An RNA Activator of Subgenomic mRNA1 Transcription in Tomato Bushy Stunt Virus*

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Many (+)-strand RNA viruses transcribe small subgenomic (sg) mRNAs that allow for regulated expression of a subset of their genes. Tomato bushy stunt virus (TBSV) transcribes two such messages and here we report the identification of a long-distance RNA-RNA interaction that is essential for the efficient accumulation of capsid protein-encoding sg mRNA1. The relevant base pairing interaction occurs within the TBSV genome between a 7-nucleotide (nt) long sequence, separated by just 3 nt from the downstream sg mRNA1 initiation site, and a complementary sequence positioned some ~1000 nt further upstream. Analyses of this interaction indicate that it (i) functions in the (+)-strand, (ii) modulates both (+)- and (−)-strand sg mRNA1 accumulation, (iii) specifically regulates the accumulation of sg mRNA1 (−)-strands, (iv) controls sg mRNA1 expression from an ectopic transcriptional initiation site, (v) may occur in cis and, and (vi) could nucleate the formation of a more complex RNA structure. These data are most consistent with a role for this interaction in regulating sg mRNA1 accumulation at the level of transcription.

Viral infections of eukaryotic cells are complex processes that require regulated expression of a variety of viral genes. Depending on the virus, this expression can be regulated at different levels, including transcriptional, post-transcriptional, translational, and post-translational (1). For (+)-strand RNA viruses, many utilize RNA-templated transcription of subgenomic (sg)3 mRNAs to allow for regulated expression of specific viral genes (2). The mechanism by which sg mRNAs are synthesized can vary, but the messages produced share the common property of encoding open reading frames (ORFs) that are located 3′-proximally in the viral genomes. Because such 3′-proximal ORFs are generally translationally silent within the context of these genomes, sg mRNA production provides a mode for their efficient translation as well as a mechanism to regulate the timing and amount of viral protein produced (2). Two mechanisms for sg mRNA transcription are well-established: (i) synthesis of sg mRNAs from a full-length (−)-strand genomic template via internal initiation (3, 4) and (ii) synthesis of a non-contiguous RNA product during (−)-strand synthesis, which is then used as a template for transcription of sg mRNAs (5, 6). A third possible mechanism that has been proposed involves premature termination during (−)-strand synthesis of the genome followed by use of the 3′-truncated product as a template to transcribe sg mRNAs (7–9). Although this latter model is consistent with data generated from studies on an assortment of (+)-strand RNA viruses (9–11), overwhelming evidence for this mechanism is still lacking.

Tomato bushy stunt virus (TBSV) is the prototype member of both the genus Tombusvirus and the family Tombusviridae. Its (−)-strand RNA genome of ~4.8 kb encodes five functional ORFs (Fig. 1A) (12). The viral RNA polymerase (p92) and accessory RNA replication protein (p33) are both translated from the genome, the former via translational readthrough of the amber termination codon of the latter (Fig. 1A) (12, 13). In contrast, the more 3′-proximal coat protein (CP) ORF (p41) and the overlapping ORFs encoding the movement (p22) and defense (p19) proteins are expressed from two sg mRNAs that are synthesized during TBSV infections (Fig. 1A) (14–16). The transcriptional regulation of the smaller of the two sg mRNAs, sg mRNA2, has been studied previously and RNA sequences important for efficient production of this message have been identified both proximal and distal to its initiation site (17). Efficient accumulation of this sg mRNA requires a long-distance base pairing interaction between a sequence within the core element (CE), located just 5′ to the sg mRNA2 initiation site, and a complementary sequence within the distal element (DE) some ~1100 nucleotides (nt) upstream (Fig. 1A) (17). This interaction functions in the (−)-strand of the viral genome and is proposed to mediate the proper positioning of other subelements within the DE and CE (10). Additionally, the production of (−)-strand sg mRNA2 occurs independently of (−)-strand sg mRNA2 accumulation (10). This latter finding, along with the observed (−)-strand activity of the CE/DE interaction, is consistent with a premature termination mechanism for sg mRNA2 transcription.

