Published in final edited form as:
Annu Rev Phytopathol. 2006 ; 44: 447–467.

Long-Distance RNA-RNA Interactions in Plant Virus Gene Expression and Replication

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Abstract

The vast majority of plant and animal viruses have RNA genomes. Viral gene expression and replication are controlled by *cis*-acting elements in the viral genome, which have been viewed conventionally as localized structures. However, recent research has altered this perception and provided compelling evidence for cooperative activity involving distantly positioned RNA elements. This chapter focuses on viral RNA elements that interact across hundreds or thousands of intervening nucleotides to control translation, genomic RNA synthesis, and subgenomic mRNA transcription. We discuss evidence supporting the existence and function of the interactions, and speculate on the regulatory roles that such long-distance interactions play in the virus life cycle. We emphasize viruses in the *Tombusviridae* and *Luteoviridae* families in which long-distance interactions are best characterized, but similar phenomena in other viruses are also discussed. Many more examples likely remain undiscovered.

Keywords

tombusvirus; luteovirus; kissing stem-loop; subgenomic mRNA; cap-independent translation

INTRODUCTION

Plant viruses utilize a variety of strategies in the regulation of their gene expression and replication. For RNA viruses, specific RNA sequences, structures, and interactions play critical roles in modulating these processes. Although often viewed as one- or two-dimensional structures, RNAs are three-dimensional molecules with complex tertiary interactions that may span long distances relative to the linear arrangement of nucleotides (2). Viral RNAs are actually four-dimensional, because coordinated changes in the structure and hence function of a given region occur over time in order to achieve productive infection. In many cases, the communication required between distal RNA elements is provided by RNA-RNA interactions. Here we provide an update and perspective on important advances involving long-distance RNA-RNA interactions that control plant virus translation, genome replication, and subgenomic (sg) mRNA transcription.

CAP-INDEPENDENT TRANSLATION ELEMENTS

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A new cap-independent translation element was identified recently in the 3′ UTR of *Panicum mosaic virus* of the *Panicovirus* genus (*Tombusviridae*). It is predicted to contain a Y-shaped stem-loop structure capable of a six-base-pair kissing interaction with a stem-loop in the 5′ UTR (Batten et al., 2006). Batten JS, Desvoyes B, Yamamura Y, Scholthof K-B. 2006. A translational enhancer element on the 3′ proximal end of the *Panicum mosaic virus* genome. *FEBS Lett*. 580:2591–97

Cap-independent translation elements in the 3′ UTR are unique to plant viruses … so far. They fall into at least three distinct structural classes, with more likely to be discovered:

- The translation enhancer domain (TED) of *Satellite tobacco necrosis virus*, which forms a long, bulged stem-loop.
- The BYDV-like cap-independent translation element (BTE) of the *Luteovirus, Dianthovirus and Necrovirus* genera. This element features multiple stem-loops and the conserved sequence GGAUCCUGGGAAACAGG.
- The cap-independent translation element (CITE) of the *Tombusvirus* genus and some, but not all, other genera of the *Tombusviridae*. This is a long branched structure.

A bulged stem-loop in the 3′ end of Maize necrotic streak virus RNA (Tombusviridae) may represent a fourth class of 3′ cap-independent translation element. All of the elements have loops with potential to base pair to the 5′ UTR. This long-distance base pairing has been demonstrated for the BTE and CITE elements.

TRANSLATION

Initiation in *Luteoviridae* and *Tombusviridae*

According to the scanning model of start codon recognition on mRNA, the 40S ribosomal subunit, along with associated factors, is recruited by the 5′ m^7GpppN cap structure to the 5′ terminus of the mRNA where it scans in the 3′ direction until it reaches the first AUG, at which the 60S subunit joins and translation ensues (5,39,61). Thus, many viral and cellular mRNAs contain *cis*-acting signals in the 5′ untranslated region (UTR) that affect translation efficiency (17). These include the translation enhancers such as the omega sequence of *Tobacco mosaic virus* (TMV) (75) and the 5′ UTR of *Alfalfa mosaic virus* (AMV) RNA 4 (31), and sequences that attenuate translation initiation, such as the 5′ UTR of *Brome mosaic virus* RNA 2 (56).

The 3′ UTR can also be a repository of translational control signals in host and viral mRNAs (25,77). A ubiquitous example is the poly(A) tail, which is required for efficient translation initiation (20,77). Thus, while the basic scanning model still holds (60), regulation by the 3′ UTR requires additional mechanisms to explain how the ribosome is recruited to the mRNA. Long-distance interactions between the 5′ and 3′ ends are probably essential for efficient translation of all eukaryotic mRNAs (68) including viral RNAs (18). With no known exceptions, all plant-encoded mRNAs contain a 5′ cap (m^7GpppN), and a 3′ poly(A) tail. These moieties act synergistically to stimulate translation of mRNAs (20) because they play key roles in formation of the mRNA initiation complex that recruits the ribosome (26). The poly(A) tail attracts poly(A)-binding protein (PABP), the 5′ cap binds eukaryotic initiation factor (eIF) 4E, and both cap-bound eIF4E and poly(A)-bound PABP simultaneously bind the large scaffolding protein, eIF4G (30,40,64). This circularizes the mRNA via a multiprotein bridge (24,86) and results in recruitment of the 40S ribosomal subunit via eIF3, by a mechanism that is still being worked out (10,72).

Unlike all known plant cellular mRNAs, many plant viral RNAs lack a 5′ cap and/or a poly (A) tail, yet they are translated efficiently (21,36). Thus, they must harbor sequences that functionally replace a cap and poly(A) tail (Figure 1). RNAs of many positive-strand viruses have internal ribosome entry sites (IRESes) in their 5′ UTRs that guide ribosomes directly to initiation sites (29). The 5′ UTRs of viruses in the largest plant virus family, the *Potyviridae*, have modest IRES activity (55,96). In contrast, the viruses discussed here represent the only mRNAs known in any eukaryote to harbor cap-independent translation elements in the 3′ UTR. We place these 3′ cap-independent translation elements into at least three classes, listed in order of their discovery (36): (*i*) the translation enhancer domain (TED) in *Satellite tobacco necrosis*

virus (STNV) RNA (Figure 1b), (*ii*) the *Barley yellow dwarf virus* (BYDV) or BYDV-like cap-independent translation element (BTE) found in genus *Luteovirus* of the *Luteoviridae* family (Figure 1a) and in the *Dianthovirus* and *Necrovirus* genera of the *Tombusviridae*, and (*iii*) the *Tomato bushy stunt virus* (TBSV) cap-independent translation element (CITE) found in the genus *Tombusvirus* and probably other genera of the *Tombusviridae*. A fourth type of 3′ element may exist in the 3′ UTR of *Maize necrotic streak virus* (MNeSV) (70). The sequences and secondary structures of these elements bear no obvious resemblance to each other (Figure 2), but the TED and BTE behave very similarly, conferring strong stimulation of cap-independent translation in vitro and in vivo. In contrast, the CITE-mediated cap-independent translation is detectable only in vivo (93). However, a feature that they all share, and which is relevant to this review, is the presence of short sequence tracts known or predicted to base-pair to the 5′ UTR that mediate translation initiation at the 5′-proximal AUG on the viral mRNAs.

