5’-3’ RNA-RNA Interaction Facilitates Cap- and Poly(A)-Tail-independent Translation of Tomato Bushy Stunt Virus mRNA

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Tomato bushy stunt virus (TBSV) is the prototypical member of the genus Tombusvirus in the family Tombusviridae. The (+)-strand RNA genome of TBSV lacks both a 5’ cap and a 3’ poly(A) tail and instead contains a 3’-terminal RNA sequence that acts as a cap-independent translational enhancer (3’ CITE). In this study, we have determined the RNA secondary structure of the translation-specific central segment of the 3’ CITE, termed region 3.5 (R3.5). MFOLD structural modeling combined with solution structure mapping and comparative sequence analysis indicate that R3.5 adopts a branched structure that contains three major helices. Deletion and substitution studies revealed that two of these extended stem-loop (SL) structures are essential for 3’ CITE activity in vivo. In particular, the terminal loop of one of these SLs, SL-B, was found to be critical for translation. Compensatory mutational analysis showed that SL-B functions by base pairing with another SL, SL-3, in the 5’ untranslated region of the TBSV genome. Thus, efficient translation of TBSV mRNA in vivo requires a 5’-3’ RNA-RNA interaction that effectively circularizes the message. Similar types of interactions are also predicted to occur in TBSV subgenomic mRNAs between their 5’ untranslated regions and the 3’ CITE, and both genomic and subgenomic 5’-3’ interactions are well conserved in all members of the genus Tombusvirus. In addition, a survey of other genera in Tombusviridae revealed the potential for similar 5’-3’ RNA-RNA-based interactions in their viral mRNAs, suggesting that this mechanism extends throughout this large virus family.

One of the earliest steps in the reproductive cycle of (+)-strand RNA viruses is translation of their encoded proteins by the translational machinery of the host (1, 2). In eukaryotes, most cellular mRNAs contain both a 5’ cap and a 3’ poly(A) tail, and these terminal structures act synergistically to stimulate translation (3–5). This translational enhancement depends on a protein bridge formed between these two structures by eukaryotic initiation factor 4E binding to the 5’ cap, poly(A)-binding protein binding to the poly(A) tail, and eukaryotic initiation factor 4G binding simultaneously to both of these proteins (6). Assembly of this complex results in the formation of a closed loop (5) and there is growing evidence that message circularization is a fundamental requirement for efficient translation of eukaryotic mRNAs (7–10).

Eukaryotic (+)-strand RNA viruses must either possess a 5’ cap and 3’ poly(A) tail or adopt alternative strategies that allow for efficient translation of their encoded viral proteins (1, 2). In this regard, translationally active viral RNA genomes have been identified that do not contain one or both of these terminal mRNAs structures. Examples of the latter case include (+)-strand RNA genomes in the families Tombusviridae (11) and Luteoviridae (12, 13). To compensate for the absence of both a 5’ cap and a 3’ poly(A) tail (11, 14, 15), BYDV (genus Luteovirus) (16, 17) and several members of the large family Tombusviridae, including STNV (genus Necrovirus) (18, 19), TBSV (genus Tombusvirus) (20, 21), TCV (genus Carmovirus) (22), Hibiscus chlorotic ringspot virus (genus Carmovirus) (23), and RCNMV (genus Dianthovirus) (24) contain RNA sequences in the 3’ region of their genomes that function as translational enhancers (TEs). For BYDV and STNV, it has been proposed that their TEs function to recruit the translational machinery of the host (25, 26). Consistent with this notion, the TEs of BYDV and STNV have been reported to interact in vitro with canonical translation initiation factors (10, 27). The recruitment of translation factors to a 3’-proximal TE would then require their subsequent delivery to the 5’ end of the viral mRNA, where initiation of translation occurs (10). Accordingly, it has been shown that a 5’-3’ RNA-RNA interaction is required for efficient translation of BYDV mRNA, but not for STNV mRNA (10, 28). The BYDV interaction is formed by a kissing-loop structure involving an RNA hairpin in the 5’ untranslated region (UTR) of the viral genome and another hairpin in the 3’ TE (10). This interaction acts to circulate the message, as occurs in cellular mRNAs, and could potentially deliver translation factors to the 5’ end of the viral message (10).

