Conformational organization of the 3' untranslated region in the tomato bushy stunt virus genome

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ABSTRACT

The 3' untranslated regions (UTRs) of positive-strand RNA viruses often form complex structures that facilitate various viral processes. We have examined the RNA conformation of the 352 nucleotide (nt) long 3' UTR of the Tomato bushy stunt virus (TBSV) genome with the goal of defining both local and global structures that are important for virus viability. Gel mobility analyses of a 3'-terminal 81 nt segment of the 3' UTR revealed that it is able to form a compact RNA domain (or closed conformation) that is stabilized by a previously proposed tertiary interaction. RNA–RNA gel shift assays were used to provide the first physical evidence for the formation of this tertiary interaction and revealed that it represents the dominant or “default” structure in the TBSV genome. Further analysis showed that the tertiary interaction involves five base pairs, each of which contributes differently to overall complex stability. Just upstream from the 3'-terminal domain, a long-distance RNA–RNA interaction involving 3' UTR sequences was found to be required for efficient viral RNA accumulation *in vivo* and to also contribute to the formation of the 3'-terminal domain *in vitro*. Collectively, these results provide a comprehensive overview of the conformational and functional organization of the 3' UTR of the TBSV genome.

Keywords: RNA replication; RNA structure; plant virus; tombusvirus; tertiary structure; promoter

INTRODUCTION

The compact nature of positive-strand RNA virus genomes often forces them to utilize the same sequence for multiple functions. For example, RNA elements involved in genome replication can be located in coding regions, thereby directing both translational and replication functions (McKnight and Lemon 1998; Goodfellow et al. 2000; You et al. 2004; Monkewich et al. 2005; Pogany et al. 2005). Even when a distinct RNA element is used for one specific function, it is often highly integrated with other operative RNA elements. This concept holds true for the 3' untranslated regions (UTR) of many positive-strand RNA viruses. The most common RNA elements to share the 3' UTR are those that mediate translation and replication (Dreher 1999; Kao et al. 2001; Dreher and Miller 2006; Kneller et al. 2006).

The 3' UTR of the Tomato bushy stunt virus (TBSV) positive-strand RNA genome is 352 nucleotides (nt) long and includes RNA structures and sequences involved in both viral RNA replication and protein translation (Hearne et al. 1990; White and Nagy 2004). The sequence can be

divided into three contiguous regions based on function. The central region 3.5 (R3.5) is translation specific (Oster et al. 1998; Wu and White 1999; Fabian and White 2004, 2006), whereas flanking RIII and RIV are important for both replication and translation (Fabian et al. 2003; Panavas and Nagy 2003; Pogany et al. 2003; Ray and White 2003; Na and White 2006) (Fig. 1A,B). The negative strand of RIII acts as an enhancer for positive-strand synthesis (Panavas and Nagy 2003; Ray and White 2003), while the extreme 3' end of RIV contains the promoter for negative-strand synthesis (Pogany et al. 2003). RIV also contains several stem-loop (SL) structures in its 3'-proximal half that mediate replication (Fig. 1C; Fabian et al. 2003; Na and White 2006).

For TBSV, much of what is known about the replication functions of the 3' UTR has come from studies utilizing defective interfering (DI) RNAs (Fig. 1B; White 1996). These small noncoding genome-derived replicons allow for the analysis of replication-specific activities in a context that is independent of protein-coding functions (White and Nagy 2004). Genetic evidence from the analysis of a TBSV DI RNA containing compensatory mutations suggested that a functionally relevant tertiary base-pairing interaction occurs between 3'-terminal residues (termed the 3' complementary silencer sequence; 3'CSS) and residues within an internal loop in SL3 (termed the replication silencer element; RSE) (Pogany et al. 2003). This requirement was predicted because the potential for base-pairing between the RSE and 3'CSS

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FIGURE 1. TBSV RNAs. (A) The TBSV RNA genome (T100) is represented linearly with coding regions depicted as boxes. Translation-specific region 3.5 (3.5) in the 3' UTR is indicated by the bracket. (B) Schematic representation of a typical TBSV DI RNA, DI-72. Regions of the genome present in this DI RNA (I through IV) are indicated by shaded boxes that are delineated by vertical dotted lines. (C) Region IV has been expanded to show its RNA sequence and known secondary structures (Fabian et al. 2003). The “open” conformation of the 3' half of RIV is shown. The nucleotides in the replication silencer element (RSE) and the 3' complementary silencer sequence (3'CSS) are white on black circles. (D) The proposed “closed” conformation of the 3' half of RIV is shown (Na and White 2006). The transition between the open and closed conformations is indicated by the double-headed arrow.

was a prerequisite for accumulation of the DI RNA in plant protoplasts (Pogany et al. 2003). The RSE–3'CSS interaction has been proposed to convert the 3'-end region from an “open” conformation to a more compact or “closed” conformation (Fig. 1C,D; Na and White 2006). Similar RSE–3'CSS interactions are also proposed for Turnip crinkle virus (TCV) (Zhang et al. 2004) and are predicted in other genera in the family Tombusviridae (Koev et al. 2002; Na and White 2006). For TBSV (genus *Tombusvirus*, family *Tombusviridae*), this interaction mediates replicase complex formation in vivo and modulates negative-strand synthesis in vitro (Panaviene et al. 2005; Pogany et al. 2003). The latter activity has also been demonstrated in vitro for TCV (genus *Carmovirus*, family *Tombusviridae*) and the interaction is required for optimal virus accumulation in vivo (Zhang et al. 2004, 2006b).

The TBSV genome lacks both a 5'-cap structure and a 3'-poly(A) tail (Hearne et al. 1990). Consequently, it utilizes an alternative mechanism to mediate translation of its viral proteins that involves both its 5' and 3' UTRs

(Fabian and White 2004). The working model for TBSV translation is one where the translational machinery is recruited to the 3' UTR and then delivered to the 5' UTR via a 5'–3' RNA–RNA interaction between SL3 in the 5' UTR and SL-B in R3.5 (Fabian and White 2006). There is both genetic and physical evidence to support the SL3–SL-B interaction, and other RNA elements in R3.5 have been implicated in the recruitment of translation factors (Fabian and White 2004, 2006). The RNA secondary structures of R3.5 as well as the 3' half of RIV have been investigated previously (Fabian and White 2004, 2006; Fabian et al. 2003; Na and White 2006); however, the overall conformation of the 3' UTR (which includes RIII, R3.5, and RIV) and the structural relationship between replication and translation elements remain unclear.