The uncapped, nonpolyadenylated RNA of STNV harbors a 120-nt translation enhancer domain (TED) in the 5′ end of the 620-nt 3′ UTR (Figure 1b) (16,78). The TED is necessary for cap-independent translation initiation at the 5′ proximal AUG on the mRNA. This translation also requires 38 bases of the 5′ UTR. The TED element is predicted to form a long, rather loose, bulged stem-loop structure (Figure 2a). A different sequence of approximately 100 nt (BTE) in the 5′ end of the 869-nt 3′ UTR of BYDV confers cap-independent translation at the 5′ proximal AUG of the genome (84) and sg RNA1 (83) (Figure 1a). BYDV-like TEs are distinguished by a conserved motif, GGAUCCUGGGAAACAGG, which forms a short stem-loop by pairing of the underlined bases. BTEs also include one or more additional stem-loops, one of which has the potential to base-pair to the 5′ UTR (82) (Figure 2b). BTEs have been demonstrated functionally in *Red clover necrotic mosaic dianthovirus* (RCNMV) RNA1 (50), and *Tobacco necrosis necrovirus* (TNV) RNA, which is unrelated in sequence to its satellite RNA, STNV (Figure 2).

Both the BTE and TED elements stimulate translation of uncapped mRNA encoding a reporter gene by over 100-fold in vivo to a level that is at least as great as that conferred by a 5′ cap in the absence of the element (47,83). Thus, no viral gene products are necessary for the element to function. Also, both types of cap-independent translation element can be moved to the 5′ UTR in place of the natural 5′ UTR and still facilitate cap-independent translation in vitro (27,47). Thus, the 3′ UTR elements must be responsible for recruiting the translational machinery. The viral 5′ UTR serves only to bring the 3′ element in proximity with the 5′ end (see below).

The CITE in the 3′ UTR of TBSV RNA differs from the TED and BTE because it confers no obvious translation advantage in vitro, but it is clearly necessary for cap-independent translation in vivo (93). Using standard reporter assays and a modified assay that required translation of the viral replicase component to amplify a DI RNA, White and colleagues mapped the core region of the CITE of TBSV to a sequence spanning about 167 bases in the 3′ UTR (93; M.R. Fabian & K.A. White, unpublished data). This central segment, termed R3.5 (Figure 2c), is a translation-specific element; however, the two segments that flank R3.5, and are involved in RNA replication (i.e., RIII and RIV), are also required for CITE function (93). Thus, the TBSV CITE, as defined by activity in vivo, consists of contiguous segments RIII/3.5/IV where R3.5 is translation specific and RIII and IV play a dual function in translation and replication.

For the BTE (28) and TBSV CITE (19), directed mutagenesis revealed that kissing stem-loop base-pairing with the 5′ UTR is necessary for translation. Five bases of stem-loop III (SL-III) of the BTE were predicted to pair to 5′ bases in stem-loop D (SL-D) of the BYDV 5′ UTR (Figure 2b). A point mutation in the middle base of either of these loops reduced translation 5- to 10-fold in vitro and > 100-fold in vivo. When both mutations were combined to restore

complementarity, translation was increased many-fold compared to the mutants, although not to wild-type levels (28). The sequence of the helix of the kissing stem-loop in the 5′ UTR is not important for the interaction. However, with regard to the kissing loop bases, simple base-pairing may not be sufficient because not all compensating mutations expected to form kissing loops between SLIII and SL-D are able to facilitate translation (A. Rakotondrafara, personal communication). Sequences on either side of the BTE including the extended portion of stem-IV of the BTE (Figure 2b) comprise the promoter for sg RNA2 synthesis (38; J. Jackson, personal communication). Thus, like the TBSV DI RNA, the translation element is flanked by RNA synthesis-regulating elements.

The 3′ BYDV-like translation enhancers (BTE) of TNV-A and TNV-D RNA also base-pair to the 5′ UTRs of their mRNAs (despite their names, TNV-A and TNV-D are different virus species). Studies with chimeric mRNAs support the need for base-pairing between the 3′ BTE and the 5′ UTR of TNV-D to effect cap-independent translation. The SL-III of the TNV-D BTE is not complementary to SL-D of BYDV to which SL-III of the BYDV BTE base-pairs, but an mRNA containing the BYDV 5′ UTR and TNV-D 3′ UTR allowed substantial cap-independent translation. This is because the TNV-D BTE is complementary to SL-A (the 5′-proximal stem-loop) of the BYDV 5′ UTR, which has the same sequence as the TNV-D 5′-proximal stem-loop (71). Additionally, the Meulewaeter laboratory reported that the 3′ BTE of BYDV could pair with the 5′ UTR of TNV-A sg RNA2 to facilitate translation, although the predicted pairing appears rather weak (46). In both cases, constructs for which no base-pairing between UTRs was predicted translated very poorly. These data strengthen the concept that the TNV BTEs require this form of communication.

All members of the *Dianthovirus* genus harbor a tract in the 3′ UTR of RNA1 that resembles the conserved BTE sequence GGAUCCUGGGAAACCAG at 15 to 16 of the 17 bases (82). This sequence motif is part of a 137-nt element in the 3′ UTR of *Red clover necrotic mosaic dianthovirus* (RC-NMV) RNA1 that Okuno and colleagues call 3′ TE-DR1 (50). Like the BYDV BTE, 3′ TE-DR1 confers substantial translation in vivo, and in its absence no translation is detected unless a 5′ cap is added, which restores translation fully. Unlike the BYDV BTE, 3′ TE-DR1 contains a total of five stem-loops (Figure 2b). Also, the authors reported that base-pairing to the 5′ UTR was unnecessary for translation in vitro, but Fabian & White (19) predicted that one of the stem-loops in the 3′ TE-DR1 can potentially form a kissing stem-loop interaction with stem-loops in the 5′ UTRs of both genomic RNA1 and sg RNA1, that arises from genomic RNA1 (Figure 2b). Given the striking conservation of predicted base-pairing between known or predicted 3′ cap-independent translation elements throughout the *Tombusviridae* family, and given the requirement of the BYDV element for base-pairing, it is likely that the BYDV-like elements in the dianthoviruses also must base-pair to the 5′ UTR.

The TED and BTE elements were analyzed by monitoring expression of a reporter flanked by viral UTRs, with little or no viral coding region present. Fabian & White (19) used a different approach involving replicating RNA. They employed a *trans*-complementation system in which one of the genes essential for TBSV RNA replication, p33, was expressed from a nonreplicating mRNA containing the UTRs of interest. Translation of p33 from this “reporter” mRNA was revealed indirectly by the accumulation of a coinoculated defective interfering RNA (DI-72), the replication of which requires both TBSV replication proteins p33 and p92. (p92 was co-expressed from a separate mRNA.) Using this assay, several sets of point mutations to disrupt and restore base-pairing between the TBSV 3′ CITE and 5′ UTR clearly demonstrated a requirement for kissing stem-loops for cap-independent translation in infected cells (19,93).