Tomato bushy stunt virus (TBSV) is the prototypical member of the genus Tombusvirus in the family Tombusviridae (11). Its 4.8-kb long (+)-strand RNA genome lacks both a 5’ cap and 3’ poly(A) tail and encodes five functional proteins (11, 29) (Fig. 1). The 5’-proximal p33 and its read through product p92 are translated directly from the genome and both are essential for viral RNA synthesis (20, 30). The larger p92 is the viral RdRp, which is required for viral RNA replication (31, 32). The 3’ terminal sequence of TBSV mRNA (p92) is highly structured and exhibits a CAA with a specific CAA binds poly(A) tail.
whereas p33 is a critical accessory replication protein. The
fragment (994–H11032) in DI-83 construct was sequenced across its entire PCR-derived segment.

**BspEI/SphI fragment (994–H11032)** in DI-83 combination with standard recombinant DNA cloning techniques. Each 21, 29, 42). All mutant constructs described below were generated using

PMF5–3′B (5′-GCGCCGCGCCGAGTCAGCTTCTTGCTCTTAAACACATATGATCGAGC-3′) and P9. Mutant 5b construction involved replacement of a SallII/SphI fragment (4501–4766) in DI-83II with the SallII/SphI-digested PCR product generated with primer pairs PMF5–3′B (5′-GCGCCGCGCCGAGTCAGCTTCTTGCTCTTAAACACATATGATCGAGC-3′) and P9. Mutant 5c construction involved replacement of the SallII/SphI fragment (4501–4766) of 5a with the SallII/SphI-digested PCR product

**PMF49 (5′-BspEI and**

**PMFrIdSL2/3 (5′-BspEI**

**TGAATTGGGCCCTAATACGACTCACTATAGGAAATTCTCCAGGAT-3′)** and P9. Mutant SL3-UUCG construction involved replacement of a BstXI/MfeI fragment (1–60°C) with the BstXI/MfeI-digested PCR product generated with primer pairs PMF48 (5′-GCGCCGCGCCGAGTCAGCTTCTTGCTCTTAAACACATATGATCGAGC-3′) and P9, respectively. Similarly, mutant V/TBSV3 was carried out as described above except that generation of Va and Vb used primer pairs vGOF5 (5′-GGCGCGCCGAGTCAGCTTCTTGCTCTTAAACACATATGATCGAGC-3′) and P9, respectively.

**Computer-aided Analysis of Viral RNA**—The nucleotide sequences of TBSV (NC_001554), TBSV’s (AJ249740), TBSVp (U80935), AMCV (X62493), CBLV (AY163842), cymbidium ringspot virus (X51551), cucumber necrosis virus (M25270), carnation Italian ringspot virus (X58521), pear latent virus (AJ100482), pelargonium necrotic spot virus (NC_005285), lettuce necrotic stunt virus (AJ288944), potato latent virus (X57115), oat chlorotic stunt virus (NC_003635), pea stem necrosis virus (NC_004995), maize chlorotic mottle virus (NC_003827), TNV (M33002), PMV (NC_002588), SPMV (M17182), and RCNMV RNA-1 (NC_003756) were obtained from the National Center for Biotechnology Information GenBank™ genetic sequence data base. RNA alignments were carried out using ClustalW (44) and RNA secondary structures were predicted at 37°C by using MFOLD version 3.1 (45, 46).

**In Vitro Transcription**—Viral RNA transcripts were generated in vitro using an AmpliScribe T7 transcription kit (Epicenter Technologies) with SmaI-digested DNA constructs as templates as described previously (42). Transcript concentrations were determined spectrophotometrically and their integrity was confirmed via ethidium bromide staining after agarose gel electrophoresis.

**RNA Secondary Structure Probing**—For in vitro analysis of RNA secondary structures, transcripts of DI-83II (3 µg) were added with yeast RNA (3 µg) to modification buffer (25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM MgCl₂, and 1 mM EDTA), equilibrated (60°C for 2 min, 60°C for 10 min, and 37°C for 10 min), treated with RNA structure probing chemicals diethyl pyrocarbonate and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide or enzymes RNase V1 and RNase T1, and analyzed by primer extension using primers PMF5′S (5′-GATTTCATCATATCCAGTGGAGATGG) and PMF3′S (5′-GATTTCATATCCAGTGGAGATGG) as described previously (47).