Using in vitro and in vivo investigative approaches we have examined the structure and function of RNA elements in the 3' UTR of TBSV. Our results provide the first physical evidence for the RSE–3'CSS interaction and suggest that the 3'-terminal segment of RIV constitutes an RNA domain. Additionally, we show that the upstream half of RIV interacts with the 3' portion of RIII, and that this interface is functionally relevant for DI RNA accumulation in vivo and for formation of the 3' domain in vitro. When integrated with what is known about translational elements in this region, the results provide a comprehensive overview of the conformational organization of replication and translational elements in the 3' UTR of the TBSV genome.

RESULTS

Conformational analysis of the 3' half of region IV

The 3'-terminal 82 nt of the TBSV genome has been proposed to adopt two different conformations, termed open and closed, based on the absence or presence of the RSE–3'CSS interaction, respectively (Fig. 1C,D; Na and White 2006). In certain cases, conformational changes in an RNA can be detected by assessing its gel electrophoretic mobility under nondenaturing conditions. To investigate the proposed open and closed conformations, different 5'-radio end-labeled RNA transcripts corresponding to this 3'-terminal segment were generated in vitro and analyzed by acrylamide gel electrophoresis under denaturing and nondenaturing conditions (Fig. 2). Initially, the mobility of wild-type HN52–1 was compared to mutants HN246 and HN52–3, containing either a tandem substitution in the 3'CSS or a deletion of the three 3'-terminal cytidylates, respectively (Fig. 2A). Both of these mutations were predicted to inhibit formation of the RSE–3'CSS interaction. Under denaturing conditions HN52–1 and HN246 co-migrated, whereas HN52–3 exhibited a faster migration rate (Fig. 2B). This result was anticipated, as HN52–3 is less massive than the other two transcripts by three residues. In contrast, under nondenaturing conditions, HN246 and HN52–3 had

FIGURE 2. Gel mobility analysis of the 3' half of RIV. (A) Wild-type and mutant 81-nt segments of TBSV viral RNA corresponding to the 3' half of RIV. The positions altered by substitution or deletion (D) are indicated for each mutant. The black star in this and other figures indicates that the 5' terminus of the RNA is labeled with ^{32}P . (B) Analysis of ^{32}P -5'-end-labeled viral RNA transcripts under denaturing (upper panel) or non-denaturing (lower panel) conditions. The position of wild-type HN52-1 is indicated on the left and a horizontal white line (with arrowhead) provides a point of reference in the non-denaturing analysis. The identities of the transcripts separated in the gels are indicated above each lane.

similar migration rates, while HN52-1 moved more rapidly. The faster rate of migration of HN52-1 is consistent with a more compact structure (i.e., a closed conformation), whereas the slower migration rates for HN246 and HN52-3 are in line with a more relaxed structure (i.e., an open conformation).

Next, the same set of compensatory mutations that was assessed previously by genetic approaches (involving C_{57} and G_4 ; Pogany et al. 2003) was introduced into the 81 nt long 3'-terminal segment to create HN254, HN255, and HN256 (Fig. 2A). When tested along with wild-type HN52-1, all transcripts exhibited similar mobilities under denaturing conditions; however, under non-denaturing conditions HN52-1 co-migrated with HN256 and HN254 with HN255 (Fig. 2B). Thus, a similar correlation between increased mobility and the potential to form the RSE-3'CSS interaction was observed. These results support the formation of a more compact or closed

structure that is mediated by the RSE-3'CSS interaction (Fig. 1D).

A bimolecular system confirms the RSE-3'CSS interaction

Evidence supporting the formation of the RSE-3'CSS interaction is based largely on *in vivo* genetic studies that showed a positive correlation between the complementarity of these sequences and the accumulation of a TBSV DI RNA in plant protoplasts (Pogany et al. 2003). The conformational analysis described above indicated a major structural transition mediated by RSE and 3'CSS; however, we sought more compelling physical data to substantiate the existence of the RSE-3'CSS interaction. To provide this evidence, a bimolecular system consisting of 5' and 3' halves of the 81-nt segment was developed. The 3'-proximal fragment included the two 3'-terminal stem-loops (SL1 and SL2) as well as the 3'CSS, while the 5'-proximal fragment contained SL3 along with the RSE (Fig. 3A,B). When increasing concentrations of wild-type HN123 were mixed with 4 nM of 5'-radio end-labeled wild-type HN122, upward shifts in mobility relative to the free HN122 were observed (Fig. 3C, upper panel). Interestingly, at high concentrations of HN123 (i.e., 10,000–15,000 nM) a single dominant complex was observed, but at lower concentrations this complex was accompanied by a second band of greater and variable mobility (Fig. 3C, upper panel). This additional band could indicate that the RNA undergoes an intermediate conformational change after the two RNA bind to each other. Alternatively, this band could represent bound HN122 that dissociates during electrophoresis and is not

FIGURE 3. A bimolecular system to study the RSE-3'CSS interaction. (A) Radiolabeled 5'-proximal wild-type viral RNA segment containing the RSE element. (B) Unlabeled 3'-proximal wild-type viral RNA segment containing the 3'CSS element and a mutant derivative. (C) Analysis of RNA-RNA complex formation by non-denaturing gel electrophoresis. Increasing concentrations of unlabeled 3'-proximal fragment was mixed with ^{32}P -labeled 5'-proximal fragment (4 nM). The concentrations (in nM) of 3'-proximal fragment used are indicated at the top of each lane. Following incubation, the mixture was separated in a non-denaturing acrylamide gel and the labeled products were detected by phosphor-imaging. The positions of free (F) and bound (B) 5'-proximal fragment are indicated on the left and the RNAs analyzed are indicated on the right.

rebound by HN123 at lower concentrations. However, as the concentration of HN123 increases, it would be able to more efficiently rebind any dissociated HN122 in the gel and retard its mobility in a proportional manner. Eventually, this increased proficiency at higher concentrations would result in the single band observed. Irrespective of the nature of the intermediate band, the overall interaction was found to be specific and 3'CSS dependent, as mixing wild-type HN122 with increasing concentrations of mutant HN147 (containing a tandem substitution in the 3'CSS; Fig. 3B) did not generate any shifted complexes (Fig. 3C, lower panel).