Essentially nothing is known about the regulatory RNA elements involved in the synthesis of sg mRNA1. This message is critical for a successful viral infection because it allows for the efficient expression of CP, 180 subunits of which are present in each assembled particle (14). Currently, all that is known about sg mRNA1 transcription is that it can be effectively eliminated by the introduction of substitutions at and immediately 5′ to its site of initiation (17). In the present study, we have sought to define RNA sequences and higher order structures within the TBSV genome that are important for sg mRNA1 transcription. Our results provide evidence for a long-distance RNA-RNA interaction that mediates the accumulation of sg mRNA1. The data also indicate that this interaction occurs in the (−)-strand, likely acts in cis, specifically mediates the accumulation of (−)-strand sg mRNA1, and is responsible...
for regulating expression from an ectopic transcriptional initiation site. The properties of this long-distance RNA-RNA interaction are compared and contrasted with those of the DE/CE interaction involved in regulating sg mRNA2 accumulation. Collectively, these data provide additional insight into how sg mRNAs are transcribed in TBSV.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The construct T100, containing a full-length cdNA copy of the wild type (WT) TBSV genome (12), and mutant ΔPs1g (17) were used in this study. The AS1m1 and RS1m1 constructs used in this study were generated by standard recombinant DNA cloning techniques and PCR-based oligonucleotide-mediated mutagenesis (18) and were sequenced across all PCR-derived regions. The mutant AS1m1 was generated from a T100 derivative containing a neutral single base substitution (C→U) at position 1686 of the genome (12) that introduced a unique AflIII restriction enzyme site. Construction of AS1m1 involved replacement of an AflIII/SstI fragment (1059–1668) with the AflIII/SstI-digested PCR product generated with primer pairs PL4 (5’-GTCGCGGCGACTGAGGCGCTTCAATTTGCT-3’) and PK15 (5’-CCCTTCCTAATGCCTTCGACGAGGATGGGCTCAC-3’), and a SfiI-digested PCR product generated with primer pairs PG-18 (5’-AAACACCAACGGGATCTCAGC-3’) and PK19 (5’-CTCTGACGATGGGATCTCGGCA-3’) and a SfiI-digested PCR product generated with primer pairs PG-18 (5’-AAACACCAACGGGATCTCAGC-3’) and P77 (5’-TCAACGATGGGCGATCCATCCATTATCTCGAC-3’) to replace the corresponding tombusvirus sequences examined (data not shown). Interest-ingly, RS1 corresponds to a portion of the sequence shown previously to be important for sg mRNA1 accumulation (17) (described above). RS1 is also conserved precisely in all tombusvirus genomes were analyzed (data not shown). Interestingly, RS1 corresponds to a portion of the sequence shown previously to be important for sg mRNA1 accumulation (17) (described above). RS1 is also conserved precisely in all tombusvirus genomes were analyzed (data not shown). Additionally, an attempt to free RS1 from its coding duty by introducing a premature stop codon 5’ to the initiation site 3’ of the downstream sg mRNA1 sequence (data not shown). Consequently, this sequence is important for sg mRNA1 accumulation, its specific role and the possible involvement of other sequences was not investigated.

Based on the previous observation that a long-distance DE/CE base pairing interaction was important for sg mRNA2 transcription (10, 17), we investigated the possibility of a similar type of functional RNA-RNA interaction for sg mRNA1. The program MFOLD, which computes minimal free energy RNA secondary structures based on thermodynamic parameters (20, 21), was used to identify possible RNA base pairing interactions within the TBSV genome that could potentially participate in regulating sg mRNA1 transcription. When the first 3000 nt of the TBSV genome was analyzed, a long-range base pairing interaction was identified between a 7-nt long sequence, termed receptor sequence 1 (RS1), separated by just 3 nt from the downstream sg mRNA1 initiation site, and a complementary sequence, termed activator sequence 1 (AS1), located ~1000 nt upstream (Fig. 1B). This long-distance interaction, observed in the optimal RNA secondary structure for TBSV (Fig. 1B), was also predicted in optimal and near-optimal structures when corresponding sequences from six other tombusvirus genomes were analyzed (data not shown). Interestingly, RS1 corresponds to a portion of the sequence shown previously to be important for sg mRNA1 accumulation (17) (described above). RS1 is also conserved precisely in all tombusviruses, unlike AS1, which contains an A→G substitution at position 3 (i.e. 5’-CUACGGC→CUCCGGC, the substitution is underlined) in cucumber necrosis virus (CNV) and TBSV-S (static isolate). However, this alteration would not significantly disrupt the predicted AS1/RS1 interaction, because a GU base pair would replace the AU base pair.