In contrast to the BTE and TBSV CITE, mutation of the bases in the TED of STNV-2 (TED_2) sequence predicted to form a kissing loop interaction with bases in the 5′ UTR (Figure

1b) reduced translation by only 50%, and compensating bases predicted to restore base-pairing did not restore translation to wild-type levels (45). This does not rule out a role for long-distance base-pairing between these regions. First, the mutants were tested only in vitro, where conditions are less stringent than in vivo, and significant translation of any mRNA can occur in the absence of a 5′ cap. Second, lack of restoration of translation by compensating mutants may be due to unpredicted folding of the mutants. Third, additional regions of complementarity between the TED and 5′ UTR (Figure 2a) were not investigated, although the kissing-loops are the most likely sequences to be available for long-distance base-pairing.

Recently, the 3′ UTR of MNeSV RNA was shown to be necessary for cap-independent translation (70). It is predicted to form a bulged stem-loop that can form a kissing stem-loop interaction with the 5′ UTR. The role of this interaction in translation is supported by the observation that mutations in the predicted kissing bases of the 3′ UTR reduced translation (70). The secondary structure of the MNeSV 5′ UTR, including kissing loop, but not the 3′ element, closely resembles that of TBSV RNA. The 3′ element of MNeSV also differs from the TBSV CITE in that it strongly stimulates cap-independent translation in wheat germ extract (70). The MNeSV 3′ UTR bears no obvious resemblance to the above three classes of cap-independent translation element, and thus may represent a fourth class of element.

Recoding in *Luteoviridae* and *Tombusviridae*

Long-distance base-pairing can also control translational events that occur after initiation. Ribosomal frameshifting in the minus one (−1) direction (ribosomes change reading frames by slipping back one base relative to the initial ORF being translated) is required for translation of the polymerase genes of the luteoviruses, sobemoviruses, and dianthoviruses of plants, as well as most retroviruses (e.g., HIV) and all nidoviruses (e.g., SARS CoV) of animals (7). Frameshifting is programmed by *cis*-acting signals that include the seven-base shifty site at which the ribosomes change frames. This is followed by a highly structured region, usually consisting of a pseudoknot located 5 to 6 nt downstream. The second loop of the pseudoknot can be quite large (8). By far the most extreme case is BYDV RNA, in which a stem-loop located 4 kb downstream of the frameshift site base-pairs with a bulge in the stem-loop adjacent to the frameshift site (Figure 1a). This kissing interaction is necessary for frameshifting (4). How this structure brings about the ribosomal frameshift remains unknown, but frameshifter pseudo-knots may interact with the ribosome in a way that transiently blocks the ribosomal helicase from melting secondary structure, causing a pause in ribosome movement to allow the -1 frameshift (76). RCNMV RNA has a shifty site and bulged stem-loop very similar to that of BYDV (35), but there is no evidence that the latter requires base-pairing to a downstream sequence for frameshifting. The long-distance kissing interaction is predicted in the frameshift signals of all other viruses in the *Luteovirus* genus (4) but not in any other virus genus. However, the effects of such long-distance sequences on frameshifting, or even their potential presence, may not have been examined for many viruses.

Another example of a long-distance interaction controlling recoding is the sequences that effect readthrough of the coat protein gene stop codon of luteoviruses. This allows expression of a C-terminal extension to the coat protein that is required for aphid transmission (6,12). Unlike frameshifting, many different types of *cis*-acting signals can cause in-frame readthrough of stop codons. For example, the consensus sequence CARYYA, following the TMV p126 ORF stop codon (R = purine, Y = pyrimidine), allows about 5% of ribosomes to read through and continue translating the p183 ORF that encodes the TMV RNA-dependent RNA polymerase (54,74). In contrast, the readthrough domain of the luteovirus coat protein gene requires a $[CCXXXX]_8$ motif beginning about 20 nt downstream of the stop codon and another sequence located about 700 to 750 nt downstream (9) (Figure 1a). In artificial constructs this distal element can function when moved two kilobases downstream into the untranslated region

beyond the readthrough ORF (9). Although some sequence complementarity between this region and the sequence near the readthrough site is present in BYDV RNA, it remains to be determined whether this base-pairing occurs.

Other Viruses: Tospoviruses

Bunyaviruses, including the economically important Tospovirus *Tomato spotted wilt virus* (TSWV), have ambisense RNAs with complementary termini. Circularization of TSWV nucleocapsids has been visualized by atomic force microscopy (32). Presumably these "panhandle" rings are formed by long-distance base-pairing between the complementary bases at the ends of the viral RNAs. Van Knippenberg and colleagues propose that a structure in the 3′ end of TSWV RNAs may function as a translation enhancer by interacting with the 5′ end (79,80).

Utility of Interactions to Translation

Why would a virus harbor a translation element in the 3′ UTR? First, a 3′ element prevents translation initiation on incomplete, 3′-truncated mRNAs. Second, the positioning of the complex translation element at the 3′ end avoids the need for multiple copies of the element in the genome to service each of the sg mRNAs, as would be necessary if it were located at their 5′ ends. Instead, by using this 3′-positioning strategy only one copy of the 3′ TE is necessary that functions with simple complementary adapter sequences in the 5′ UTR of each sg mRNA (e.g., Figure 1a).

Having a 3′ TE also allows the 5′ end of the mRNA to be sensitive to events at the 3′ end. This 3′–5′ communication may prevent nonproductive collisions between ribosomes translating the genome and viral replicase molecules copying the same RNA molecule (4). The first event upon uncoating of positive-strand viral RNA is translation of the viral replicase. As the replicase protein accumulates, it recognizes the 3′ end and begins synthesizing negative strand while moving in the 5′ direction on the genomic RNA template. Gamarnik & Andino (22) showed that on poliovirus RNA, no negative-strand synthesis could occur in the presence of translating ribosomes on the same RNA. During replication of viral RNAs with 3′ TEs, as the 5′-moving replicase copies the 3′ TE, the replicase would disrupt the element's structure and its ability to base-pair to the 5′ end (Figure 3). This in turn would shut off translation and free the upstream portion of the genome of ribosomes, hence allowing synthesis of full-length negative strand. The negative strand would in turn serve as template for synthesis of positive strands that could eventually outnumber replicase molecules, and thus be available for translation, and the cycle would repeat (4). This provides an elegant feedback mechanism to assure a productive balance of translation and RNA synthesis (Figure 3).

REPLICATION

Interactions in Tombusviruses

RNA virus genomes are replicated by virally encoded RNA-dependent RNA polymerases (RdRps) (11). For positive-strand viruses, the genome serves as a template for generation of a full-length negative-strand RNA intermediate. This minus strand then acts as a template for the synthesis of progeny positive-strand full-length genomes. Promoter elements reside at the 3′ termini of positive and negative genomic strands and function to mediate proper initiation at these sites. However, additional RNA elements located at different and sometimes distal positions in the genome can play important roles in the RNA replication process. These other elements are found in either the positive or negative strands and can act in concert with, or independently of, the core promoter elements. In some cases, communication between these different *cis*-acting RNA elements via RNA-RNA interactions is required.

The RNA replication elements in TBSV are among the best characterized (91). The advances have been facilitated greatly by the existence of DI RNAs that are small viral replicons derived from the TBSV genome (Figure 4) (89). TBSV DI RNAs do not encode any proteins but maintain *cis*-acting RNA elements that allow them to be amplified efficiently when the viral replication proteins p33 and p92 are provided in *trans*. Several noncontiguous segments of the genome are maintained in a typical TBSV DI RNA (termed regions I through IV or RI-RIV). RI and RIV are derived from the termini (where promoters are predicted to reside) and the other two, RII and RIII, correspond to internal positions. Analyses of these conserved RNA regions revealed new insights into the structure and function of these RNA elements and have determined that both intra- and interregional RNA-RNA interactions are important to their activities (91).