Next, to determine if the RSE–3'CSS interaction was responsible for the complex formation, the compensatory mutation set involving C₅₇ and G₄ was introduced into the two segments (Fig. 4A,B). Efficient complex formation was observed only when RSE and 3'CSS segments in the two RNAs were complementary (Fig. 4C), supporting the importance of the RSE–3'CSS interaction. Interestingly, the relative level of complex formation for the mutant compensatory pair (HN242+179) was consistently lower than that for the wild-type pair (HN122+123) (Fig. 4C), suggesting reduced binding capacity in the former pair.

As the contexts of the RSE and 3'CSS have been proposed to influence the interaction (Na and White 2006), the importance of SL1 and the apical stem of SL3 were also

assessed using this system (Fig. 4D,E). Disruption of the terminal stem of SL3 abolished complex formation, whereas disruption of SL1 did not (Fig. 4F), thereby implicating SL3 in mediating the RSE–3'CSS interaction. Additionally, SL2 was not necessary for the RSE–3'CSS interaction, as disruption of this SL had no appreciable effect on complex formation (data not shown).

Probing the RSE–3'CSS interaction in a DI RNA

Having obtained physical evidence to support the formation of the RSE–3'CSS interaction using small RNA fragments, we next turned our focus on its proposed formation in the larger context of a prototypical DI RNA. The approach developed to assess this involved a bimolecular system utilizing transcripts of full-length DI RNA and 5'-radiolabeled HN123 (Fig. 5A,B). Formation of the RSE–3'CSS interaction in the DI RNA was assessed by monitoring the efficiency with which HN123 (containing a 3'CSS) was able to bind to the RSE in the DI RNA. The principle being that formation of the RSE–3'CSS interaction in the DI RNA would inhibit HN123 binding. Conversely, if the RSE was not paired intramolecularly in the DI RNA, then it would be accessible and bound by HN123.

The validity of this approach was examined by assessing HN123 binding to wild-type DI-72, DI-147 (in which the 3'CSS was mutated), or DI-149 (where the RSE was mutated) (Fig. 5A). Relative binding levels of HN123 to the different DI RNAs were consistent over a large concentration range, with DI-147 binding best, followed by wild-type DI-72, and finally DI-149 (Fig. 5C,D). These results are consistent with the predictions that (1) “unmasking” the RSE in DI-147 results in the best relative binding, (2) formation of the RSE–3'CSS interaction in wild-type DI-RNA reduces binding through intramolecular competition, and (3) the least efficient binding occurs when the RSE in the DI RNA is mutated to be noncomplementary to the 3'CSS in HN123.

This system was then used to examine the C₅₇ and G₄ compensatory mutation set in a DI RNA context (Fig. 6A,B). When the wild-type DI-72 and mutants DI-61 and DI-62 were tested with wild-type HN123, a low–high–low relative binding profile, respectively, was observed (Fig. 6C). When mutants DI-61, DI-62, and DI-63 were tested with mutant HN179, a similar low–high–low profile, respectively, was observed (Fig. 6C). In both cases, the 3' probes bound most efficiently only when their complementary target RSEs were unmasked in DI-61 or DI-62. These results indicate that the RSE–3'CSS interaction does, indeed, occur in the context of the DI RNA.

This system was also used to determine the relative contribution of each nucleotide to the formation of the RSE–3'CSS interaction in the DI RNA. Each of the 5 nt in the 3'CSS of the DI RNA was individually substituted with its complementary residue and the effect on HN123

FIGURE 4. Analysis of the RSE–3'CSS interaction. (A) Radiolabeled 5'-proximal wild-type viral RNA segment containing the RSE element and a mutant derivative. (B) Unlabeled 3'-proximal wild-type viral RNA segment containing the 3'CSS element and a mutant derivative. (C) Analysis of RNA–RNA complex formation by nondenaturing gel electrophoresis. Unlabeled 3'-proximal fragment (12,000 nM) was mixed with ³²P-labeled 5'-proximal fragment (4 nM). Following incubation, the mixture was separated in a nondenaturing acrylamide gel and the labeled products were detected by phosphor imaging. The positions of free (F) and bound (B) 5'-proximal fragment are indicated on the left and the RNAs analyzed are indicated above the lanes. D–F are as described in A, B, and C, respectively.

FIGURE 5. Probing the RSE–3'CSS interaction in a DI RNA. (A) Unlabeled wild-type DI RNA and mutant derivatives. The shaded *inset* indicates that the RNA being analyzed is a full-length DI RNA. (B) Radiolabeled 3'-proximal wild-type viral RNA segment containing the 3'CSS element. (C) Analysis of RNA–RNA complex formation by nondenaturing gel electrophoresis. Increasing concentrations of unlabeled DI RNA was mixed with 32 P-labeled 3'-proximal fragment (4 nM). The concentrations (in nM) of DI RNA used are indicated at the *top* of each lane. Following incubation, the mixture was separated in a nondenaturing acrylamide gel and the labeled products were detected by phosphor-imaging. The positions of free (F) and bound (B) 3'-proximal fragment are indicated on the *left* and the RNAs analyzed are indicated on the *right*. (D) The fraction bound for each RNA pair in C was quantified at the different DI RNA concentrations tested.

binding was monitored (Fig. 7). The results revealed that substitution of C₂ was most detrimental to the intramolecular RSE–3'CSS interaction, followed by C₃, (C₁, G₄), and finally A₅ (Fig. 7C). Thus, all residues appear to contribute to formation of the RSE–3'CSS interaction in the DI RNA, but they do so to different degrees.