Further examination of the predicted secondary structure for TBSV revealed the presence of a prominent stem-loop (SL) structure positioned just 5’ to RS1 (denoted by the dashed bracket in Fig. 1B). This SL structure, termed SL1sg1, has a low p-num value, which indicates that it is well-defined within the context of the sequence analyzed (20, 21). Additionally, it is present in the optimal structures predicted for all other corresponding tombusvirus sequences examined (data not shown). Its formation and functional relevance is further supported by comparative sequence analysis, which revealed both mono- and co-variation of predicted base pairs in the structures predicted (data not shown).

The AS1/RS1 Interaction Mediates sg mRNA1 Accumulation—To determine whether the predicted AS1/RS1 interaction plays any role in regulating the accumulation of sg mRNA1, we carried out mutational analysis on the nucleotides implicated in forming this 7-bp long helix. Options for mutagenesis via nt substitution were limited due to the fact that both AS1 and RS1 reside in the p92 coding region. Efforts to reposition these sequences into non-coding viral replicons, where they could be modified freely, were unsuccessful (i.e. sg mRNA1 transcription and/or accumulation did not occur in these new contexts). Additionally, an attempt to free RS1 from its coding duty by introducing a premature stop codon 5’ to it in the p92 ORF, which created a C-terminal truncation of 10 amino acids, resulted in a non-viable genome (data not shown). Consequently,
Regulation of TBSV Subgenomic mRNA1 Transcription

Fig. 1. Schematic representation of the TBSV genome and a predicted long-distance base pairing interaction. A, the TBSV RNA genome is shown as a horizontal line with coding regions depicted as boxes with approximate molecular mass values (in thousands) of encoded proteins. Bent arrows indicate the positions of the transcriptional initiation sites for sg mRNAs and the corresponding structures of sg mRNAs 1 and 2 are shown below as horizontal arrows. The relative positions of RNA sequences that interact via base pairing to regulate sg mRNA2 levels (DE, distal element; CE, core element) and sg mRNA1 levels (AS1, activator sequence 1; RS1, receptor sequence 1) are indicated above the genome. B, relevant portion of optimal RNA secondary structure predicted by MFOLD for the first 3000 nt of the TBSV genome. AS1 and RS1 are indicated by solid brackets, and the sg mRNA1 transcriptional initiation site is denoted by an arrow. An adjacent stem-loop structure, SL1sg1, is delineated by a dashed bracket. The coordinates correspond to those of the TBSV genome (12).

Modifications had to be introduced into coding regions of the genome, however these mutations were designed so as to minimize changes to the WT amino acid coding sequence (summarized in Fig. 2). Mutant genomes harboring p92 products with minimally modified C termini (i.e. RS1m1 and RS1-GU2 in Fig. 2) were tested in vivo. Both were found to direct WT levels of genome replication (data not shown) and thus were judged suitable for comparative data analysis.

Various mutant viral genomes were analyzed by inoculating their corresponding in vitro generated transcripts into cucumber protoplasts and monitoring viral RNA accumulation by Northern blot analysis. The levels of sg mRNA1 accumulation observed for different mutants were quantified, relative to the levels of corresponding parental genomes, and are summarized in Fig. 2. Substitutions introduced into either AS1 or RS1 (mutants AS1m1 and RS1m1, respectively), which were predicted to disrupt the base pairing interaction, dramatically reduced the relative levels of sg mRNA1 to ~2 and 0% that of WT T100, respectively (Figs. 2 and 3A). Interestingly, for RS1m1, reduced levels of sg mRNA2 accumulation were also observed consistently (Fig. 3A). When both of these mutations were combined in the mutant A/Rm1, which regenerated base pairing, sg mRNA1 accumulation was restored to ~26% that of WT (Fig. 2); however, there was no restoration of sg mRNA2 levels (Fig. 3A). For sg mRNA1, the reduced recovery may be related to the minor modification of the C terminus of p92 and/or the conversion of two GC base pairs in the WT interaction in T100 to weaker AU base pairs in A/Rm1. The changes in base pair type were necessary to preserve as best as possible the coding of p92. Even with these complications, the observed >10-fold level of restoration of sg mRNA1 accumulation is compelling and supports a functional role for the base pairing interaction between AS1 and RS1. This result does not, however, preclude a role for primary structure in this activity.