Important intradomain interactions that span smaller segments of RNA have been identified in RI and RIV (53,62,66). These interactions occur in the plus strand of the DI RNA and also likely occur in the larger context of the TBSV genome. RIV contains the core promoter for negative-strand synthesis and an intradomain interaction between the 3′ terminal 5 nts (that is part of the core promoter) and a complementary sequence (termed silencer) located in an internal loop of a 5′-proximal stem-loop (SL) structure is required for DI RNA replication (62) (Figure 4). In vitro, formation of this interaction (that spans ~ 50 nts) results in downregulation of viral RdRp-mediated negative-strand synthesis. Conversely, the disruption of the interaction caused increased levels of transcription, and these results led to the proposal that the interaction is important for regulating minus-strand synthesis by controlling access to the 3′-terminal initiation site. This interaction is also important in vivo for detectable accumulation of DI RNA, supporting its function in the viral RNA replication process. Similar 3′ end-silencer interactions have been identified in a variety of genera in *Tombusviridae*, and in luteoviruses, underscoring the importance and prevalence of this type of structure in viral RNA replication (37,53,99).

A second major intraregional interaction occurs in RI (67). This region of the DI RNA corresponds to the 5′ UTR of the TBSV genome, and it folds into discrete and functional domains. The 5′-proximal T-shaped domain (TSD) (65,92) is separated from the 3′-proximal downstream domain (DSD) by a stable hairpin structure. A base-pairing interaction (termed PK-TD1) between the TSD and DSD (that spans ~ 100 nts) is required for optimal replication of the DI RNA (Figure 4). The precise role of this interaction remains to be determined. Formation of the TSD-DSD complex appears to be an elementary feature of RNA replication because similar functional complexes are also present in tombusvirus satellite (Sat) RNAs (13), albeit traversing a significantly larger segment of RNA (spanning 137 nts). This bridge colocalizes the TSD and DSD in the sat RNA, thereby allowing formation of the necessary interdomain interaction, PK-TD1 (13) (Figure 4).

Interregional interactions that occur between the conserved DI RNA regions and cover greater distances have been proposed. RII is absolutely essential for DI RNA replication. It is bound by p33 that recruits the RNA template to the viral replication complex (51,63). Additionally, both RII and RIV are required simultaneously for the formation of an active replication complex (59). The necessity for both RII and RIV at the same time suggested that the two regions may need to communicate in order to function. RII and RIV contain two complementary single-stranded regions that could mediate an interaction (59) (Figure 4); however, compensatory mutational analysis did not confirm existence of this proposed interface. Thus the relevance of these potential contacts remains unknown.

A second interregional interaction has been identified between RI and RIII (Figure 4). However, in this case, the base-pairing is believed to occur in the minus strand (59). RIII(−) contains small hairpins in the minus strand that act as replication enhancers in vivo and in vitro

(57,66). It was proposed that these regions are bound by the viral RdRp, thereby recruiting it to the minus-strand template. Indeed, interaction between short sequences near the promoter at the 3′ end of the (−) strand and the RIII(−) enhancer was shown to be necessary for enhanced DI RNA replication in vitro and in vivo (58). In the DI RNA this interregional interaction spans ~ 400 nts; however, if the same interaction also operates in the context of the viral genome, it would traverse a distance of more than 4 kb.

Other Viruses: Potex-, Tospo-, and Flaviviruses

Potexviruses—Long-distance base-pairing interactions involved in replication have also been identified in the Potexvirus *Potato virus X* (34). Here these interactions were assessed in the context of the complete 6.4-kb plus-strand RNA genome. Sequence elements in the 5′ UTR and just upstream from the initiation sites of two major sg mRNAs are key players in the long-distance interactions. The terminal element is complementary to both sg mRNA elements. Efficient positive-strand synthesis of the genome required base-pairing, in the full-length genomic minus strand, between the 3′-terminal and the sg mRNA proximal elements (34). Disrupting the ability of the 3′-terminal element to base-pair with one sg mRNA element did not significantly affect synthesis of the plus-strand genome. However, disruption of both sets of interactions simultaneously markedly reduced positive strand genome synthesis. It was concluded that interaction between the terminal element and at least one downstream sg mRNA element is required for efficient initiation of plus-strand genome synthesis (34).

Tospo- and Rhabdoviruses—Long-distance RNA-RNA interactions may also be important for genome replication in other types of RNA plant viruses. In particular, ambisense and negative-sense RNA plant viruses contain complementary termini that, when paired, form panhandle structures (32). Unfortunately, these elements have not been studied in any detail in plant viruses. However, if these terminal interactions function comparably to their animal virus counterparts such as Bunyamwera virus, then these elements may play important roles in facilitating and modulating genome replication (3,87).

Flaviviruses—In the plus-strand RNA flaviviruses, ten nucleotide conserved cyclization sequences (CS) located near each end of the positive-sense genome base-pair to each other giving a nearly 10-kb loop (33). Cyclization of viral RNAs conferred by the CS base-pairing along with flanking sequences was visualized directly by atomic force microscopy (1). The long-distance CS base-pairing is required for RNA synthesis (94) but not for translation (44). Because the 5′ CS is located in the beginning of the coding region, it has been proposed that the long-distance base-pairing shuts off translation by blocking the ribosome. However, translation may be inhibited by (proteins recruited to) a different portion of the 3′ UTR (41) and not by base-pairing per se (44).

Utility of Interactions to Replication

Long-distance interactions in viral genomes facilitate various steps in the RNA replication process. The putative interaction between the p33-binding RII and the minus-strand promoter-containing RIV in TBSV may be an example of this type of cooperation (58). These types of interactions can help two different elements communicate so that processes can be coordinated. This distal arrangement may be dictated by size, coding, evolutionary or other constraints. Thus, the elements are positioned distally at sites that are compatible with the rest of the structure and function of the genome.

A second possibility for distal elements is that a single element is split into two or more components and to be functional the element requires “assembly” via long-distance RNA-RNA interactions. This seems to be the case for viruses that contain complementary sequences at or near the termini of their genomes (e.g., flavi-, tospo-, and rhabdoviruses). These interactions

are necessary for either replication and/or transcription. The benefit of having this type of split-element could be that it conveys to the virus replication system that a complete genome segment is present (1). This strategy could thus prevent initiation of replication on terminally truncated genomes.

SUBGENOMIC mRNA TRANSCRIPTION

The Premature Termination Model in Dianthoviruses and Tombusviruses

Many RNA viruses express their genes by transcription of viral sg mRNAs (49). In positive-strand RNA viruses, sg mRNAs are 3′ coterminal with the genome but have 5′ termini that map just upstream of 3′-proximal ORFs. This positioning of downstream ORFs at the 5′ end of sg mRNAs ensures their efficient translation. Transcription of sg mRNAs can be accomplished by different mechanisms including: (*i*) internal initiation of transcription in a full-length negative strand of the genome (48), (*ii*) discontinuous transcription (DT) during negative-strand synthesis (69,81), and (*iii*) premature termination (PT) during negative-strand synthesis (73,90). In the latter two cases, the recombinant or 3′-truncated minus strands, respectively, are then used as templates for transcription of the sg mRNAs.

