged previous conclusions on the nature of the ORF translated via the frameshift. The most recent (and now apparently incorrect, see below) ICTV classification (Hull and Fargette, 2005) divides sobemoviruses into two subgroups: those in which the frameshift event shifts the ribosome out of the RdRp frame into a much shorter reading frame (ORF 3) and those in which the ribosome is shifted into the RdRp ORF (ORF 2b) as is the case with all other viruses discussed previously in this chapter.

Some of the first sobemoviruses sequenced, including southern cowpea mosaic virus (SCPMV, Genbank accession no. NC_001625), southern bean mosaic virus (SBMV, NC_004060), Rice yellow mottle virus (RYMV, NC_001575), Ryegrass mosaic virus (RGMoV, NC_003747), and Lucerne transient streak virus (LTSV, NC_001696) were thought to encode a protease, VPg, and RdRp all in ORF 2, which would have been translated as one large polyprotein of about 100 kDa (Fig. 9.5) (Tamm and Truve, 2000). A ribosomal frameshift site identified in the middle of this ORF was proposed to allow translation of a short ORF 3 of unknown function (Fig. 9.5). Other sobemoviruses, the first being cocksfoot mottle virus (CfMV, (Makinen et al., 1995)), were reported to have a more polerovirus-like genome organization with the protease and VPg encoded in ORF 2a and the RdRp translated from the overlapping ORF2b via -1 frameshift (Fig. 9.5). The amino acid sequences just downstream of the frameshift site were shown to be homologous between the two groups of viruses, i.e., ORF 3 of the viruses with the SCPMV-like organization was

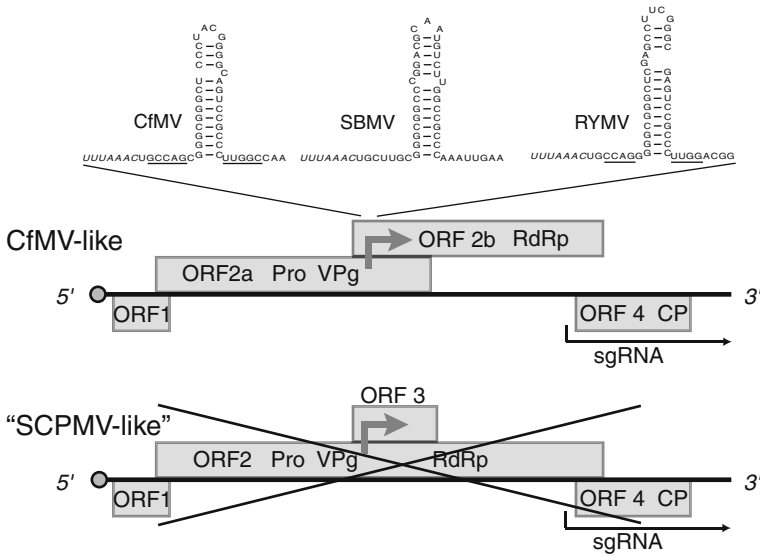


Fig. 9.5 Corrected genome organization of sobemoviruses and examples of -1 frameshift sequences. *Bottom*: Genome organizations of the two apparent subgroups of sobemoviruses are shown. Erroneous SCPMV-like organization is crossed out. All sobemoviruses have the CfMV-like organization (Meier and Truve, 2007). *Top*: Predicted secondary structures of the indicated sobemoviral RNAs at the -1 frameshift site (Makinen et al., 1995). Slippery site is in italics. Underlined bases extend the helix in selected viruses but this additional base pairing is not phylogenetically conserved. Frameshifting has been demonstrated only for CfMV and no data exist to support or refute the secondary structures

shown to be homologous to the portion of the 2b-encoded protein (RdRp) immediately downstream of the frameshift site in the CfMV-like viruses (Dwyer et al., 2003). Why would some viruses use frameshifting to produce a truncated version of the RdRp (those with ORF 3), while others use frameshifting to produce the entire RdRp (those with ORF 2b)? This puzzle was resolved recently by resequencing of all of the so-called SCPMV-like viral genomes which revealed that they all have CfMV-like genome organizations (Balke et al., 2007; Fargette et al., 2004; Lokesh et al., 2001; Meier and Truve, 2007). Meier and Truve (2007) showed that sequencing errors in the form of a single base insertion in the region of ORF 2–ORF 3 overlap led to the appearance that the RdRp coding region was in the same ORF as the upstream protease coding region. Thus, the sequences thought to be in “ORF 3” that were homologous to the 2b ORF are actually all encoded by ORF 2b. In short, resequencing revealed a paradigm shift on the role of the frameshift in sobemoviruses.

