The p92 Polymerase Coding Region Contains an Internal RNA Element Required at an Early Step in Tombusvirus Genome Replication

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The replication of positive-strand RNA viral genomes involves various cis-acting RNA sequences. Generally, regulatory RNA sequences are present at or near genomic termini; however, internal replication elements (IREs) also exist. Here we report the structural and functional characterization of an IRE present in the readthrough portion of the p92 polymerase gene of Tomato bushy stunt virus. Analysis of this element in the context of a noncoding defective interfering RNA revealed a functional core structure composed of two noncontiguous segments of sequence that interact with each other to form an extended helical conformation. IRE activity required maintenance of several base-paired sections as well as two distinct structural features: (i) a short, highly conserved segment that can potentially form two different and mutually exclusive structures and (ii) an internal loop that contains a critical CC mismatch. The IRE was also shown to play an essential role within the context of the viral genome. In vivo analysis with novel RNA-based temperature-sensitive genomic mutants and translationally active subgenomic viral replicons revealed the following about the IRE: (i) it is active in the positive strand, (ii) it is dispensable late in the viral RNA replication process, and (iii) it is functionally inhibited by active translation over its sequence. Together, these results suggest that IRE activity is required in the cytosol at an early step in the viral replication process, such as template recruitment and/or replicase complex assembly.

Positive-strand RNA viruses replicate their genomes via a negative-strand RNA intermediate (2). This process is catalyzed by a virally encoded RNA-dependent RNA polymerase that is the core subunit of a viral replicase complex. Replicase activity is regulated in part by that is the core subunit of a viral replicase complex. Replicase is functionally inhibited by active translation over its sequence. Together, these results suggest that IRE activity is required in the cytosol at an early step in the viral replication process, such as template recruitment and/or replicase complex assembly.

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TBSV DI RNAs contain 5’ and 3’ terminal segments from the genome, termed regions I and IV (RI and RIV, respectively) (34) (Fig. 1B). RI is important but not essential for efficient DI RNA replication (21, 27, 30, 38, 39), while RIV is critical (8, 17, 24). In addition to these terminal regions, two noncontiguous internal segments, RII and RIII, are present in DI RNAs (Fig. 1B). In vivo and in vitro studies on RIII have revealed that it functions in the negative strand and acts to promote efficient positive-strand synthesis (20, 28, 29). In contrast, no detailed information on RII structure and function has been reported. Nonetheless, RII is known to be essential since various deletions or inversion of the entire RII segment abolish DI RNA accumulation in vivo (3, 10, 35). It is unclear whether the critical role played by RII in DI RNAs also extends to the TBSV genome, where it maps to the readthrough portion of the p92 open reading frame (ORF) (Fig. 1A). If RII does function in this genomic context, its activity would need to be integrated with that of translation.

In this paper, we describe the detailed analysis of RII in the context of a TBSV DI RNA. A core functional structure for RII was established, and various properties important for its activity were determined. RII was also found to operate in the context of the TBSV genome, defining it as an internally positioned cis-acting RNA replication element that functions...
within a translationally active genomic sequence. Since RII activity was required only at early stages of viral infection, a role for this RNA element in template recruitment and/or replicase complex assembly is proposed.

**MATERIALS AND METHODS**

**Viral constructs.** Construction of full-length TBSV genome, T100 (11), DI-82, DI-83 (36), and DI-72SXP (38) have been described previously. Mutant D22-1, containing 5′, 3′, and central deletions, was generated from DI-72SXP by using standard oligonucleotide-mediated PCR mutagenesis. The basic structure of the truncated RII, containing XbaI, BamHI, and PstI sites, is shown in Fig. 1C and 2A. All other mutant derivatives of D22-1 (i.e., the SM series and WX series) were generated by PCR mutagenesis, where the modified PCR fragments were introduced into their respective constructs by using the restriction enzyme sites XbaI, BamHI, and PstI described above. Mutant series SM32 derived from DI-72SXP, VII1 series derived from T100, and the DI-82 and DI-83 derivatives were also generated by using standard PCR-based cloning strategies. The type and position of the modifications introduced into each construct are indicated in the figures depicting the mutants. For all constructs, the entire PCR-derived regions inserted were sequenced completely to ensure that only the desired mutations were present.

**In vitro transcription.** Viral transcripts were synthesized in vitro by using Smal-linearized DNA templates and an Ampliscribe T7 RNA polymerase transcription kit (Epitecten Technologies) as described previously (36). The concentrations of RNA transcripts were determined spectrophotometrically, and RNA integrity was verified by agarose gel electrophoresis.