Currently, only the internal initiation and PT models are known to occur in plant viruses. The PT mechanism has been reported in two different viruses in *Tombusviridae*, RCNMV and TBSV (88). In both cases base-pairing interactions are required in the genomes just 5′ to the transcriptional initiation sites. The first functional interaction supporting a PT mechanism was discovered in bisegmented RCNMV, which produces a single sg mRNA from RNA1 that encodes the coat protein. The required RNA-RNA interaction occurs in *trans* and involves the loop portion of a stem-loop in RNA2 (termed *trans*-activator or TA) base-pairing with a complementary sequence in RNA1 (termed TA binding site or TABS) located just upstream from the initiation site for sg mRNA transcription. It was proposed that this interaction acts as an RNA barricade that blocks progression of the viral RdRp during negative-strand synthesis of RNA1, thereby generating 3′-truncated templates for sg mRNA transcription (Figure 5).

Additional support for the PT model comes from the analyses of TBSV in which similar types of base-pairing interactions are necessary for transcription of its two sg mRNAs (15,42). Like RCNMV, the interactions occur in the positive strand; however, for TBSV they occur in *cis*. The so-called activator sequences (AS1 and AS2) involved in the interaction are located in the terminal loops of SL structures positioned in tandem in the coding region of p92 (Figure 5). Their partner receptor sequences (RS1 and RS2) are located just upstream from the initiation sites of sg mRNA1 and 2, respectively. The AS1/RS1 interaction spans a distance of approximately 1 kb while the AS2/RS2 interaction traverses about 2 kb. In both cases, interaction of an AS with its partner RS is necessary for transcription of the associated sg mRNA. An additional long-distance interaction spanning over 1 kb and involving sequences that are distal (DE) and proximal (CE) to the sg mRNA2 initiation site is also required for sg mRNA2 transcription (14,98) (Figure 5). The DE/CE interaction serves to colocalize AS2 and RS2. Thus, at least three long-distance interactions are required for transcription of the two sg mRNAs generated by TBSV. The requirement for all of these interactions in the positive strand, the positions of the AS/RS interactions just 5′ to the sg mRNA initiation sites, the ability to uncouple negative-strand sg mRNA synthesis from plus sg mRNA production, and the fact that the sequence 3′ to the AS/RS interactions can act as plus-strand promoters all support the proposal that TBSV sg mRNAs are generated by a PT mechanism (42,90).

Other Viruses: Noda- and Potexviruses

Nodaviruses—The PT mechanism of transcription may not be limited to plant viruses. *Flock house virus* (FHV), an insect nodavirus, possesses a bisegmented genome and produces one

sg mRNA during infections. Similar to the process in TBSV and RCNMV, FHV sg mRNA transcription requires a long-distance base-pairing interaction immediately upstream of its sg mRNA transcription site (43). This interaction occurs in *cis* and involves three noncontiguous segments of genome segment RNA1. The similarities in RNA structural requirements suggest that FHV may use a PT mechanism for production of its sg mRNA.

Potexviruses—The mechanism used for sg mRNA transcription of PVX is not known. For transcription to occur, long-distance interactions between the 3′ end of the full-length minus strand of the genome and the internal sites of sg mRNA initiation are necessary. In fact, because these interactions are also required for synthesis of the plus strand of the genome (as described in the preceding section), it appears that all positive-strand synthesis in PVX (i.e., genome and sg mRNA synthesis) is somehow coupled (34). Thus, assembly or activation of an RdRp complex competent for plus-strand synthesis may require the formation of these long-distance interactions.

Utility of Interactions to Transcription

The *trans* interaction between genome segments in RCNMV occurs later in the infection when relatively high levels of the two genomic RNAs are reached. The dependency of this biomolecular interaction on the concentration of its RNA components provides an elegant mechanism to transcriptionally control the timing of production of coat protein production so that it coincides with high levels of the two genomic RNAs (73).

The rationale behind long-distance regulation of transcription in other viruses such as TBSV and FHV is less evident. Unlike for RCNMV, analysis of TBSV did not detect any *trans*-activity between corresponding elements (15). One possibility regarding the origin of these elements (i.e., AS and RS) is that they originally resided in separate RNAs in a multisegment genome (i.e., similar to the arrangement in RCNMV) but, following a recombination event between genomic segments, both were united in a single RNA. If the original utility of the interaction was retained, then the virus would continue to the system in its new single-molecule context. Another possible reason for maintaining long-distance *cis*-acting interactions may be that they are simply superior to corresponding local elements. There may be some validity to this concept as replacement of long-distance interactions with local hairpin structures in TBSV still allowed for sg mRNA transcription; however, the localized hairpin elements were less effective at activating sg mRNA transcription (42). Thus, the long-distance interactions may have been selected for and maintained over time because of their superior ability to activate sg mRNA transcription.

PERSPECTIVE

The long-distance interactions discussed in this chapter raise many questions. For example, how common are such interactions in viral and (for translation) nonviral RNAs? We predict that long-distance base-pairing interactions are far more widespread than is currently appreciated. Given the fact that as few as four base pairs can bridge a genome-size loop for translation (71), it is possible that such tracts of complementarity with a role in gene expression or replication are common. The challenge, of course, is in identifying those interactions that really do take place. This also prompts the intriguing questions of how short complementary sequences are able to find each other, interact stably enough to perform their function, and how base-pairing to the wrong site is avoided. It is likely that non-Watson-Crick interactions by neighboring bases enhance the kissing-loop interaction, as has been demonstrated for kissing stem-loops that dimerize HIV RNA (52). Indeed, kissing stem-loops are known to base-pair with higher stability than simple linear oligomers of the same sequence (85). Non-Watson-Crick interactions and/or protein-mediated stabilization may also explain how small base-pairing interactions (6–7 bps) are able to cause the viral RdRp to terminate transcription in sg

mRNA synthesis of *Tombusviridae*. Further detailed analysis of the RNA complexes involved will help to unravel this paradox.

Also unanswered is the possible role of proteins in mediating the long-distance interactions. Proteins, such as the Sm-like protein Hfq, required for bacteriophage Qβ replication, are known to enhance RNA base-pairing interactions specifically (97). Clearly, the BTE and STNV TED do not require viral proteins because they function on reporter constructs that lack viral coding sequence. Additionally, structure-probing data indicate that long-distance kissing interactions can occur on BTE-containing mRNA in the absence of protein (28). The TED (23) and the BTE (E. Pettit Kneller & E. Allen, unpublished data) interact directly with eIF4F, presumably to recruit the ribosome. However, the role of any host protein in facilitating the long-distance interaction per se is unknown.

The growing numbers of different interactions now catalogued have helped to change our view of RNA genomes as linear molecules. Instead, RNA genomes can now be viewed as three-dimensional or globular structures in which functionally relevant long-distance in-tramolecular interactions occur (2). The ultimate example of this is the ribosome (95). This chapter also provides a cautionary message to researchers mapping *cis*-acting elements: A wide-ranging view of RNA regulatory elements must be considered because key RNA elements can be located far from the site of activity. Based on their growing prevalence, we anticipate that, in the future, many more such long-distance interactions will be uncovered in RNAs of pathogens and hosts.