Structure and function of the 5' half of region IV

The 3' half of RIV has been the subject of several studies (Fabian et al. 2003; Pogany et al. 2003; Na and White 2006). In contrast, very little is known about the structure or importance of the 5' half of RIV. Previously, nested-deletion analysis of RIV in a TBSV DI RNA revealed that removal of 16, 34, or 47 5'-proximal residues resulted in accumulation levels in protoplasts of 110%, 70%, and ~0%, respectively (Fig. 8A; Fabian et al. 2003). This result implicated the RIV sequence closest to SL3, i.e., residues 97–85, as being most important. Mfold analysis of this region in DI-72 suggested the possibility of an RIII–RIV interaction, termed S–Z (Fig. 8A). Based on the results from the deletion analysis, the lower stem was predicted to be most important in the proposed structure. To test this hypothesis, compensatory mutational analysis was carried out on the bottom stem (Fig. 8A). Mutant DI RNAs were

co-inoculated with helper TBSV genome into protoplasts, and DI RNA accumulation was quantified 22 h post-infection (Fig. 8B). Disruption of the predicted stem essentially abolished DI RNA accumulation, while its restoration led to ~60% recovery (Fig. 8B), confirming the importance of the predicted structure for DI RNA viability *in vivo*.

The bottom of S–Z is separated from SL3 by a single adenylate residue, A₈₃ (Fig. 1C). This close proximity suggested that S–Z could potentially influence the formation of the RSE–3'CSS interaction. To examine this possibility, DI RNAs containing the compensatory set of mutations in S–Z were tested for their ability to bind to HN123 *in vitro*. When S–Z was disrupted in mutants 3/4S1-A and 3/4S1-B, HN123 binding was increased compared to when S–Z was present or restored in wild-type DI-72 and mutant 3/4S1-C, respectively (Fig. 8C). The positive correlation between the formation of S–Z and the RSE–3'CSS interaction suggests that the former contributes to establishment of the latter.

Formation and importance of the RSE–3'CSS interaction in the TBSV genome

In previous studies, the RSE–3'CSS interaction was studied only in the simpler context of a DI RNA (Pogany et al. 2003; Na and White 2006). The results with the DI RNA prompted two questions: (1) Does this interaction also occur in the TBSV genome? (2) Is the interaction necessary for genome viability? To address the first question, an *in vitro* approach similar to that used to assess the RSE–3'CSS interaction in the DI RNA was used. Full-length transcripts of the wild-type genome or mutants containing substitutions at G₄ or C₅₇ (mutants G-250 and G-251, respectively) were tested for their ability to bind to HN123 (Fig. 9A). Consistent and statistically significant differences were observed (Fig. 9B), supporting formation of the RSE–3'CSS interaction in the TBSV genome.

Next, to determine if the interaction contributed to the accumulation of the TBSV genome *in vivo*, a full set of compensatory mutations involving C₅₇ and G₄ were analyzed in plant protoplasts. Infected protoplasts were incubated at 22°C for 22 h, after which the yield of virus genome was determined (Fig. 9C). G-250 showed dramatically reduced levels of accumulation to ~10% that of the wild-type genome. In contrast, G-251 and G-252 (double mutant) both accumulated relatively well, to ~60% and

FIGURE 6. Analysis of the RSE-3'CSS interaction in a DI RNA. (A) Unlabeled wild-type DI RNA and mutant derivatives. The shaded *inset* indicates that the RNA being analyzed is a full-length DI RNA. (B) Radiolabeled 3'-proximal wild-type viral RNA segment containing the 3'CSS element and a mutant derivative. (C) Analysis of RNA–RNA complex formation. Unlabeled DI RNA (2000 nM) was mixed with 32 P-labeled 3'-proximal fragment (4 nM). Following incubation, the mixture was separated in a nondenaturing acrylamide gel and the labeled products were detected and quantified by phosphor-imaging. The values correspond to means and the error bars represent standard deviation from three experiments.

75%, respectively (Fig. 9C). However, when the incubation temperature was increased to 28°C, only G-252 was able to accumulate notably, achieving a level of ~55% (Fig. 9D). Collectively, these results indicate that the RSE-3'CSS interaction also forms in the TBSV genome and is important for its viability.

DISCUSSION

Biochemical analyses were undertaken to provide physical evidence for a base-pairing interaction between the RSE and 3'CSS. The results indicate that this interaction does, indeed, occur and that it mediates the formation of a compact 3'-terminal RNA domain. When combined with other structural and *in vivo* data on adjacent upstream regions, the overall conformational organization for the 3' UTR of TBSV was deduced. These findings offer important new insights into virus genome structure and function in TBSV.

The RSE-3'CSS Interaction

Although previous genetic evidence suggested that the RSE-3'CSS interaction occurs (Pogany et al. 2003), there was no compelling physical evidence to verify this notion. By keeping the two key elements in their natural local contexts and positioning them in two separate RNAs, we were able to unambiguously show that formation of a stable bimolecular RNA–RNA complex is dependent on complementarity between the RSE and 3'CSS. Other approaches, using small RNA probes to assess intramolecular RSE-3'CSS interactions, provided persuasive evidence that the same interaction also occurs in both the DI RNA and TBSV genome. Collectively, these results build a strong case for the existence of the RSE-3'CSS interaction in various TBSV RNA contexts. Moreover, these physical findings validate the interpretation of previous genetic studies (Pogany et al. 2003), since mutations proposed to disrupt and then restore the interaction *in vivo* were confirmed to operate as predicted *in vitro*.

FIGURE 7. Contribution of individual base pairs to the RSE-3'CSS interaction. (A) Unlabeled wild-type DI RNA and mutant derivatives. The shaded *inset* indicates that the RNA being analyzed is a full-length DI RNA. (B) Radiolabeled 3'-proximal wild-type viral RNA segment containing the 3'CSS element. (C) RNA–RNA complex formation by nondenaturing gel electrophoresis was analyzed as described in the legend of Figure 6C. The 3'CSS is shown at the *top* along with five different substitutions (in gray), each of which corresponds to the column directly below it. The values correspond to means and error bars represent standard deviation from three experiments.