The AS1/RS1 Interaction Specifically Mediates (−)-Strand sg mRNA1 Accumulation—Disruption and restoration of the AS1/RS1 interaction also led to corresponding decreases and increases in (−)-strand sg mRNA1 levels (Fig. 3A). (−)-Strand sg mRNAs have been observed previously in TBSV infections (22), however, it is not known whether they play any role in sg mRNA transcription. For sg mRNA2, the accumulation of corresponding (−)-strands can be uncoupled from (−)-strand accumulation by substitution of its initiating nucleotide, a guanylate, with any of the other three residues (10). To determine if the same would apply for sg mRNA1, a single base substitution (G→A) was introduced into the initiating nucleotide of sg mRNA1, generating SG1-A, and maintaining a properly positioned termination codon for the p92 ORF (Fig. 2). Very low levels of (−)-strand sg mRNA1 were observed for SG1-A, however a significant increase in (−)-strand sg mRNA1 was apparent (Fig. 3B). To address whether the AS1/RS1 interaction was essential for this preferential (−)-strand sg mRNA1 accumulation, the AS1 disruption in AS1m1 was introduced into SG1-A, thereby creating AS1m1-A. This disruption of the AS1/RS1 interaction eliminated the high levels of (−)-strand sg mRNA1 observed for SG1-A, suggesting that this interaction specifically mediates (−)-strand sg mRNA1 accumulation (Fig. 2B).

sg mRNA1 Accumulation Persists When the AS1/RS1 Interaction Is Preferentially Destabilized in the (−)-Strand—Due to the canonical nature of the base pairs in the AS1/RS1 interaction, the association could potentially occur in either the (−)- or (−)-strand. In an attempt to determine the functional polarity of this interaction, GU base pairs were created by introducing substitutions into AS1, RS1, or both. The presence of GU base pairs in the (−)-strand is predicted to be less destabilizing to the (−)-strand interaction than to the complementary (−)-strand interaction (10). When two GU base pairs were introduced by substitutions into either AS1 or RS1 (mutants AS1-GU2 and RS1-GU2), sg mRNA1 levels were ~28% and ~21% that of WT, respectively (Figs. 2 and 4). In A/R-GU4, which contained a total of four GU base pairs, ~9% activity was observed (Figs. 2 and 4). This latter activity was 4-fold above the level determined for AS1m1 (~2%) and even more so for that of RS1m1 (0%) (Fig. 2). This notably higher level of accumulation for A/R-GU4 is relevant, because the very defective mutants AS1m1 and RS1m1 each contain two and three mismatches, respectively, in their (−)-strands. Based on this observation, the four mismatches in A/R-GU4 would disrupt the (−)-strand interaction very efficiently and, if the AS1/RS1 interaction was resident in the (−)-strand, would effectively block sg mRNA1 accumulation. However, the analysis of A/R-GU4 revealed moderately low sg mRNA1 levels. This result therefore does not support the idea of (−)-strand activity and instead is more consistent with the maintenance of a weak, but functional, AS1/RS1 interaction in the (−)-strand—mediated by non-canonical GU base pairing. This concept of (−)-strand ac-
activity is further supported by MFOLD analysis that predicts this interaction in the (+)-strand and by the naturally occurring GU base pair present in the AS1/RS1 interaction in CNV and TBSV-S genomes.