**Isolation and Transfection of Protoplasts**—Protoplasts were prepared from 6–8-day-old cucumber cotyledons (var. Straight 8) as described previously (42). Quantification was carried out by bright field microscopy using a hemacytometer. Purified protoplasts (~3 × 10^5) were inoculated as described (42) with viral RNA transcripts (1 µg for DI-72 transcripts and 5 µg each for HS157 and DI-83II) and were incubated in a growth chamber under fluorescent lighting at 22°C for the periods of time specified.

**Analysis of Viral RNA**—Total nucleic acids were harvested from protoplasts as described previously (42). Equal aliquots of the total nucleic acid were separated in 1.2% agarose gel. The gels were stained with ethidium bromide and excised from the gel not degraded and were loaded evenly. Viral RNAs were detected by electrophoretic transfer to nylon membrane followed by Northern blot analysis using a 32P-end-labeled oligonucleotide probe complementary to the 3′ truncated of the TBSV genome (P9). Quantification of the bound viral RNAs was performed by radioanalytical scanning of the blot images. InstantImager (InstantImager Instruments Co., Ltd). Stability assays were carried out as described above except that samples were harvested at different time intervals and the levels of DI-83II or its mutant derivatives were quantified.

**RESULTS**

**RNA Secondary Structure of R3.5**—The 3′ CITE is composed of three contiguous regions that were defined on the basis of their presence (RII and RI) or absence (R3.5) in prototypical TBSV DI RNAs (Fig. 1) (20). The RNA secondary structures of RII and RI have been defined previously, but that for R3.5
has not (38, 40). To determine the structure of R3.5, solution structure probing in vitro was performed and the results were plotted on the most stable MFOLD-predicted RNA secondary structure (45, 46) (Fig. 2). There was a strong correlation between the reactivity of three different single-strand specific reagents (1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide, diethyl pyrocarbonate, and RNase T1) and residues predicted to be unpaired (Fig. 2B). Also, RNase V1, which has a substrate preference for base paired and stacked residues, cleaved within several of the predicted helical portions of R3.5. These data

Fig. 1. The TBSV genome and a prototypical DI RNA. The TBSV RNA genome (T-100) is shown in the middle as a thick horizontal line with coding regions depicted as boxes (29). The transcription initiation sites for subgenomic mRNAs 1 and 2 are indicated by arrows below the genome. Domains and regions in the 5’ and 3’ UTRs, respectively, are delineated and expanded above the genome. A prototypical DI RNA, DI-72, is shown below the genome. Shaded boxes in this molecule correspond to non-contiguous regions (I through IV) derived from the genome, whereas lines correspond to segments that are absent.

Fig. 2. RNA secondary structure of R3.5. A, solution structure probing of R3.5 in a full-length DI-83ΔII transcript. Following treatment with modifying enzymes (RNase T1 and RNase V1) or chemicals (1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide (CMCT) and diethyl pyrocarbonate (DEPC)) the RNA was subjected to primer extension and the products were separated in a sequencing gel. A sequencing ladder of the region analyzed was also generated and is shown to the left in each panel. The 5’ and 3’ boundaries of R3.5 are indicated on the gels along with coordinates the correspond to those indicated in panel B. B, solution structure probing results mapped onto the MFOLD-predicted RNA secondary structure of R3.5. The three major stems predicted are labeled in the structure and the 5’ and 3’ boundaries of R3.5 are indicated by arrows. Different reactivities with residues are indicated by various symbols that are defined in the box.
CymRSV, carnation Italian ringspot virus; CNV, cucumber neocrocis virus; CymRSV, cymbidium ringspot virus; LNSV, lettuce necrotic stunt virus; PeLV, pelargonium necrotic spot virus.