FIGURE 8. An RIII–RIV interaction contributes to DI RNA accumulation and 3' domain formation. (A) Sequence and Mfold-predicted RNA secondary structure of the 3' half of RIII and the 5' half of RIV in a DI RNA. The shaded inset indicates that the RNA being analyzed is a full-length DI RNA. An NsiI restriction enzyme site separates RIII from RIV and S-Z is the predicted stem formed by the RIII–RIV interaction. Previous deletion analysis of the 5' portion of RIV is indicated to the right by vertical lines and corresponding arrowheads (Fabian et al. 2003). The percentages shown in gray boxes represent the relative in vivo accumulation levels of DI RNAs containing the deletions. Compensatory mutations introduced into the DI RNA are indicated on the left. (B) DI RNAs containing compensatory mutations in S-Z were tested by coinfectioning each with TBSV helper genome (T100) into protoplasts. Following a 22-h infection at 22°C, DI RNA levels were determined by radio analytical quantification of Northern blots. The values provided represent means (with standard deviations) from three separate infections that were normalized to wild-type DI RNA levels (set at 100%). (C) DI RNA–HN123 complex formation analyzed by nondenaturing gel electrophoresis as described in the legend of Figure 6. The fraction bound for each RNA pair was quantified. The values correspond to means and the error bars represent standard deviation from three experiments.

Systematic mutational analysis of residues proposed to be involved in the interaction showed that all five of the predicted base pairs contribute to the formation of the interaction. However, substitutions disrupting middle base pairs were more detrimental, consistent with the general concept that internal disruptions of helices are more destabilizing. As each of the predicted pairs contributes to some degree to stability, the RSE–3'CSS interaction can be defined to involve all five base pairs. In addition to these key base pairs, the interaction was also shown to be influenced by the structural contexts of the partner sequences. Interestingly, secondary structure flanking the RSE (i.e., the apical stem of SL3) was important, while that involving the 3'CSS (i.e., SL1) was not. However, in protoplast infections, both of these mutations cause severe accumulation defects in DI RNAs (Na and White 2006). For the SL3 mutation, our in vitro results support the in vivo effect

being linked to a defect in formation of the RSE–3'CSS interaction. Conversely, the SL1 mutation appears not to be related to the RSE–3'CSS interaction, and thus must be linked to some other important function. A likely role for SL1 is as a key structural element of the promoter for negative-strand synthesis, as has been shown for TCV (Song and Simon 1995).

Previous in vivo genetic analysis of the C₅₇ and G₄ compensatory mutation set in the context of a DI RNA at 22°C led to significantly reduced levels for the single mutants (~2%), but only a modest recovery in accumulation for the double mutant (~18%) (Pogany et al. 2003). This left open the possibility that (1) the interaction was not reforming efficiently in the double mutant or/and (2) the identity of the RSE or/and 3'CSS was important. Our in vitro analyses of the double mutant interaction suggest that the RSE–3'CSS interaction does occur, but with reduced efficiency compared to the wild-type interaction (Figs. 4C, 6C). Thus, the inability of the DI RNA double mutant to attain wild-type levels of accumulation is likely due, at least

FIGURE 9. In vitro and in vivo analysis of the RSE–3'CSS interaction in the TBSV genome. (A) The 3' half of RIV in a TBSV genome. The shaded inset indicates that the RNA being analyzed is a full-length TBSV genome. Mutations that were introduced into this region are indicated. (B) Analysis of genome–HN123 complex formation by nondenaturing gel electrophoresis. Unlabeled genome (150 nM) was mixed with ³²P-labeled 3'-proximal fragment HN123 (4 nM). Following incubation, the mixture was separated in a nondenaturing acrylamide gel and the labeled products were detected and quantified by phosphor-imaging. The values correspond to means and the error bars represent standard deviation from eight experiments. The differences observed between all means were calculated to be statistically significant ($P \leq 0.01$) using the Student's *t*-test. (C) TBSV genomes containing the mutations shown in A were tested by protoplast infections (incubated at 22°C for 22 h). Genome levels were determined by radio analytical quantification of Northern blots. The values provided represent means (with standard deviations) from three separate infections that were normalized to the wild-type genome (T-100) (set at 100%). (D) Analysis as in C except that infected protoplasts were incubated at 28°C.

in part, to reduced stability of the RSE–3'CSS interaction. Nonetheless, some contribution for nucleotide identity to the *in vivo* defect still cannot be discounted.

Interestingly, when the same compensatory mutation set was introduced into the TBSV genome and tested at 22°C, relative accumulation levels for the single mutant G-251 (containing G₅₇) and the double mutant were significantly greater than those observed for their DI RNA counterparts. Even at the more stringent 28°C incubation temperature, the double mutant genome maintained ~55% accumulation level; however, that of the G-251 mutant was significantly depressed. The efficient accumulation of the G-251 mutant at 22°C (~60%) versus 28°C (~5%) is likely related to the stable formation of a noncanonical GG base pair at the lower temperature. Importantly, this temperature effect on G-251 convincingly demonstrates the necessity for pairing between the RSE and 3'CSS in a manner that is independent of nucleotide identity.

The *in vivo* compensatory analysis in the genome clearly established the importance of the RSE–3'CSS interaction for virus viability. The proportionally better recovery of the double mutant of the genome (~75%) versus that of the DI RNA (~18%) at 22°C (Pogany et al. 2003) may be due to the absence of competition in the former infection. In co-infections, the DI RNA must compete with its helper genome that contains a wild-type RSE–3'CSS interaction, whereas in genome-only infections no such heterologous competition exists. The consequence of this would be further “attenuation” of mutant DI RNA accumulation levels when compared with corresponding mutant genome levels.