AS1 Acts in Cis and Activates Transcription from an Ectopic Transcriptional Initiation Site

To investigate whether a functional AS1/RS1 interaction could occur intermolecularly, mutants AS1m1 and RS1m1 were co-inoculated to determine if their defects could be complemented in trans. Formation of a WT AS1/RS1 interaction between these two mutant genomes is possible, because AS1m1 contains a WT RS1 and RS1m1 a WT AS1. Co-inoculations were performed at concentrations shown previously to allow for efficient coinfection of protoplasts (13, FIG. 3. Mutational analysis of the AS1/RS1 interaction. A, schematic representation of relevant portions of mutants containing substitutions (specified by black boxes) in AS1 and/or RS1 are shown to the left. Northern blot analysis showing viral RNA accumulation for mutant genomes containing various modifications in AS1 and/or RS1 is shown on the right. The identity of the transcript used in the infection is shown above each lane, and the positions of viral RNAs (g, genome; sg1 and sg2, subgenomic mRNA1 and 2, respectively) are indicated. Total nucleic acids were isolated from inoculated cucumber protoplasts after a 24-h incubation and analyzed as described under “Experimental Procedures.” The upper panel represents detection of (+)-strand viral RNAs, whereas the (−) symbols indicate detection of (−)-strands in the lower panel. B, substitution of the initiating nucleotide of sg mRNA1. Schematic representation of relevant portions of mutants containing substitutions (specified by black boxes) in the initiating nucleotide and AS1 are shown to the left. Northern blot analysis, as described above, showing viral RNA accumulation for mutant genomes containing various modifications are to the right.

FIG. 4. Analysis of the functional polarity of the AS1/RS1 interaction. A, schematic representation of relevant portions of mutants containing substitutions (specified by black boxes) in AS1 and/or RS1. B, Northern blot analysis showing viral RNA accumulation for mutant genomes containing various modifications in AS1 and/or RS1 is shown in the lower panel. The identity of the transcript used in the infection is shown above each lane, and the positions of viral RNAs (g, genome; sg1 and sg2, subgenomic mRNA1 and 2, respectively) are indicated to the left. Analysis was performed as described in the legend to Fig. 3.
23). Co-inoculation of 5 or 10 µg each of AS1m1 and RS1m1 (Fig. 5, lanes 5 and 6, respectively) did not result in any detectable increase in sg mRNA1 accumulation. This result suggests that the interaction between AS1m1 and RS1m1 occurs primarily in cis and is consistent with the MFOLD-predicted intramolecular nature of this interaction.

Previous studies have shown that sg mRNA1 transcription can be inactivated by substitutions at its initiation site (17). However, when a second WT copy of the initiation site and its flanking sequences (−100/+61) was inserted into this context at a new location (mutant ΔPs1g1−1, Fig. 6A), sg mRNA1 transcription from this ectopic position occurred, albeit at notably lower levels (17) (Fig. 6B). One possible explanation for this reduced activity may be that the AS1/RS1 interaction is required but does not occur efficiently in the modified context. To assess this possibility, the same modification in AS1m1 was introduced into ΔPs1g1+1, creating ΔPs1g1+1-AS1m1. This mutant was no longer able to mediate detectable levels of sg mRNA1 (Fig. 6B), suggesting that AS1 is responsible for promoting sg mRNA expression from this ectopic initiation site and further supporting a functional role for the AS1/RS1 interaction.

**DISCUSSION**

Our analysis of the TBSV genome has allowed us to identify sequences and structures that are important for sg mRNA1 accumulation. Specifically, two sequences, AS1 and RS1, were found to be essential for efficient sg mRNA1 accumulation, and the data support a functional requirement for their base pairing in the (+)-strand. Additionally, the AS1/RS1 interaction was found to be important specifically for (−)-strand sg mRNA1 accumulation and was shown to regulate sg mRNA1 production from an ectopic initiation site. These data support a role for this long-distance RNA-RNA interaction in regulating transcriptional activity.

**Structural and Functional Features of the AS1/RS1 Interaction**—The distant positioning and comparatively small size of AS1 and RS1 prompt the following questions: (i) how do these sequences find each other in the context of the genome? and (ii) could this interaction be facilitated and/or stabilized by other RNA elements? Our MFOLD analysis of tombusvirus sequences suggests that the global folding of the genome likely assists in the formation of this base paired segment in cis via colocalizing the participating sequences. Additionally, local structures could also be involved in mediating this interaction.