Frameshifting has been demonstrated for CfMV (Makinen et al., 1995). The portion of the genome that confers -1 ribosomal frameshifting was narrowed to a sequence that includes a simple imperfect stem-loop with one or two unpaired bases interrupting the helix (Lucchesi et al., 2000). No mutagenesis or structure probing

has been performed to verify the structure, but it contains a highly stable, extremely GC-rich stem-loop predicted to be stabilized by a UNCG or GNRA tetraloop at the end (Fig. 9.5). It is conserved in all sobemoviruses, and no alternative structures such as a pseudoknot have been reported or predicted. This imperfect stem-loop superficially resembles the bipartite stem-loop that stimulates -1 frameshifting of HIV-1 and SIV RNAs (Gaudin et al., 2005; Marcheschi et al., 2007; Staple and Butcher, 2005).

Lucchesi et al. (2000) observed about a 10% frameshift rate by CfMV RNA in wheat germ extract. When a stop codon was placed downstream in the -1 ORF to mimic translation of the ORF 3 in what they thought was the situation in the SCPMV-like viruses, the authors found frameshift rates of about 50%. They proposed that this stop codon (which could be 53–462 nt in the -1 frame downstream of the shifty site) enhanced stalling of ribosomes on the shifty site. However, similarly placed stop codons downstream in the zero frame (ORF 2a frame) did not enhance frameshifting.

In yeast cells, using a β -galactosidase–firefly luciferase dual reporter assay designed so that expression of a luciferase gene required the CfMV -1 frameshift sequence, frameshifting efficiencies varied wildly. The small stem-loop alone gave 14% frameshifting, while a region that included about 750 nt of viral sequence spanning upstream and downstream, beyond the region of ORF overlap, gave a 26% frameshift rate (Makelainen and Makinen, 2005). Surprisingly, a subset of that ORF overlap sequence gave a whopping, apparent 68% frameshift rate in yeast. Using a dual luciferase reporter in wheat germ extract, the rates with the same inserts were 25%, 36%, and 28%, respectively (Makelainen and Makinen, 2005). Frameshift rates were less than 6% in bacterial cells but frameshifting was not tested in plant cells. The authors conclude that “reliable estimates for -1 programmed ribosomal frameshifting . . . can be obtained only by using full-length viral sequences.” Importantly, altering the UUUAAC frameshift sequence to UUUAAGC or removal of the sequence containing the predicted adjacent stem-loop sequence totally abolished frameshifting in wheat germ extract (Lucchesi et al., 2000).

Unlike most studies, Makelainen and Makinen (2005) tested the effects of viral proteins on frameshifting efficiencies in yeast. They found that the P1 protein encoded by the pre-shift (zero frame) ORF, called p27, appeared to reduce frameshifting as measured by its negative effect on luciferase expression in yeast cells. This finding suggests a possible frameshift regulatory role for p27 in viral infection. Its significance would be improved if the results can be corroborated by studies in plant cells and with related viruses.

9.2.6 *Closteroviruses*

Viruses in the Closteroviridae family of positive sense RNA viruses have the largest genomic RNA of any plant virus, ranging from 12 to 20 kb. The genome organization and expression strategy resemble that of viruses in the Nidovirales order of

animal viruses (see Chapter 7): The 5' half to two-thirds of the genome encodes proteins involved in replication (Karasev, 2000). The 3' portion of the genome contains several ORFs expressed from a nested set of 3' co-terminal subgenomic RNAs. In closteroviruses, the protease and RNA replication genes, encoded by ORFs 1a and 1b, have similarities to those in the alphavirus-like supergroup, including putative helicase, methyltransferase, and two proteases encoded by ORF 1a followed by the RdRp encoded by ORF 1b (Karasev, 2000). Unlike the Nidovirales, expression of the RdRp (ORF1b) is predicted to require a +1, instead of a -1, reading frame change to be expressed (Agranovsky et al., 1994; Karasev et al., 1995).