Conformational organization of the TBSV 3' UTR

The 3' UTRs of RNA virus genomes often contain elaborate secondary and tertiary RNA structures involved in fundamental viral processes, such as RNA replication and protein translation (Dreher 1999; Kao et al. 2001; Dreher and Miller 2006; Kneller et al. 2006). Previous analysis of the TBSV genome revealed that R3.5 is a translation-specific region that forms a Y-shaped structure (Fig. 10; Fabian and White 2004, 2006). In the present study we have shown that the 81-nt 3'-terminal segment of the TBSV genome is able to fold into a compact or closed structure that is mediated by the RSE–3'CSS interaction. We propose that this segment represents a distinct domain in the 3' UTR, which we term the RSE–3'CSS domain. Formation of this domain must be transient, since it has to unfold to be copied into a complementary strand during negative-strand synthesis. This concept is in agreement with *in vivo* results, which indicated that the potential to form both conformations is essential (Fig. 9; Pogany et al. 2003; Na and White 2006). *In vitro*, disruption of the RSE–3'CSS interaction resulted in the generation of a more relaxed or open

FIGURE 10. Structural and functional organization of the 3' UTR of TBSV. Schematic representation of secondary and tertiary RNA structures in the 3' UTR of the TBSV genome. The 3' half of RIV that is important for replication is depicted in its default closed conformation (dashed oval). A crucial region of the long-distance interaction between the 3' half of RIII and the 5' half of RIV (i.e., S-Z) is delineated by the dotted rectangle. Translation-specific R3.5 is bounded by a solid line rectangle with SL-B indicated by a black box. The two opposing gradients indicate the general importance, with respect to replication (Fabian et al. 2003; this study) and translation (Fabian and White 2006), of different parts of S-Z based on the analysis of mutants with modifications in these regions. However, it cannot be precluded that some of the defects observed may have been caused by general, rather than local, structural effects.

structure. Therefore, this tertiary interaction is central to the folding and stability of this domain, and could represent a key element controlling conformational conversions. Indeed, access to the 3'CSS (where RNA synthesis initiates) would be an early requirement for negative-strand synthesis, which in turn would concomitantly lead to disassembly of the closed conformation.

According to our results, the closed conformer appears to be the default structure in both the DI RNA and the TBSV genome. This contrasts the results from the structural analysis of TCV satellite RNA C (SatC) where the corresponding RSE–3'CSS interaction did not represent the default conformation (Zhang et al. 2006a). For SatC, it was proposed that a preactive conformation exists that must be converted to include the RSE–3'CSS interaction before replication can occur (Zhang et al. 2006a). A corresponding RSE–3'CSS interaction in the TCV genome has also been found to be important for virus viability; however, it is possible that the preactive conformation in SatC is unique to this subviral RNA and does not extend to its helper genome (Zhang et al. 2006b). Based on our results with TBSV, the closed structure is likely to be the conformation present in invading and newly synthesized viral genomes. As previously suggested, this “default” structure could help to protect the nonpolyadenylated 3' end of the genome from exonucleases (Pogany et al. 2003).

Additionally, this structure was recently shown to be important for formation of tombusvirus replication complexes (Panaviene et al. 2005). Thus, the closed default structure may be important for both genome protection and early replication events. However, eventually conversion to the open conformation would be required to allow negative-strand synthesis to occur. Indeed, the confines of the replication complex, an environment that is apt to be free of cellular exonucleases, could provide a safe locale for unmasking of the 3' end. Exactly how the structure would be converted from a closed to open conformation remains unknown. Disruption of the RSE–3'CSS interaction could involve an RNA helicase or chaperone function provided by either viral or host proteins. Regardless of the source of such activity, it would need to be well coordinated so as to occur at the proper location and time in the replication process.

Our study has also uncovered the structure and a function of the 5' half of RIV. The data indicate that the 5' half of RIV interacts with the 3' portion of RIII and that this interregion interaction facilitates DI RNA accumulation *in vivo*. Interestingly, the extended stem formed, S-Z, was recently shown also to be important for translation of TBSV proteins (Fabian and White 2006). However, for translation the upper portion of S-Z was found to be more relevant, which contrasts the requirement for DI RNA accumulation where the lower portion was more significant (Fig. 8). For translation, it was proposed that the upper portion of S-Z acts to stabilize the formation of R3.5, which is critical for translation (Fabian and White 2006). We have shown that the lower portion of S-Z facilitates DI RNA accumulation in protoplasts and also the formation of the RSE–3'CSS domain *in vitro*. Therefore, the *in vivo* defects in DI RNA accumulation observed are likely due, at least in part, to reduced ability to form the RSE–3'CSS interaction. S-Z formation could act to prevent misfolding or stabilize the RSE–3'CSS domain once formed, which in turn would facilitate replication complex formation and/or 3' end protection (Pogany et al. 2003; Panaviene et al. 2005). What is interesting is that S-Z appears to be a polar dual-function element that is increasingly important for translation in its upper region and replication in its lower portion (Fig. 10). Accordingly, different selection pressures would act at either end to maintain this extended helical structure in the viral genome. Incorporating the RIII–RIV interaction with other structures in the 3' UTR provides a more complete conformational picture and reveals a high degree of integration of replication and translation elements (Fig. 10). Moreover, the structural organization depicted in Figure 10 is also predicted by Mfold to exist in some of the other genera in Tombusviridae (e.g., Necroviruses and Panicoviruses; data not shown). Thus, this general organization likely extends to other related viruses and probably represents an evolutionary remnant derived from a common progenitor of this virus family.

MATERIALS AND METHODS

Preparation of RNA templates

Construction of the full-length TBSV genome, T100 (Hearne et al. 1990) and DI-72SXP (Fabian et al. 2003) have been described previously. Mutations were introduced into PCR products by oligonucleotide-mediated PCR mutagenesis using T100 or DI-72SXP templates and incorporated into plasmids using restriction enzyme-based methods (Sambrook et al. 1989). The oligonucleotides used in this study are listed in Table 1. To generate the small 3'-proximal viral RNA segments, corresponding PCR products were cloned between the *SacI* and *SphI* restriction sites of Puc19. To construct DI RNA clones, the *NsiI* and *SphI* sites flanking RIV in DI-72SXP were used for exchange with PCR products as described previously (Fabian et al. 2003). To construct TBSV genome mutants, modified PCR fragments containing *SalI* and *SphI* restriction sites were used to replace the corresponding 3'-proximal fragment in wild-type T100. All PCR-derived regions introduced into constructs were sequenced completely to ensure that only the desired modification was present.