Results from comparative sequence analysis that included nine additional tombusvirus genomes were consistent with the secondary structure proposed (Fig. 3). With the exception of CBLV (which contained only SL-B), all maintained the basic Y-shaped structure defined for TBSV (Fig. 2B) and contained numerous mono- and covarying base pairs in each of the three major stems (Fig. 3). The terminal loop sequence of SL-B, 5'-UUGGUC, was also well conserved, except for CBLV, which had a 1-nt substitution in this sequence and pear latent virus -UUGGUC, was also well conserved, except for CBLV, which had a 1-nt substitution in this sequence and pear latent virus -UUGGUC, was also well conserved, except for CBLV, which had a 1-nt substitution in this sequence and pear latent virus -UUGGUC, was also well conserved, except for CBLV, which had a 1-nt substitution in this sequence and pear latent virus.

Support the presence of three extended stems, termed S-A, SL-B, and SL-C, centered on a 3-helix junction (Fig. 2B).

Sequences in R3.5 and the 5' UTR Are Required for Efficient Translation in Vivo—To determine whether SL-B and/or SL-C were relevant to translation we used a trans-complementation translational assay system developed previously (Fig. 4A) (20, 21). All of the viral mRNAs used in this system are uncapped and non-polyadenylated. In the assay the “test” mRNA, DI-83II, contains the p33 ORF bordered by the TBSV 5’ UTR and 3’ CITE (Fig. 4A). This message is non-replicative (as it lacks RII, an essential replication element) and thus acts solely as an mRNA for p33 (21). Importantly, use of this viral-based test message offers the advantage of limiting the possibility of mRNA misfolding, which is a concern when reporter mRNAs containing nonviral ORFs are used. In assays, DI-83II (with either WT or modified 5’UTRs and/or 3’ CITEs) is co-transfected into protoplasts along with the non-replicating HS175 mRNA, DI-72 (Fig. 4). Both p33 and p92 are necessary for replication of DI-72 and the efficiency of p33 translation from the DI-83II-based message modulates the accumulation of DI-72 (21). The level of DI-72 thus provides a sensitive and biologically relevant measure of the translational activity of DI-83II-based mRNAs.

As an initial step to investigate the structural features of R3.5 important for translation, targeted deletions and substitutions were introduced into SL-B or SL-C in DI-83II (Fig. 4B). Following their co-transfection into cucumber protoplasts along with HS175 and DI-72, the accumulation of DI-72 was quantified by Northern blot analysis (Fig. 4C). Complete deletion of either SL in mutants ΔSL-B and ΔSL-C resulted in extremely low levels of reporter DI RNA accumulation, indicating significant impair-
ment of p33 translation (Fig. 4, B and C). Next, to investigate whether the terminal loops on these SLs were important, each was substituted with a UUCG tetraloop (Fig. 4, B and C). In mutant LB-UUCG, the substitution greatly reduced reporter DI RNA accumulation (∼6% compared with WT), whereas in mutant LC-UUCG the modification led to notably increased levels (∼225%) (Fig. 4, B and C). The ability of SL-C to tolerate a terminal loop substitution is consistent with the sequence variation observed in lettuce necrotic stunt virus (Fig. 3C), whereas the critical nature of the terminal loop of SL-B is in agreement with its essentially invariant presence in all tombusviruses (Fig. 3B). Additionally, the result with LC-UUCG demonstrates that our assay system (using WT DI-83ΔII as the standard) is not “saturated” and is also capable of detecting increases in translational activity. These results indicate that SL-B and SL-C are critical for efficient translation of p33 and that their terminal loops contribute to their activities.

The clear importance of the terminal loop of SL-B, along with the presence of a U-turn motif within this loop, prompted us to search for potential RNA base pairing partners for it. Exam-
nutation of the sequences flanking the p33 ORF in DI-83ΔII led to the identification of a segment in the 5' UTR that possessed very significant complementarity to the terminal loop of the SL-B, and this complementarity extended into the stem sequences (Fig. 5). The complementary sequence in the 5' UTR was located in a previously defined 5'-terminal RNA structure called the T-shaped domain (TSD) (47) and mapped to a terminal loop, SL3, that also contained a YUNR motif (Fig. 5).