**Plant infections.** Chenopodium quinoa plants (30 days old) were inoculated with wild-type (wt) or mutant TBSV genomic RNA transcripts. Three leaves were inoculated (5 ng/leaf) on each plant, and three plants were used for each construct. The numbers of local lesions were counted at 4 days postinoculation. Protoplasts were inoculated with uncapped viral RNA transcripts (1 μg for DI RNAs and 3 μg for genomic T100 transcripts) by using polyethylene glycol-CaCl2 and were incubated in a growth chamber under fluorescent lighting at 22°C (unless otherwise noted) for the specified time periods.

**Analysis of viral RNA.** For DI RNA analysis, total nucleic acids were harvested from protoplasts as described previously (36). One-fifth of the total nucleic acid preparation was separated in denaturing 4.5% polyacrylamide gels containing 8 M urea. For analysis of genomic RNA accumulation, total nucleic acids harvested from protoplasts were denatured with glyoxal and separated in 1% agarose gels (5). All gels were stained with ethidium bromide and inspected to ensure even loading of samples. Northern blot analysis of viral RNA accumulation was conducted by transferring total nucleic acids to nylon, followed by hybridization with either negative- or positive-sense 32P-end-labeled oligonucleotide probes (28) or riboprobe (5), respectively, to detect positive or negative strands. The hybridization signals for either the viral genome or DI RNAs were quantified by radioanalytical scanning with an InstantImager (Packard Instrument Co.), and the values are presented with standard errors from three independent experiments.

**RNA secondary structure analysis.** For in vitro analysis of RNA secondary structure, D22-1 transcripts (3 μg) were added to Torula yeast RNA (3 μg) in modification buffer (25 mM Tris-Cl [pH 7.5], 200 mM NaCl, 5 mM MgCl2, and 1 mM EDTA), equilibrated (95°C for 2 min, 60°C for 10 min, and 37°C for 10 min), treated with various RNA structure-probing chemicals [diethyl pyrocarbonate (DEPC) and 1-cyclohexyl, 3-(2-morpholinoethyl)carbodiimide (CMCT)] or enzymes (RNase T1 and RNase V1), and analyzed by primer extension as described previously (38). RNA secondary structures were predicted at 37°C by using mfold version 3.1 (14, 42).

**RESULTS**

**A truncated RII core element is functional.** In a typical TBSV DI RNA, such as DI-72SXP (Fig. 1B), RII is 239 nucleotides (nt) in length (34). However, previous deletion anal-
yses of RIIs in DI RNAs from different tombusviruses, including TBSV, suggested that not all of these nucleotides are necessary for function (3, 10). Collectively, these results indicated that both terminal and central regions of RII were dispensable (3, 10). Based on these findings, we designed and constructed a DI-72SXP derivative, D22-1, in which 5′ (74 nt), 3′ (71 nt), and central (28 nt) portions of RII were deleted, leaving two noncontiguous segments, each 33 nt long (Fig. 1C).

A BamHI restriction enzyme site was substituted in place of the deleted central segment, and XbaI and PstI sites were introduced at the 5′ and 3′ borders, respectively. When D22-1 was tested for biological activity by coinoculating it with the TBSV genome (T100) into protoplasts, it accumulated to ~80% the level of wt DI-72SXP (Fig. 1D). This result confirmed the dispensability of most of the original 239-nt-long RII sequence and demonstrated that the two noncontiguous 33-nt-long segments were sufficient for productive DI RNA accumulation. D22-1, containing this active RII core element, was used for more detailed structure and function analyses.

Structure of the RII core. The mfold analysis of the RII core sequence suggested extensive base pairing between the two 33-nt segments (Fig. 2A) (14, 42). This same interaction was also predicted to occur when the original 239-nt-long RII was analyzed by mfold (data not shown). In the latter case, the deleted central sequence formed an apical hairpin that would facilitate formation of the lower helical regions (Fig. 2B).
RII core sequence is predicted to form an extended helix that is interrupted by three unpaired segments. The four stem (S) and three loop (L) elements in this structure are labeled numerically, starting from its base (Fig. 2A). The terminal loop and its closing base pair in this core structure correspond to the introduced BamHI site (Fig. 2A).

Solution structure probing was performed on the RII core sequence present in D22-1 in an attempt to acquire support for the extended stem-loop structure predicted by mfold (Fig. 2C). The results from this analysis were mapped onto the mfold-determined RII core structure and were found to be in general agreement with the proposed arrangement (Fig. 2A). RNase V1, which targets double-stranded and stacked regions, cleaved primarily in the positions predicted to be helical (Fig. 2A and C). In contrast, the single-strand-specific enzyme (RNase T1) and chemicals (DEPC and CMCT) reacted mostly with residues determined by mfold to be unpaired (Fig. 2A and C). The proposed secondary structure model of the RII core is, therefore, consistent with the results of this analysis (Fig. 2A).