It must be pointed out that frameshifting has yet to be experimentally demonstrated for closteroviruses. The +1 frameshift was proposed to occur either at the stop codon of the 1a ORF in beet yellow virus (BYV) (Agranovsky et al., 1994) or at a rare codon at the homologous position in citrus tristeza virus (CTV) (Karasev et al., 1995). Either codon would facilitate frameshifting by inducing ribosomes to pause, analogous to the mechanism of +1 frameshifting transposable elements in yeast (Belcourt and Farabaugh, 1990). Sequencing of additional closterovirids revealed no obvious conserved frameshift site. Similar to BYV, pineapple mealybug wilt-associated virus-1 (PMWaV-1) has a putative +1 frameshift site, GUUUAGC, in which underlined bases indicate the ORF 1a stop codon (UAA in BYV) and italics indicate first codon (asparagine) of the 1b ORF (Melzer et al., 2008). In contrast closteroviruses PMWaV-2 and grapevine leafroll-associated virus-3 (GLRaV-3) have an entirely different sequence at this location in the genome, UUUCGAG (proposed first codon of ORF 1b in italics), at which the +1 shift is proposed to occur (Melzer et al., 2008). We await experimental evidence to determine if either of these sites is the actual frameshift site.

Cevik et al. (2008) detected the RdRp of CTV in infected cells and by cell-free *in vitro* translation. Enigmatically, western blots probed with antibodies against the 57-kDa RdRp domain (ORF 1b product) of CTV revealed only a ~50-kDa product, a ~10-kDa fragment but no 400-kDa product expected of the whole 1a–1b transframe protein. This led the authors to conclude that the 57-kDa RdRp domain is proteolytically cleaved immediately from the precursor 1a–1b fusion protein into ~50- and ~10-kDa fragments. The lack of detection of the expected full-length 1a–1b protein, or a larger precursor of the 57-kDa product, leaves the mechanism of expression of the RdRp domain in doubt. Even if the RdRp is produced as a cleavage product of the 1a–1b fusion, it is not known whether it is produced by a +1 frameshift or -2, +4 shift or a larger ribosome hopping mechanism.

9.2.7 *Carlaviruses*

Potato virus M in the genus *Carlavirus* has a 12-kDa extension added to the coat protein (CP) by a novel type of -1 frameshift. The 34-kDa CP ORF terminates with the sequence AAU AGA AAA UGA, with the AUG forming a potential start codon for the 12-kDa ORF. In reticulocyte lysates, Gramstat et al. (1994) showed that the 12-kDa protein can be synthesized by initiation at the AUG, but of greater interest is that

the 12 kDa is also present fused to the C terminus of the CP via a -1 frameshift that does not require the canonical shifty heptanucleotide, nor does it require a downstream structured element. Instead, this frameshift requires only a homotetramer of AAAA or UUUU immediately upstream of an adjacent stop codon. The overlapping AUG in the 12-kDa ORF frame is not necessary for frameshifting. The authors propose a model in which ribosome slippage occurs with the tRNA:mRNA base-paired only at the P-site, rather than the canonical simultaneous A- and P-site slippage, because the A-site would be at the stop codon (Gramstat et al., 1994). Readers should be aware that the analysis of the frameshift signal was performed using only rabbit reticulocyte lysates which often have reduced translational fidelity relative to wheat germ extract (Dinesh-Kumar et al., 1992; Kozak, 1990) or, most importantly, compared to the environment *in vivo*. The evidence that this frameshift takes place *in vivo* is identification of a low-abundance protein of the expected size using antisera targeted to either the product of the 34-kDa CP ORF or the 12-kDa ORF. We are unaware of any subsequent research that confirms the proposed frameshift process *in vivo*.

9.2.8 Potyviruses

Viruses in the large, economically important Potyviridae family were thought to contain a single ORF encoding a ~ 340 – 395 -kDa polyprotein that is cleaved by viral proteases into the functional proteins, like viruses in the Picornavirales (Berger et al., 2005; Le Gall et al., 2008). However, a small overlapping ORF called *pipo* was discovered recently using bioinformatic methods (Chung et al., 2008). This ORF, which ranges in size from 60 to 115 codons depending on the virus, is in the $+2$ (or -1) frame relative to the polyprotein ORF and overlaps with the polyprotein ORF region coding for the P3 protein (Fig. 9.6). In turnip mosaic potyvirus (TuMV), the amino acid sequence (referred to in uppercase as PIPO) encoded by *pipo* has a molecular weight of 7 kDa, but antibodies against this protein detected only a ~ 25 -kDa protein in TuMV-infected plants (Chung et al., 2008). A protein of this size is consistent with a fusion product of the amino terminal portion of the P3 protein (after proteolytic cleavage of P3 from the polyprotein) fused to PIPO and terminating at the *pipo* stop codon (Fig. 9.6). Thus, PIPO does not appear to be translated independently.