Therefore, we analyzed AS1 and its flanking sequence by MFOLD for potential structures that could facilitate the AS1/RS1 interaction. Different conformations of a SL structure were predicted depending on the parameters used for the analysis, however, a common feature in each was the presence of all or most of AS1 within a terminal loop (Fig. 7). This positioning, in a predicted single-stranded region, could facilitate the presentation of AS1 for base pairing with RS1. Surprisingly, one of the predicted conformers (ΔG = −7.9 at 22 °C) is strikingly similar in general structure to the trans-acting hairpin activator of sg mRNA transcription in the bipartite (+)-strand virus red clover necrotic mosaic virus (RCNMV) (Fig. 7) (9). In RCNMV, the nucleotides in the terminal loop of the hairpin activator in genomic RNA-2 interact in trans with a sequence just 5′ to the sg mRNA transcriptional initiation site in genomic RNA-1, thereby facilitating sg mRNA accumulation. It was proposed that, as with other bimolecular interactions, the base pairing of these elements would be favored by high concentrations of RNA-1 and RNA-2, which occur late in the infection (9).

This is also the time at which CP, which is translated from the induced RCNMV sg mRNA, is required in large quantities for encapsidation of progeny RNA genomes. The sg mRNA1 of TBSV also encodes CP, therefore, a similar concentration-dependent mechanism for controlling appropriate timing of induction of CP (i.e. late in the infection when progeny genomes are abundant) could also apply. Consistent with this notion are the observed accumulation profiles of sg mRNA1 and sg mRNA2.
mRNA2 for TBSV and other tombusviruses during 24-h protoplast infections (17, 24, 25). sg mRNA2 is detectable earliest in the infection, however, its accumulation then levels out at intermediate time points. In contrast, sg mRNA1 accumulation is low early in the infection but increases at later time points. The lack of functional complementation in co-infections with AS1- and RS1-defective mutants suggests that these elements do not function efficiently in trans. However, because the two mutants could conceivably localize to different replication sites within coinfected cells, these results do not preclude the involvement of intermolecular interactions in WT infections.

Formation of the AS1/RS1 helix is clearly essential for efficient sg mRNA1 accumulation, however, the precise nature of this interaction is unknown. It is possible that the base pairing interaction between AS1 and RS1 nucleates the formation of a larger more complex structure. For the RS1 context, the existence of an adjacent secondary structure, SL1sg1, is supported by MFOLD and comparative sequence analysis (Fig. 1B). The formation of such a structure could further stabilize the AS1/RS1 helix through coaxial stacking (Fig. 8A). Stabilization of higher order RNA structures by coaxial stacking of helices is quite common (26) and may be involved in stabilizing other RNA structures within the TBSV genome (27). For the AS1/RS1 interaction, additional types of stabilizing interactions could also exist. For instance, the sequence just 3' to AS1 is complementary to the sequence just 5' to SL1sg1, thus base pairing of these sequences could further stabilize the AS1/RS1 helix (Fig. 8A). The above examples illustrate the potential for this RNA interaction to be more complex and these putative structural features are being investigated.

**Comparison of the AS1/RS1 Interaction with the DE/CE Interaction and Mechanistic Insights**—The AS1/RS1 interaction is the second long-distance interaction shown to be involved in sg mRNA synthesis in TBSV. The previously characterized DE/CE interaction, which is required for efficient sg mRNA2 production, shares both similarities and differences with the AS1/RS1 interaction (Fig. 8, compare A with B). Similarities between the predicted structures include: (i) the key base pairing interactions involve sequences just 5' to the sites of initiation; (ii) the long-distance interactions span similar distances (−1000–1100 nt); (iii) the functional interactions occur in the (+)-strand and; (iv) similar sequences surround the initiating nucleotides (underlined, 5'-CUUGA(C/A)CAAGA). There are, however, some distinct differences: (i) the overall RNA structures predicted share no striking similarities, other than those listed above; (ii) the AS1/RS1 base pairing interaction is smaller than the DE/CE base pairing interaction (7 versus 12 bp) and; (iii) the spacing of the base pairing interaction relative

![Fig. 7](http://example.com/fig7.png)

**Fig. 7.** MFOLD analysis of AS1 and its flanking sequences predicts the formation of stem-loop structures. Analysis of AS1 and its immediately adjacent sequences by MFOLD predicts stem-loop structures in which all or most of the AS1 nucleotides (in bold) reside in the terminal loop. Different optimal and suboptimal conformations are predicted at 37°C using MFOLD version 3.1 or at 22°C using MFOLD version 2.3, and the corresponding free energy values are indicated below each structure. The sequence shown is entirely conserved in sequenced tombusviruses, except for an adenylate (denoted by an asterisk) that is a guanylate in CNV and TBSV-S. For comparison, the predicted structure of the RCNMV trans-activator hairpin is shown to the right (9).