To determine whether SL3 in the TSD was important for translational activity, this structure, along with some flanking sequences, was deleted from DI-83ΔII to create ΔSL2/3 (Fig. 4D). The lack of detectable accumulation of the reporter DI RNA in co-transfections with this mutant indicated that the deletion severely compromised translation (Fig. 4E). To more precisely target the complementary sequence in SL3, the 6-nt long loop sequence was replaced with a 4-nt long tetraloop, 5'-UUCG (Fig. 4D). Although still detectable, the more localized modification in mutant L3-UUCG led to a 5-fold reduction in reporter DI RNA accumulation (Fig. 4E). Therefore, efficient translation requires terminal loop sequences in SL3 and in SL-B, which are complementary to each other (Fig. 5).

Base Complementarity between SL3 and SL-B Is Essential for Efficient Translation in Vivo—Compensatory mutational analysis, in which base pairing between complementary sequences is first disrupted and then restored, is a common strategy used to provide evidence for a functional interaction between two RNA sequences. This approach was used to test the functional relevance of the proposed interaction between SL3 and SL-B.

The initial set of compensatory mutations analyzed, 5a, 5b, and 5c, contained tandem substitutions that would maintain the YUNR motifs in both loops (Fig. 6A, i). When these mutants were assayed, the substitutions in mutants 5a and 5b had different effects (Fig. 6B, i). Mutant 5a showed a moderate increase in DI RNA accumulation (~125%), whereas 5b exhibited a substantial decrease (~15%) (Fig. 6B, i). This result is not surprising as the substitutions in mutant 5a would still allow for the interaction to occur via tandem UG base pairs, whereas those in 5b would cause tandem CA mismatches (Fig. 6A, i). In the compensatory mutant 5c, where the substitutions in 5a and 5b were combined so as to restore base pairing via canonical tandem UA base pairs, DI RNA accumulation levels were similar to those of the WT (~110%) (Fig. 6, A, i, and B, i). These results are consistent with the requirement for base pairing between SL3 and SL-B, because substitutions that maintained complementarity (i.e. 5a and 5c) supported efficient translation, whereas those that compromised complementarity (i.e. 5b) did not.

To acquire further evidence for the importance of the proposed interaction, two additional sets of compensatory mutations were generated. In the second set of mutants, several nucleotides within the loops of SL3 and SL-B were substituted (Fig. 6A, ii). Mutants PMA and PMB, containing disruptive substitutions, mediated lower DI RNA accumulation (~20 and ~60%, respectively), whereas the restorative mutant PMc promoted very efficient accumulation (~160%) (Fig. 6B, ii). The strong correlation between disruption/restoration of base pairing and weak/strong activity supports an important functional role for the interaction in mediating efficient translation. In addition, partial disruption of the YUNR motifs in these mutants suggests that a canonical form of the motif is not essential (Fig. 6A, ii).

In the third set of mutants, different 12-nt long segments encompassing the two SLs were substituted singly or reciprocally (Fig. 6A, iii). Mutants Va and Vb contained identical loops (and adjacent stems) from either SL-B or SL3, respectively, and both showed reduced activity to ~20–30% that of WT (Fig. 6B, iii). In contrast, mutant Vc, which contained reciprocal segment substitutions, was restored to near WT levels, ~90% (Fig. 6B, iii). This latter exchange not only supports the functional-
ity of the interaction, it also demonstrates that there is no strict requirement for the respective sequences at their normal 5' and 3' positions. Importantly, similar physical stabilities were observed in vivo for all three sets of mutants (Fig. 6C). This suggests that the differences in activity observed were related primarily to modulation of translational efficiency.

**Potential 5'-3' RNA-RNA Interactions in Genomic and Subgenomic mRNAs Are Conserved in Tombusvirus Genomes—R3.5 is also present in the two 3'-coterminal TBSV subgenomic mRNAs, thus it could potentially facilitate translation in these additional viral contexts (Fig. 1). In agreement with this concept, sequences complementary to the terminal loop of SL-B are present in the 5' UTRs of both subgenomic mRNAs (Fig. 5, shaded box). For subgenomic mRNA2, the putative interacting sequence is present in a predicted terminal loop, whereas for subgenomic mRNA1, the complementary sequence corresponds to a linear 5'-terminal segment. Inspection of other tombusvirus sequences revealed that both the genomic and subgenomic
mRNA 5'-3' interactions described for TBSV are strictly conserved and, importantly, include a number of mono- and co-variations within predicted base paired regions (Fig. 7). The maintenance of corresponding complementary sequences in all tombusvirus mRNAs further supports the functional relevance of our experimental findings.