SUMMARY POINTS

1. A variety of different long-distance RNA-RNA interactions, spanning hundreds or thousands of intervening bases, have been discovered in plant RNA viruses in recent years.
2. These interactions play critical roles in facilitating and regulating a variety of viral processes including translation, replication, and sg mRNA transcription.
3. Although general properties have been investigated, the details of precisely how these interactions function remain to be determined.
4. Their effectiveness as regulatory elements suggests that many more long-distance RNA-RNA interactions are likely to be uncovered in the future.

Acknowledgements

Research in the authors' laboratories is funded by NSERC, PREA, CRC, CFI, and a Steacie Fellowship (KAW), and NIH grant no. GM067104 (WAM). The authors apologize to those colleagues whose work was not presented due to space restrictions.

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Glossary

sg
: subgenomic

UTR
: untranslated region

TMV
: Tobacco mosaic virus

AMV
: Alfalfa mosaic virus

PABP
: poly(A)-binding protein

IRES
: internal ribosome entry site

TED
: translation enhancer domain

BYDV
: Barley yellow dwarf virus

BTE
: BYDV-like cap-independent translation element

TBSV
: Tomato bushy stunt virus

CITE
: cap-independent translational enhancer

MNeSV
: Maize necrotic streak virus

RCNMV
Red clover necrotic mosaic virus

TNV
Tobacco necrosis virus

SL
stem loop

TSWV
Tomato spotted wilt virus

RdRp
RNA-dependent RNA polymerase

TSD
T-shaped domain

DSD
downstream domain

CS
cyclization sequence

PT
premature termination

TA
trans-activator

TABS
trans-activator binding site

AS
activator sequence

RS
receptor sequence

FHV
Flock house virus

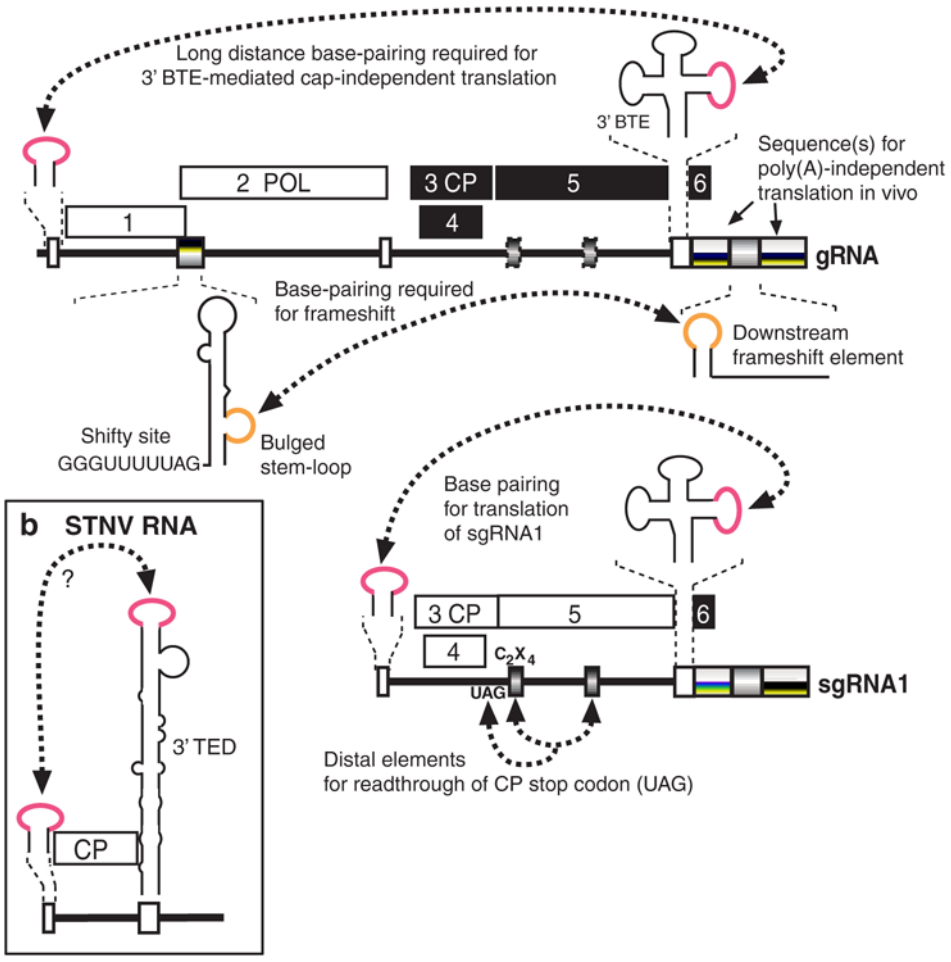

Figure 1.

Genome organizations of BYDV and STNV showing secondary structures of long-distance interacting regions. Bold lines represent genomic or subgenomic RNAs on which boxes indicate translational control elements. Labeled open boxes above the RNA indicate translatable ORFs. Black boxes indicate ORFs not translatable from the RNA shown. Colored lines connected by dashed, double-headed arrows indicate bases that pair over long distances. Question mark indicates predicted base-pairing that has not been demonstrated. For BYDV, sgRNAs 2 and 3 are not shown, because no long-distance base-pairing is known or predicted within these RNAs.