To gain further insight into the RII core structure, corresponding RII sequences from other tombusviruses were examined. Comparative sequence analysis revealed a number of interesting features about this RNA element (Fig. 3): (i) the primarily helical nature of the RII core was well supported by the numerous mono- and covariations that maintained base-paired segments; (ii) several of the residues in the internal loop regions, as well as S2, were strongly conserved in all species; and (iii) the presence of several GU base pairs in the TBSV structure, and in different positions in other tombusviruses, suggested that the element forms and functions in the positive strand (as GU base pairs would correspond to disruptive CA pairs in the minus strand). Overall, this analysis supports the existence of the basic structural framework of the TBSV RII core in all tombusvirus species examined and suggests a common and important function for it in members of this genus.

**Functional analysis of the RII core in D22-1.** Mutational analysis of the RII core in D22-1 was performed to identify structural properties important for its activity. Initially, the role
of each of the four stem regions was investigated by using a compensatory mutational strategy that first disrupted and then restored the helices (Fig. 4A). The mutants (series SM24, SM1, SM28, SM29, SM30, SM20, and SM31) were tested by coinoculating each along with the TBSV genome into protoplasts and then quantifying their accumulation levels relative to wt D22-1. A clear correlation between disruption of base pairing and a decrease in DI RNA accumulation was observed for all four stems (Fig. 4A). In contrast, the restoration of base pairing allowed for nearly wt levels of recovery for S1, S3, and S4 but not S2 (Fig. 4A). This indicates that maintaining the helical nature of S1, S3, and S4 is essential for RII core function, whereas formation of S2 does not seem to be required. Additional mutational analysis revealed that the critical C1384G1426 base pair at the bottom of S1 needs to form in the positive strand, because converting it to a U1384G1426 (SM25) allowed for ∼42% accumulation, while changing it to C1384A1426 (SM26) essentially abolished accumulation (Fig. 4A). This result supports the earlier proposal, based on comparative sequence analysis, that the RII element functions in the positive strand.

Based on compensatory mutational analysis, S2 was not necessary for RII function (Fig. 4A, SM30 series). However, closer examination of the sequences in the S2 region revealed a possible alternative base pairing scheme. The alternative stem, S2A, involved a 1-nt shift in base pairing partners relative to S2 and included one additional base pair (Fig. 4B). Formation of S2A would change L2 into a bulge (B2) and the asymmetrical internal loop L1 into a symmetrical internal loop (L1A) (Fig. 4B). This alternative conformation was not predicted by mfold in any of its computed suboptimal structures. However, based on comparative sequence analysis (Fig. 3), S2A could potentially form in all the tombusviruses examined. Furthermore, the S2A structure in TBSV was also compatible with our solution structure probing data (Fig. 2A). In order to address its possible structural significance, S2A was disrupted and then restored in mutant series SM36 (Fig. 4B). All modifications in S2A led to drastically reduced DI RNA accumulation; thus, like S2 formation, S2A formation is not sufficient to confer function to the RII core.

In addition to the S2/S2A region, another section of interest was L3. The high level of conservation of a number of residues in this internal loop hinted that it could represent a specificity determinant. Interestingly, the comparative sequence analysis suggested that in Cucumber Bulgarian latent virus (CBLV) most of L3 is paired (Fig. 3). This alternative organization would be mediated by the A1380G1381 to U1380U1381 substitutions in CBLV that would cause more extensive base pairing in L4 (Fig.
3). To test whether such increased base pairing would also be tolerated in L3 of TBSV, A\textsubscript{1380}G\textsubscript{1381} to U\textsubscript{1380}U\textsubscript{1381} substitutions were introduced into D22-1, creating WX1 (Fig. 4B). This mutant accumulated to ~60% the level of wt, indicating that the lower portion of L4 can be base paired and still maintain a considerable level of function. Thus, the residues in question do not have to exist as mismatches and could possibly base pair via non-Watson-Crick interactions in the wt structure (i.e., A\textsubscript{1380} with A\textsubscript{1430} and G\textsubscript{1381} with A\textsubscript{1429}) (Fig. 3).