A possible mechanism for expression of PIPO is by frameshifting of some ribosomes from the P3 coding region into the -1 ($= +2$) frame at the beginning of the *pipo* ORF. A likely frameshift site is the $G_{1-2}A_{6-7}$ motif which is the only well-conserved sequence in *pipo* and is predicted to be the start of the *pipo* ORF based on the presence of in-frame stop codons upstream in some potyviruses (Chung et al., 2008). GAA AAA A (gaps separate codons in the polyprotein ORF) does not fit the canonical X XXY YYZ shifty sequence at the -1 frameshift site used by other viruses, although it is conceivable that ribosomes could shift reading frame in the homopolymeric A tract. It is noteworthy that the potyvirus $G_{1-2}A_{6-7}$ motif resembles the AGA AAA UGA sequence at the reading frame

change site in carlaviruses (above), suggesting that a possible common frameshift mechanism may be employed by these unrelated virus groups. No conserved secondary structures, such as those required for conventional -1 frameshifting, have been detected downstream of the $G_{1-2}A_{6-7}$ motif in potyviral RNAs (Chung et al., 2008). Thus, if -1 or $+2$ frameshifting or a type of ribosome hop that leads to a net change of $+2$ in reading frame takes place, it is likely to be via a mechanism different from that of “canonical” -1 frameshifting. Other nontranslational mechanisms can explain the production of a 25-kDa protein containing PIPO epitopes, so further experimentation is necessary to determine whether potyviruses employ frameshifting.

9.3 Summary

In most cases, the biological role and basic mechanism of -1 ribosome frameshifting are likely the same in plant viruses as in animal viruses. While some of the shifty heptanucleotide sites are the same in plant and animal viruses, the specific downstream structures that facilitate -1 frameshifting differ significantly between viruses of the two kingdoms. No ALIL-like structures that base pair with a stem-loop kilobases downstream are known outside of the plant viruses. At the other extreme, the polero- and enamoviruses have the smallest known frameshift pseudoknots, enabling the highest levels of structural characterization of any frameshift-inducing structure. As these structures facilitate frameshifting in rabbit reticulocyte lysates (Cornish et al., 2005; Kim et al., 1999, 2000; Nixon et al., 2002), the reason why they exist only in plant viruses is not likely due to special features unique to plant ribosomes. These plant virus frameshift signals add to the large and diverse pool of eukaryotic frameshift elements, which should perhaps help us solve the question of why some RNA structures, but not others of similar apparent stability, interact with the ribosome in a way that brings about a -1 frameshift. The apparent $+1$ frameshift of closteroviruses appears to be confined to the plant virus world as are the possible -1 frameshift signals in carlaviruses and potyviruses. Thus, we eagerly

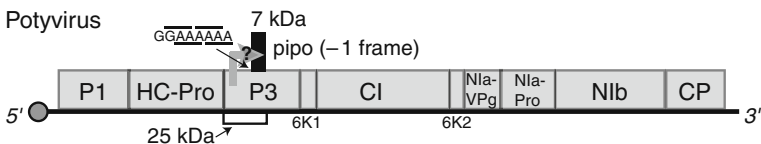


Fig. 9.6 Potyvirus genome organization. *Gray boxes* indicate individual proteins generated by proteolytic cleavage of the polyprotein. *Black box* above P3 represents the pipo ORF in the -1 frame. *Large gray arrow* with question mark indicates possible frameshift event. An example of the conserved $A_{1-2}G_{6-7}$ site is shown at the start of the pipo ORF with lines under the codons in the zero frame (polyprotein ORF) and lines over the codons in the -1 frame (pipo ORF). The 25-kDa protein that could be generated by a frameshift followed by cleavage at the HC-Pro/P3 cleavage site is indicated (Chung et al., 2008)

anticipate results of future research and structural analysis to determine how these diverse plant viral RNAs induce ribosomes to change reading frames by what may be novel mechanisms.

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