The small segments of viral RNA corresponding to SL1, 2, and 3 of RIV, i.e., HN52–1, HN52–3, HN246, HN254, HN255, and HN256, were constructed using primer pairs PHN52–1/P9, PHN52–1/PMF34, PHN52–1/PHN132, PHN52–1/PHN191, PHN194/P9, and PHN194/PHN191, respectively (Table 1). Small RNA segments containing SL3 of RIV, i.e., HN122, HN242, and HN162, were constructed using primer pairs PHN52–1/PHN52–2, PHN134/PHN186a, and PHN145/PHN146, respectively. Small RNA segments containing SL1 and 2 of RIV, i.e., HN123, HN147, HN179, and HN266, were constructed using primer pairs PHN110X/P9, PHN110X/PHN132, PHN110X/PMF61, and PHN110X/PHN86, respectively.

DI RNA mutants DI-147, DI-149, DI-61, DI-62, and DI-63 were constructed using primer pairs PR21/PHN132, PHN133/P9, PR21/PHN191, PR21/PHN192, and PR21/PHN193, respectively. DI RNAs 3/4S1-A, 3/4S1-B, and 3/4S1-C were constructed using three-part blunt-end ligation strategy as described previously (Fabian et al. 2003). For 3/4S1-A, two inserted fragments obtained from primer pairs 5'-PstI-III/III-IV-1 and III-IV-2/M13R were digested with *PstI* and *HindIII*, respectively. For 3/4S1-B, two fragments obtained from primer pairs 5'-PstI-III/III-IV-3 and III-IV-4/M13R were digested with *PstI* and *HindIII*, respectively. For 3/4S1-C, clone 3/4S1-B was used as template for two primer pairs 5'-PstI-III/III-IV-1 and III-IV-2/M13R to produce two PCR fragments that were digested by *PstI* and *HindIII*, respectively. DI-259, DI-260, DI-261, DI-262, and DI-263 RNA templates were obtained directly from amplified PCR products using primer pairs PF7/PHN195, PF7/PHN196, PF7/PHN197, PF7/PHN198, and PF7/PHN199, respectively.

Genome mutants G-250, G-251, and G-252 were constructed using primer pairs PHN188/PHN191, PHN188/PHN192, and PHN188/PHN193, respectively.

In vitro transcription and purification

Viral RNAs were generated by *in vitro* transcription using T7 RNA polymerase as described previously (White and Morris 1994). The small segment RNAs used in binding analysis were gel purified. The DI RNAs and genome RNAs were purified using MicroSpin

TABLE 1. Oligonucleotides used in this study

Oligo	Position ^a	RE ^b site	Sequence ^c	Sense ^d
PF7	0001–0021	SacI	5-GGCG <u>GAGCTC</u> TAATACGACTCACTATAGGAAATTCCTCAGGATTCTC	+
P9	4746–4776	SphI/SmaI	5-GGCGGCCC <u>GCATGCCC</u> GGGCTGCATTCTGCAATGTTCC	–
PR21	4645–4668	NsiI	5'-CCGCGC <u>ATGCAT</u> TATTCCTGTTACGAAAGTTAGG	+
PR36	4437–4467		5'-GTAACCTGTATGCTATGCCAGATTACACTCATCTCCTGCA	–
PHN52–1	4695–4729	SacI	5'-GGCG <u>GAGCTC</u> TAATACGACTCACTATAGGTTGATCTCACCCCTTCGGG GGGGCTATAGAGATC	+
PHN52–2	4706–4733	SphI/NaeI	5'-GGCGGCCC <u>GCATGCCC</u> GGGCTGCTATAGCCCCCGAAGGGT	–
PHN86	4746–4776	SphI/SmaI	5'-GGCGGCCC <u>GCATGCCC</u> GGGCTGATTCTGCAATGTTCCGGTGTCC	–
PHN110X	4733–4752	SacI	5'-GGCG <u>GAGCTC</u> TAATACGACTCACTATAGGAAGCACTACCGGACAACC	+
PHN132	4752–4776	SphI/SfiI	5'-GGCGGCCC <u>GCATGCCC</u> GGGCTGCATTCTGCAATGTTCCGG	–
PHN133	4691–4743		5'-GACACGGTTGATCTCACCCCTTCGGGGGCCCTATA GAGATCGCTGGAAGCACTA	+
PHN134	4695–4715	SacI	5'-GGCG <u>GAGCTC</u> TAATACGACTCACTATAGGTTGATCTCACCCCTTCGGGG	+
PHN145	4695–4725	SacI	5'-GGCG <u>GAGCTC</u> TAATACGACTCACTATAGGTTGATCTCACCCCTTC GGAGGGGCTATAT	+
PHN146	4706–4734	SphI/NaeI	5'-GGCGGCCC <u>GCATGCCC</u> GGGCTGCTATAGCCCCCTCCGAAGGGTG	–
PH N186a	4706–4733	SphI/NaeI	5'-GGCGGCCC <u>GCATGCCC</u> GGGCTGCTATACCCCCCGAAGGGT	–
PHN191	4758–4776	SphI/SmaI	5'-GGCGGCCC <u>GCATGCCC</u> GGGCTGCATTCTGCAATG	–
PHN192	4706–4776	SphI/SmaI	5'-CCGCGC <u>GCATGCCC</u> GGGCTGCATTCTGCAATGTTCCGG TTGTCCGGTAGTGCTCCAGCGATCTCTATACCCCCCGAAGGG	–
PHN193	4706–4776	SphI/SmaI	5'-CCGCGC <u>GCATGCCC</u> GGGCTGCATTCTGCAATGTTCCGGTGTG CGGTAGTGCTTCAGCGATCTCTATACCCCCCGAAGGG	–
PHN194	4695–4729	SacI	5'-GGCG <u>GAGCTC</u> TAATACGACTC ACTATAGGTTGATCTCACCCCTTCGGGGGGGTATAGAGATC	–
PHN195	4752–4776		5'-CGGCTGCATTCTGCAATGTTCCGG	–
PHN196	4752–4776		5'-CGGCTGCATTCTGCAATGTTCCGG	–
PHN197	4752–4776		5'-GGCCTGCATTCTGCAATGTTCCGG	–
PHN198	4752–4776		5'-GGGGTGCATTCTGCAATGTTCCGG	–
PHN199	4749–4776		5'-GGGAGCATTCTGCTATGTTCCGGTTG	–
PMF34	4764–4776	PvuII/SphI	5'-GGCGGCCC <u>GCATGCCC</u> AGCTGCATTCTGC	–
III-IV-1	4399–4419		5'-TGAAGAGTCTGTCTTACTCGC	–
III-IV-2	4420–4452		5'-GTGTGAGAACTGGAGATGAGTGTAATCTGGC	+
III-IV-3	4667–4683		5'-TTCCACAAGTGACACC	–
III-IV-4	4683–4711		5'-GGTTTCCACACACGGTTGATCTCACCC	+
5'-PstI-III	4398–4415	PstI	5'-GGCGCCCGC <u>CTGCAGAGCGAGTAAGACAGACTC</u>	+
M13R			5'-AGCGGATAACAATTTACACAGGA	–