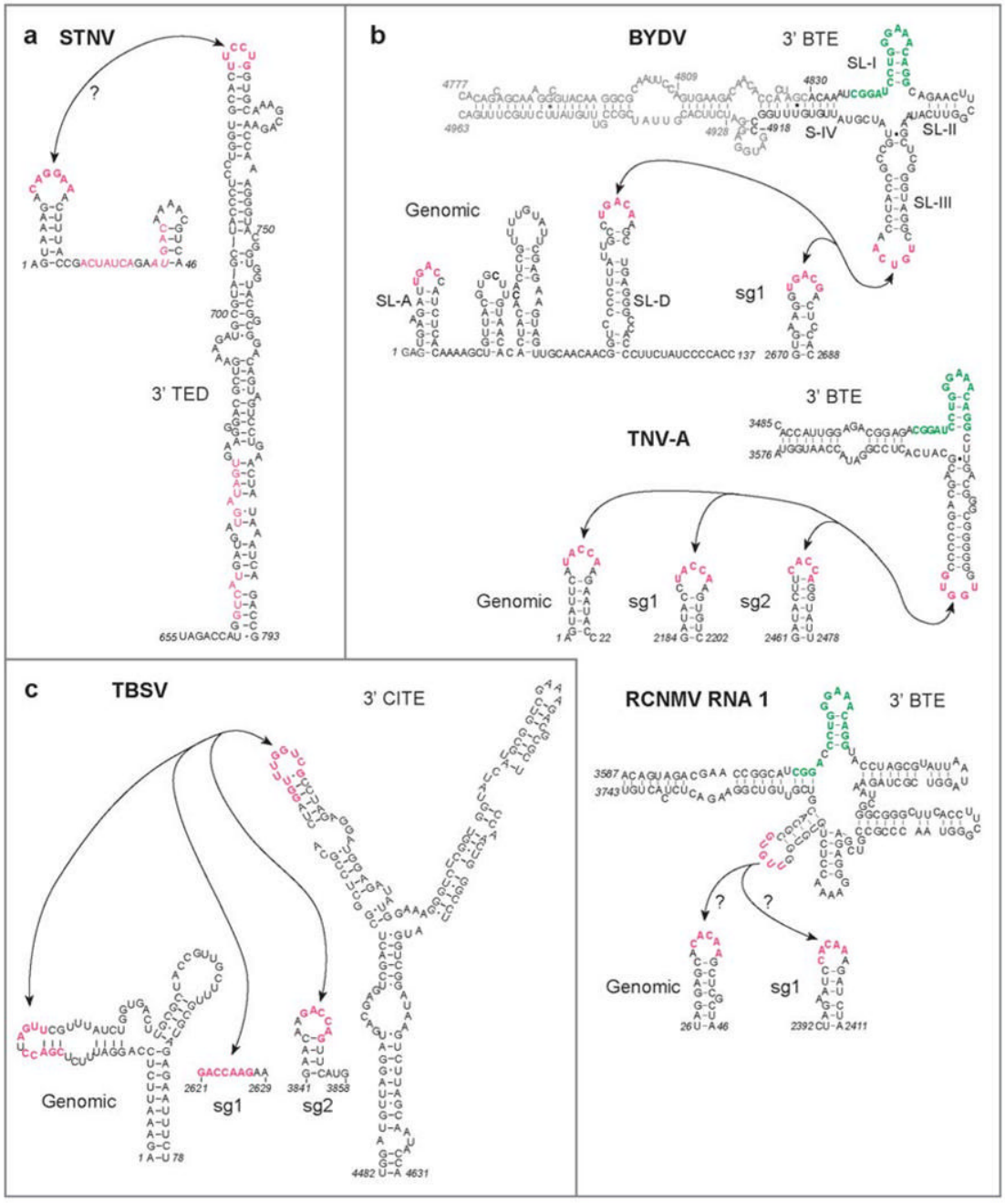

Figure 2.

Sequences and secondary structures of long-distance interacting cap-independent translation elements. (See Figures 1 and 4 for their positions in the genome.) Numbering indicates position of bases in the viral genomic RNA. All sg RNAs are shown beginning at their 5′ ends. Bases in magenta connected by arrows are known or suspected to participate in long-distance base-pairing. Question marks indicate interactions that are supported only by phylogenetic comparisons. Magenta bases not connected by arrows indicate alternative possible base-pairing. Green indicates 17-nt motif conserved in BTEs. Bases shaded in gray on BYDV BTE (*panel b*) are not necessary for cap-independent translation in vitro (27), but represent the maximum sequence needed for translation in vivo (E. Pettit Kneller, personal communication).

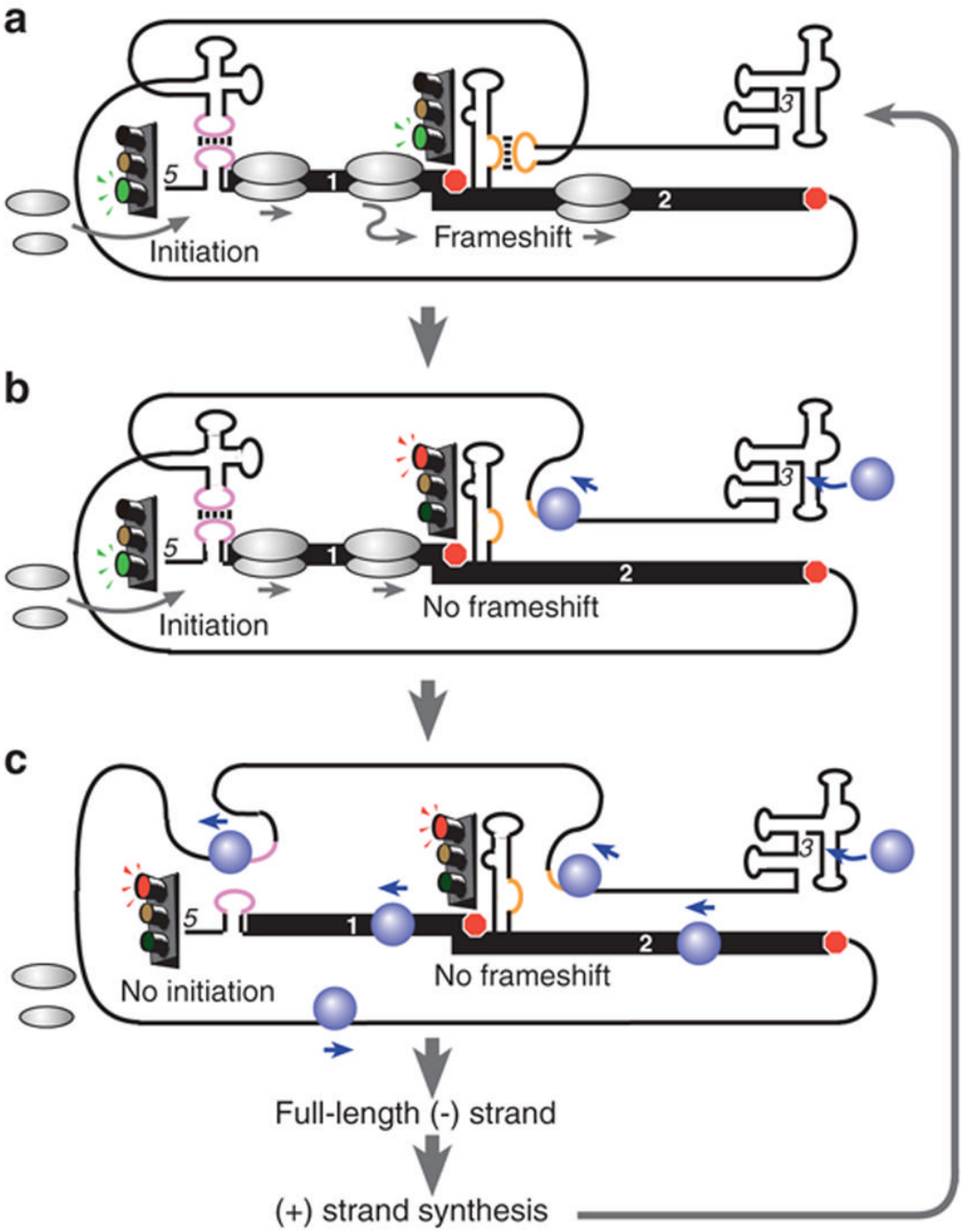

Figure 3.
RNA traffic signal model for regulation of BYDV translation and replication [modified from Figure 5 in Reference (4)]. Black line indicates BYDV genomic RNA (not to scale) with relevant long-distance interactions color-coded as in Figure 1. For simplicity, ORFs other than 1 and 2, and (−) strand RNA are not shown. (*a*) Base-pairing between the 3′ BTE and 5′ UTR (*magenta*) and between the 3′ UTR long-distance frameshift element and bulged stem-loop adjacent to the frameshift site (*gold*) allow ribosomes (*gray ovals*) to translate ORF 1 and ORF 2 (*green lights*). (*b*) As the viral RdRp molecules (*blue spheres*) accumulate, they initiate (−) strand synthesis at the 3′-terminal structure (37). They proceed upstream along the template and melt out the 3′ frameshift element and disrupt its ability to base-pair to the frameshift site.