The viability of WX1, containing only a C\textsubscript{1383}C\textsubscript{1427} mismatch in L4, suggested that this mismatch may be a key structural determinant in L4. This concept was tested by converting C\textsubscript{1383}C\textsubscript{1427} to either a G\textsubscript{1383}C\textsubscript{1427} base pair or an A\textsubscript{1383}C\textsubscript{1427} mismatch in WX4 and WX5, respectively (Fig. 4B). Both mutants were unable to accumulate to detectable levels, implicating the C\textsubscript{1383}C\textsubscript{1427} mismatch as an essential element for function (Fig. 4B). The C\textsubscript{1383}G\textsubscript{1427} base pair located directly above the C\textsubscript{1383}C\textsubscript{1427} mismatch was shown to be important for RII activity (Fig. 4A, mutant series SM1). Additionally, formation of the base pair immediately below the C\textsubscript{1383}C\textsubscript{1427} mismatch is also likely important, since mutations that disrupt it (SM2a and SM27) inhibited efficient DI RNA accumulation (Fig. 4B). A compensatory mutant for SM2a and SM27 was not tested as transposition of the residues involved (i.e., C\textsubscript{1382} and G\textsubscript{1426}) is predicted by mfold to promote a misfolded structure that would disrupt the CC mismatch (i.e., G\textsubscript{1381}G\textsubscript{1382} would base pair with C\textsubscript{1427}C\textsubscript{1428}). Collectively, these results suggest that CG base pairs flank the crucial C\textsubscript{1383}C\textsubscript{1427} mismatch and act to arrange it in a functionally relevant configuration.

**RII functions in the viral genome.** Having demonstrated the importance of the RII core in a DI RNA, we next sought to investigate its possible function in the TBSV genome. In these experiments the additional coding role of the RII sequence needed to be factored into the design of mutants. Before testing RII function in the genome, wobble positions mapping to S1 were substituted in DI-72SXP (Fig. 5A). S1 was chosen as the target because it was shown to be important for DI RNA accumulation (Fig. 4A), and it was the only helix in the RII core into which compensatory mutations could be introduced at directly opposing wobble positions (Fig. 5A). S1 was chosen as the target because it was shown to be important for DI RNA accumulation (Fig. 4A), and it was the only helix in the RII core into which compensatory mutations could be introduced at directly opposing wobble positions (Fig. 5A). DI-72SXP was used instead of D22-1 because its larger 239-nt RII more closely resembled the RII core context in the genome (Fig. 1). We reasoned that if DI-72SXP accumulation was affected by these modifications at wobble positions in S1, then the same mutations would probably have some effect if introduced into the genome. The SM32 series of compensatory mutations in
DI-72SXP were tested by coinoculating them with T100 into protoplasts, and the desired loss and recovery of DI RNA accumulation were observed (Fig. 5B). When negative strands were monitored, they were found to accumulate to similar relative levels as their positive-strand counterparts (Fig. 5B). Additionally, no significant differences in RNA decay rates were observed when in vivo stability assays were performed on these DI RNAs (data not shown).

These same compensatory mutations in S1, which were silent with respect to p92 coding, were then introduced into the TBSV genome to generate the VII1 genomic series (Fig. 5A). The effects of these modifications were first tested by whole plant infections of the local lesion host plant C. quinoa. As-saying lesion numbers on this host provides a quantitative evaluation of any effects on virus infectivity. When inoculated onto the leaves of this host plant, mutants VII1a and VII1b (containing the S1 disruptions) did not induce any lesions, while VII1c (with S1 restored) caused lesions at levels greater than wt levels (Fig. 5C). These results demonstrate that RII is clearly functional in the viral genome, because it is critical for survival of the virus in a host plant.

Next, the genomic VII1 mutant series was tested in protoplasts (Fig. 5D). At 22°C, the disruptive substitutions in mutants a and b had surprisingly little effect on genome accumulation in comparison to their effect on DI-72SXP levels (compare Fig. 5D, left panel, with Fig. 5B). However, when the temperature was raised to 28°C, dramatic decreases in accumulation were observed for the disruptive a and b genomic mutants for both positive and negative strands, while VII1c (with S1 restored) caused lesions at levels greater than wt levels (Fig. 5C). These results demonstrate that RII is clearly functional in the viral genome, because it is critical for survival of the virus in a host plant.