Tombusviridae Members Contain Complementarity in Their 5' and 3' UTRs—The family Tombusviridae consists of a large group of viruses currently divided into eight genera (15). These viruses share various properties, including (i) icosahedral particles, (ii) RdRps belonging to supergroup II, and (iii) genomes lacking 5' caps and 3' poly(A) tails (15). Based on the latter feature, we wondered if the 5'-3' base pairing requirement observed for TBSV extended to other genera in Tombusviridae. Indeed, at least one other member of this family, TNV (Genus Necrovirus) has been reported previously to contain 5'-3' interactions in its subgenomic mRNAs (10). In agreement with these properties, complementary sequences in the 5' and 3' UTRs of different genera were located in predicted terminal loops and the presence of similar potential interactions in corresponding subgenomic mRNAs (10). In agreement with these properties, complementary sequences in the 5' and 3' UTRs of different genera were located in predicted terminal loops and the presence of similar potential interactions in corresponding subgenomic mRNAs (10). In agreement with these properties, complementary sequences in the 5' and 3' UTRs of different genera were located in predicted terminal loops and the presence of similar potential interactions in corresponding subgenomic mRNAs (10).

To investigate whether this phenomenon was in fact more widespread, we analyzed genomic sequences from members of the six other genera in Tombusviridae (Aureovirus, Avenavirus, Carmovirus, Dianthovirus, Machlomovirus, and Panicovirus). Complementary segments between genomic 5' and 3' UTRs were identified in various members and an example for each genus is provided in Fig. 8. It should be noted that there were some genomes that lacked compelling 5'-3' interactions. For example, TCV and several other carmoviruses contain 5' UTRs that are largely unstructured and show no significant complementarity to their respective 3' UTR sequences (data not shown). Notwithstanding, we were able to identify plausible interactions in the carmovirus, pea stem necrosis virus (Fig. 8). Thus, it is possible that only some members of a particular genus utilize a 5'-3' RNA-RNA-based communication mechanism. Furthermore, in the bi-segmented genome of RCNMV (genus Dianthovirus), RNA1 and its subgenomic mRNA possessed a potential interaction, whereas RNA2 did not (Fig. 8). This implies that the mechanism of 5'-3' communication within the genome of a single virus may vary.

Common features of the translationally relevant 5'-3' RNA-RNA interactions in TBSV and BYDV are the location of the interacting sequences in terminal loops and the presence of similar potential interactions in corresponding subgenomic mRNAs (10). In agreement with these properties, complementary sequences in the 5' and 3' UTRs of different genera were located in predicted terminal loops and the presence of similar potential 5'-3' interactions were also identified in their subgenomic mRNAs, and in an associated satellite virus of PMV. The presence of these characteristic features add strength to the concept that this mechanism extends throughout the family Tombusviridae.

DISCUSSION

Structural Properties of R3.5—A combination of approaches was used to define the structure of R3.5. Our current working model for this region is one in which it forms a branched 3-helix structure containing at least two functional SLs, SL-B and SL-C. The sequence covariation observed within the three helical regions in different tombusviruses suggests that their primary role is nonspecific structural support. However, some of the internal loops and bulges in these stems are highly conserved and could potentially function in a sequence-dependent manner.

The terminal loop of SL-B is essentially invariant in tombusviruses. However, we have shown that this sequence, and
that of its loop partner SL3, can tolerate substantial modifications without major effects on translational activity, as long as complementary between them is maintained. This apparent flexibility in sequence identity is in stark contrast to that observed for the equivalent 5'-3' loop-loop interaction in BYDV (10). MCMV, conveying mottle virus; OCSV, oat chlorotic stunt virus; PoLV, pothos latent virus; PMV, panicum mosaic virus; PSNV, pea stem necrosis virus; TNV, tobacco necrosis virus.