![Fig. 8](http://example.com/fig8.png)

**Fig. 8.** RNA secondary structure models for the sequences involved in modulating sg mRNA1 and sg mRNA2 transcription in TBSV. The structures presented are based on a combination of compensatory-type mutational analyses, comparative sequence analyses, as well as MFOLD-based structural modeling. A, predicted RNA secondary structure for sg mRNA1 modulating sequences. The AS1/RS1 helix is in **boldface**, and the predicted adjacent SL1sg1 is delineated by a **dotted bracket**. A putative helix formed by base pairing of sequences 5' to SL1sg1 and 3' to AS1 is indicated by a **parenthesis** with a **question sign**. The initiation site for sg mRNA1 is indicated by a **bent arrow**. B, predicted RNA secondary structure for sg mRNA2 modulating sequences. The subelements of the DE and CE are delineated by **thick horizontal and vertical lines**. The base pairing nucleotides in A and B subelements of both DE and CE are in **boldface**. The initiation site for sg mRNA2 is indicated by a **bent arrow**. The 5' boundary of the DE/CE RNA complex is indicated by a **diagonal dashed line** that traverses the contiguous sequence connecting the two structures.
to the initiation nucleotides are different (3 versus 11 nt). It is possible that this latter difference reflects unique structural features that contribute to distinct activities for these RNA complexes (e.g. timing and/or amount of sg mRNA synthesized). Alternatively, the structure currently proposed for sg mRNA2 may be incomplete and could be lacking, for example, an additional base pairing interaction involving the CE-C sequence that would correspond to the AS1/RS1 helix. In potato virus X (PVX) long-distance base pairing interactions involving the 5’ terminus of the genome and sequences just 5’ to the initiation sites of its two sg mRNAs act to regulate sg mRNA accumulation (28, 29). The spacing between the end of the helix and the initiating nucleotides in both of these cases is 11 nt. In contrast, the corresponding spacing in RCNMV is 2 nt. Considering that RCNMV, but not PVX, is closely related to TBSV, and that the spacing for sg mRNA1 in TBSV is 3 nt, the latter of the two possibilities described above seems more plausible and is being explored.

We have now defined two working RNA secondary structural models for RNA complexes involved in regulating sg mRNA accumulation in TBSV (Fig. 8). Our data indicate that these structures function in the (+)-strand of the genome and likely regulate steps in the transcriptional process. It is interesting to note that, if both long-distance interactions were to occur simultaneously, the RNA complexes would be in close proximity to each other (Fig. 8). Such an association may be relevant to their function and could represent a type of multicomplex center where both structures are conveniently serviced by colocalized cis- and/or trans-acting elements. Some overlap in sequence function is suggested by the dependence of both sg mRNA1 and sg mRNA2 on RS1 (Fig. 3A). However, if sg mRNA2 is directly dependent on the sequence of RS1 (as opposed to its coding capacity, see below), it does not appear to rely on the AS1/RS1 interaction, because there was no recovery of sg mRNA2 levels when this helix was restored (Fig. 3A). Alternatively, it is possible that the minor modification to the extreme C terminus of p92 in RS1m1 is responsible for the observed defect. Indeed, modifications of viral RNA replication proteins have been found to specifically affect sg mRNA levels (30, 31).

Viewed collectively, the information gathered on sg mRNA regulatory RNA elements in TBSV are consistent with their involvement in transcriptional regulation via a premature termination mechanism (10, 17): (i) Accumulation of sg mRNA (+)-strands occurs independently of complementary (+)-strand accumulation, as would be expected if (+)-strands are synthesized first and then function as templates for (+)-strand synthesis. (ii) The key secondary structures function in the (+)-strand of the genome, as would be predicted for elements implicated in modulating (+)-strand accumulation. (iii) The AS1/RS1 interaction is required specifically for mediating (+)-strand sg mRNA1 accumulation, in accordance with a putative role for this element in promoting premature termination of (+)-strand synthesis. Although these data are consistent with a premature mechanism, they do not preclude alternative transcriptional models. Therefore, further studies will be necessary to determine conclusively the mechanism(s) utilized by TBSV for sg mRNA transcription.