This prevents frameshifting (*red light at frameshift site*), clearing ORF 2 of ribosomes. (*c*) Next, the RdRp disrupts the 3′ BTE structure and its base-pairing to the 5′ UTR, preventing translation initiation (*red light at 5′ end*). This clears ORF1 and the entire RNA of ribosomes, allowing the RdRps to continue to the 5′ end, and synthesize full-length negative-strand RNA. Subsequent rounds of replication would cause (−) strand RNA to accumulate in excess of RdRp molecules, returning the process to panel *a* (*long arrow*).

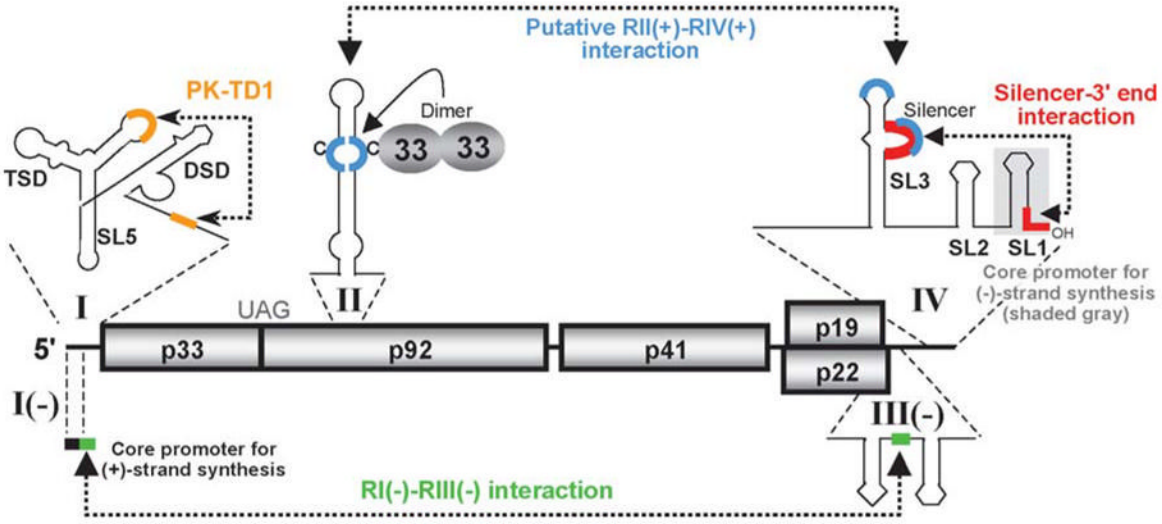

Figure 4.

Long-distance interactions regulating replication of the TBSV genome. DI RNA regions I through IV and their structures are expanded above and below the schematic of the TBSV genome. Plus strand structures are shown above whereas minus strand structures are shown below. The intraregional interactions that occur in either region I or region IV are shown in gold and red, respectively. The interregional interactions involving regions II and IV or regions I(−) and III(−) are shown in blue and green, respectively. See text for details.

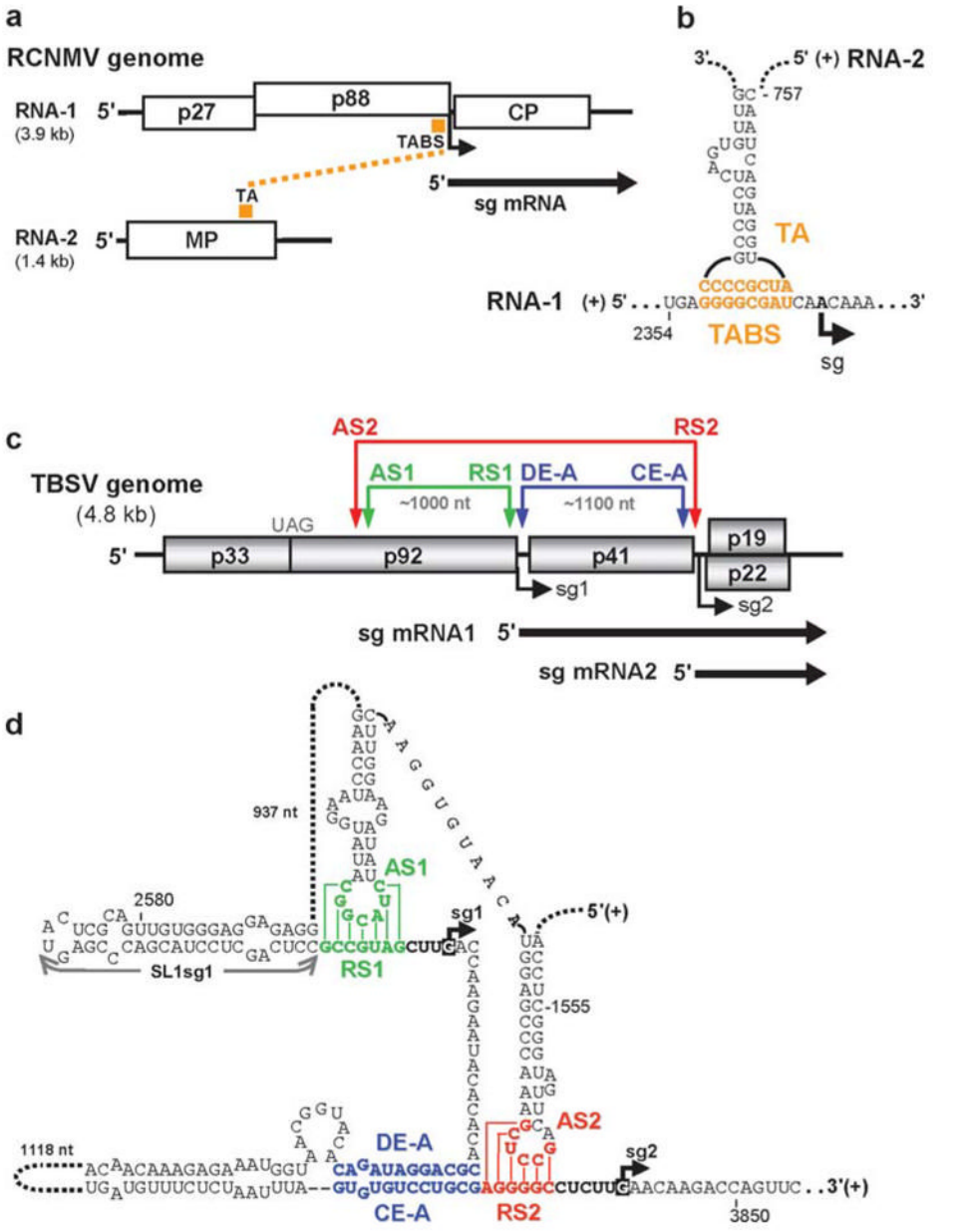

Figure 5.

Long-distance interactions controlling sg mRNA synthesis in RCNMV and TBSV. (*a*) Schematic of the RCNMV genome showing the TA-TABS *trans*-interaction (*gold*) between genome segments RNA1 and RNA2. (*b*) Details of the TA-TABS *trans*-interaction showing the base-pairing interaction involved (73). (*c*) Schematic of the TBSV genome showing the different long-distance interactions involved in regulating sg RNA1 (AS1/RS1, green) and sg mRNA2 (DE-A/CE-A, *blue* and AS2/RS2, *red*). (*d*) Details of the three long-distance interactions showing the different base-pairing interactions involved (42). See text for details.