Further structural differences between the TBSV 3' CITE and the BYDV 3' TE, include (i) different sized terminal loops in the relevant hairpins (6 versus 5 nt, respectively); (ii) different loop lengths of complementarity for the interacting sequences (9 versus 5 bp); (iii) the absence in TBSV of a 17-nt tract of sequence that is conserved in the TEs of BYDV, necroviruses, and dianthoviruses (17); (iv) the location of the 3' CITE between RNA replication elements (i.e., RII and RIV); (v) a more 3' genomic position for the 3' CITE; (vi) the presence of the 3' CITE at the 3' end of all TBSV mRNAs (in BYDV its 3' TE is present in the 5' UTR of subgenomic mRNA2 and absent in subgenomic mRNA3); and (vii) different overall secondary structures for the two RNA domains (25). Some of these differences likely account for the observation that, in side-by-side comparisons, the TBSV 3' CITE is not functional in conventional wheat germ in vitro translation systems, whereas the BYDV 3' TE is fully active (21).

Role of the 5'-3' SL3/SL-B Interaction—The results from analyses of three different sets of compensatory mutants support a functional base pairing interaction between SL3 and SL-B. Long distance RNA-RNA base pairing interactions have been shown to function in different fundamental processes in (+)-strand RNA virus reproduction. These include, genome replication (51, 52), subgenic mRNA transcription (31–33, 53–55) and viral mRNA translation (10, 56). In TBSV, the SL3/SL-B interaction is clearly not essential for viral RNA replication, as DI RNAs lacking R3.5 are able to replicate very efficiently (42). As for transcription, other sets of long distance RNA-RNA interactions have been found to promote transcription of TBSV subgenic mRNAs, making it less likely that the SL3/SL-B interaction functions directly in this process (31–33). Therefore, the role of this interaction is likely limited to mediating translation of viral proteins.

In terms of translation, one effect of the 5'-3' RNA-RNA-based interaction is to circularize viral messages (10). For cellular mRNAs, protein-based circularization of messages is thought to (i) facilitate stabilization of ribonucleoprotein complexes involved in translation initiation; (ii) help protect the message from decay; (iii) mediate ribosome recycling; and/or (iv) promote preferential translation of full-length messages (3, 5, 6, 57). Some or all of these activities, which would act to increase translation, could also be facilitated by the RNA-RNA interaction in TBSV. However, an additional critical role, mediating delivery of recruited factors to the 5' end of the message, has been postulated for the corresponding interaction in BYDV, and this function could also apply to TBSV (10, 25). In this case, the 3' CITE would act to recruit translation factors/machinery, whereas the SL3/SL-B interaction would mediate...
their delivery to the 5' end of the message. A recruiting role for the 3' CITE seems reasonable, as the variable nature of 5' UTRs in genomic and subgenomic mRNAs make them unlikely candidates for binding to the same factor(s). Conversely, the presence of identical copies of the 3' CITE at the 3' end of all TBSV viral messages is consistent with the binding of a common factor(s).

Regulatory Aspects of the 5'-3' Interaction—R3.5 was originally defined as an RNA segment consistently absent in prototype TBSV DI RNAs (20). Its location between replication-related RIII and RIV indicates that translation and RNA replication elements are integrated physically. Interestingly, SL3, the pairing partner for SL-B, is also nestled within a replication context. In fact, with the exception of SL3, all of the SLs in the 5'-proximal TSD play significant roles in promoting efficient viral RNA replication (47, 58).2

The close physical association of translation and replication elements in both the 5' and 3' UTR of the TBSV genome could be related to regulatory mechanisms that control these two distinct processes. During infections, the genome first serves as a message for translation and subsequently as a template for distinct processes. During infections, the genome first serves as a message for translation and subsequently as a template for replication. There is evidence from studies on poliovirus that translation is antagonistic to replication and, consequently, replication. There is evidence from studies on poliovirus that translation is antagonistic to replication and, consequently, TBSV represents the only member of the family Tombusviridae for which a translationally important 5'-3' RNA-RNA interaction has been confirmed experimentally.

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