REFERENCES

1. Maia, I. G., Seron, K., Haenni, A., and Bernardi, F. (1996) Plant Mol. Biol. 32, 367–391
2. Miller, W. A., and Koev, G. (2000) Virology 273, 1–8
3. Miller, W. A., Drehner, T. W., and Hall, T. C. (1985) Nature 313, 68–70
4. Siegel, R. W., Adkins, S., and Cas, C. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11238–11243
5. Pasternak, A. O., Gulyaev, A. P., Spaan, W. J., and Snijder, E. J. (2000) J. Virol. 74, 11642–11653
6. van Marle, G., Dobbe, J. C., Gulyaev, A. P., Luypjes, W., Spaan, W. J., and Snijder, E. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12056–12061
7. Zhong, W., and Rueckert, R. R. (1995) J. Virol. 69, 2716–2722
8. Miller, W. A., Brown, C. M., and Wang, S. (1997) Semin. Virol. 8, 3–13
9. Sit, T. L., Vaevelongs, A. A., and Lommel, S. A. (1998) Science 281, 829–832
10. Choi, I., Otvosvany, M., Zhang, G., and White, K. A. (2001) J. Biol. Chem. 276, 41761–41768
11. Price, B. D., Roeder, M., and Ahkquist, P. (2000) J. Virol. 74, 11724–11733
12. Heanne, P. Q., Knorr, D. A., Hillman, B. I., and Morris, T. J. (1990) Virology 177, 141–151
13. Oster, S. K., Wu, B., and White, K. A. (1998) J. Virol. 72, 5845–5851
14. Hillman, B. I., Heanne, P., Rochon, D., and Morris, T. J. (1998) Virology 169, 42–52
15. Schönhof, H. B., Schönhof, K. B., Kikker, M., and Jackson, A. O. (1995) Virology 213, 425–438
16. Batey, R. T., Rambo, R. P., and Doudna, J. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14147–14152
17. Zhang, G., Slowinski, V., and White, K. A. (1999) RNA (N. Y.) 5, 550–561
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. White, K. A., and Morris, T. J. (1994) J. Virol. 68, 14–24
20. Mathews, D. H., Sabina, J., Zuker, M., and Turner, D. H. (1999) J. Mol. Biol. 288, 911–940
21. Zuker, M., Mathews, D. H., and Turner, D. H. (1999) in RNA Biochemistry and Bio/Technology (Barricklowitz, J., and Clark, B. F. C., eds) pp. 11–43, NATO ASI Series, Kluwer Academic Publishers, Dordrecht, The Netherlands
22. Ray, D., and White, K. A. (1999) Virology 256, 162–171
23. Wu, B., and White, K. A. (1999) J. Virol. 73, 8982–8988
24. Johnson, J. C., and Rochon, D. M. (1995) Virology 214, 100–109
25. TavaZZa, M., LuciiZ, A., Calgero, A., Pay, A., and TavaZZa, R. (1994) J. Gen. Virol. 75, 1515–1522
26. Batyè, R. T., Rambo, R. P., and Doudna, J. A. (1999) Angew. Chem. Int. Ed. Engl. 38, 2326–2343
27. Wu, B., Vanti, W. B., and White, K. A. (2001) J. Mol. Biol. 305, 741–756
28. Kim, K. H., and Hemenway, C. L. (1997) Virology 232, 187–197
29. Kim, K. H., and Hemenway C. L. (1999) RNA (N. Y.) 5, 636–645
30. van Dinten, L. C., den Boon, J. A., Wassenaar, A. L., Spaan, W. J., and Snijder, E. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 991–996
31. van Marle, G., van Dinten, L. C., Spaan, W. J., Luypjes, W., and Snijder, E. J. (1999) J. Virol. 73, 5274–5281
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