^aCoordinates refer to those of the TBSV genome (Hearne et al. 1990).^bRestriction enzyme.^cViral sequences are underlined and correspond to the coordinates shown. Italicized and bold sequences represent restriction site(s), the identities of which are indicated.^dRefers to the sense of the oligonucleotide in references to the plus-sense viral RNA.

G-50 columns (Amersham Bioscience Inc). Purified RNAs were quantified spectrophotometrically and their integrity was verified by electrophoresis in agarose gels.

Intramolecular RNA conformational analysis by gel electrophoresis

HN52–1, HN246, HN52–3, HN254, HN255, and HN256 RNAs were dephosphorylated by treatment with calf intestinal phosphatase and then 5'-end labeled using γ -P32-ATP and polynucleotide kinase (Sambrook et al. 1989). γ -P32 labeled RNA was precipitated with 2.5 μ g yeast RNA and finally dissolved in RNA binding reaction buffer. The RNA binding reaction buffer contained 5 mM HEPES, pH 7.8, 100 mM KCl, 3.8% glycerol, 6 mM MgCl₂ (Alvarez et al. 2005). Labeled RNAs (20 nM) were incubated at 80°C for 3 min, slow cooled to room temperature,

and then placed on ice. One microliter of labeled RNA (20 nM) was mixed with 4 μ L RNA-binding buffer and 3 μ L loading buffer (composed of an equal volumes of RNA-binding buffer and glycerol) and then separated in 15% acrylamide gels under denaturing (8M urea) or nondenaturing conditions. Electrophoresis for nondenaturing acrylamide gel was carried out in standard 1 $\times$ Tris-borate buffer supplemented with 6 mM MgCl₂, and was performed at 4°C. Gels were subsequently analyzed by radioanalytical scanning using a Pharos FX plus Molecular Imager (BIO-RAD Laboratories Inc.).

RNA–RNA gel mobility shift assay

Small segment RNAs were dephosphorylated and 5'-end labeled using γ -P32-ATP as described above. Labeled RNAs were also incubated at 80°C for 3 min and then immediately cooled on ice

for 5 min. For small–small or small–DI RNA interactions, 1 μ L of labeled small RNA (20 nM) was incubated with 4 μ L (12,000 nM) unlabeled small RNA or 4 μ L (2000 nM) DI RNA in RNA-binding reaction buffer at 25°C for 30 min and then incubated at 4°C for 10 min. For small–genome interactions, 1 μ L labeled RNA (20 nM) was incubated with 4 μ L (150 nM) unlabeled genome RNA in RNA-binding reaction buffer at 25°C for 13 h followed by 4°C for 10 min. Following these incubations, samples were mixed with 3 μ L loading buffer and separated in a nondenaturing 15% polyacrylamide gel (supplemented with 5% glycerol and 6 mM MgCl_2) at 150 V for 3 h, which was prerun for 30 min at 4°C at 150 V. Gels were subsequently analyzed by radioanalytical scanning using a Pharos FX plus Molecular Imager (BIO-RAD Laboratories Inc.) and the values are presented with standard deviations from three to eight experiments.

Protoplast inoculation and viral RNA analysis

Protoplasts were prepared from 6- to 7-d-old cucumber cotyledons (var. Straight 8) and inoculated with viral RNA transcripts using polyethylene glycol (PEG)- CaCl_2 as described previously (White and Morris 1994). Approximately 3 μ g T100-derived genome transcripts or 1 μ g of DI-72-derived transcripts mixed with 3 μ g of T100 TBSV wild-type genome helper virus were used for inoculation and were incubated in a growth chamber under fluorescent lighting at 22°C or 28°C for 22 h. Total nucleic acid was harvested from protoplasts as described previously (White and Morris 1994). For DI RNA analysis, aliquots of the total nucleic acid (a fifth) was separated in denaturing 4.5% polyacrylamide gels containing 8 M urea. For genome RNA analysis, one-fifth of the total nucleic acid preparation was separated in 1.4% agarose gels. Northern blot analysis of viral RNA accumulation was conducted by transferring total nucleic acids to nylon, followed by hybridization with a ^{32}P -end-labeled PR36 oligonucleotide probe (Fabian et al. 2003). The hybridization signals were quantified by radioanalytical scanning using a Pharos FX plus Molecular Imager (BIO-RAD Laboratories Inc.) and the values are presented with standard deviations from three independent experiments.

RNA secondary structure analysis

RNA secondary structure in this study was predicted by Mfold version 3.2 (Mathews et al. 1999; Zuker et al. 1999).

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