**RII function is required at an early step in viral genome replication.** Temperature shift assays were carried out by using genomic mutant VII1a. When VII1a was incubated at 22°C for 22 h, it accumulated to ~104% of wt genome levels, while incubation at 28°C for 22 h resulted in an accumulation equivalent to only ~7% that of wt (Fig. 5E). However, when VII1a was incubated at 22°C for either 7 or 15 h, followed by a shift to 28°C for the remaining time, there was partial (~45%) and full (~112%) recovery of accumulation, respectively (in comparison to incubation at 22°C only for 22 h) (Fig. 5E). Thus, there was significant genome replication at the nonpermissive temperature if it was preceded by incubation at the permissive temperature. Similar results were also observed when the same temperature shift experiment was carried out with VII1b (data not shown). Also, there was no significant reversion during the 22°C preincubations in these experiments as the progeny generated were still fully temperature sensitive when they were inoculated into a new set of protoplasts and incubated at 28°C (data not shown). Furthermore, the permissive temperature is not predicted to be selective for revertants as the mutants replicated at wt rates at 22°C. Therefore, the “recovered” phenotypes observed for the a and b mutants in the temperature shift experiments suggest that RII functions primarily at an early step in genome accumulation and that its activity is not required at later stages of the infection.

**RII activity is inhibited by translation.** RII is located in a translationally active part of the TBSV genome, the readthrough portion of the p92 coding region. During infections both p33 and p92 are translated directly from the viral genome. Our finding that RII functions in the genomic context suggests that its activity may need to be coordinated with that of translation. Alternatively, it is possible that RII functions at a stage in the infection that does not overlap either temporally or spatially with translation.

Both DI-72SXP and D22-1 are not translationally active because they lack R3.5, the sequence between RII and RIV, which is an essential component of the viral 3′ cap-independent translational enhancer (9, 19, 40). Consequently, larger genome-derived RNA replicons were used to study the relationship between RII and translation. DI-82 and DI-83 are well-characterized genomic deletion mutants that encode p33; however, only DI-83 (which contains R3.5) is able to template the translation of p33 (9, 19, 36, 40) (Fig. 6). Both the translationally active DI-83 and translationally inactive DI-82 replicate and accumulate well in coinfections with the wt TBSV genome (19, 36) (Fig. 6). To determine if efficient translation over the RII sequence would inhibit its function, the stop codon for p33 was converted to a tyrosine in both DI-82 and DI-83, creating DI-82Y and DI-83Y, respectively (Fig. 6). We predicted that extending the p33 ORF through RII would have a significant negative effect on the accumulation of the translationally active DI-83 but little or no effect on the translationally inactive DI-82. Consistent with this prediction, coinfections with T100 revealed that DI-83Y accumulated poorly, while DI-82Y accumulated efficiently (Fig. 6).

In addition to RII, the extended p33 ORF in DI-83Y included the sequence between the introduced tyrosine codon and RII and also a short 5′-proximal segment of RIII (Fig. 6). Therefore, to ensure that translation over RII was responsible for the defect—and not translation over an RNA element upstream of RII or one located in RII—stop codons were introduced into DI-83Y immediately upstream of RII or just downstream of the RII core, creating DI-83Y1 and DI-83Y2, respectively (Fig. 6). In accordance with a translation-mediated RII-specific defect, DI-83Y1 accumulated efficiently while DI-83Y2 did not (Fig. 6). It should be noted that the helper genome replicated well in all coinfections with the defective DI-83 derivatives (data not shown); therefore, it is unlikely that the C-terminally extended p33 produced from the DI-83Y or DI-83Y2 templates possessed any dominant-negative activity that could account for the reduced replication observed.

**DISCUSSION**

**Functional properties of the RII core.** Our analysis of a TBSV DI RNA revealed that only a small core structure within the 239-nt-long RII is necessary for efficient accumulation. Analysis of various DI RNA mutants revealed that RII functions in the positive strand and that modifications causing defects affect both positive- and negative-strand RNA synthesis. However, because these mutations did not markedly influence the intrinsic stabilities of DI RNA templates, the de-
creases in RNA accumulation observed are likely due to a defect in one or more steps in viral RNA replication.

The structure of the RII core is helical, and two elements within it, the S2/S2A segment and L3, were found to be distinct determinants of function. Such irregularities within an otherwise regular helical structure can represent specific binding sites for protein factors (7). Our analysis of the S2/S2A segment revealed that formation of either S2 or S2A was not sufficient to mediate function. This result suggests that (i) both of these mutually exclusive structures are important for activity and/or (ii) sequence identity in this region is important. The precise structural features that confer activity to this RNA subelement will be defined in future studies.

In L3, the C_{1383}-C_{1427} mismatch is the best candidate for a specificity element. The potential importance of this mismatch is underscored by the function of CC mismatches in other viral systems. For instance, a CC mismatch was found to be important for (i) encapsidation of the Turnip yellow mosaic virus genome (1) and (ii) positive-strand genome synthesis in Potato virus X (15, 16). These examples illustrate the functionality of CC mismatches and support an active role for the CC mismatch in TBSV. Moreover, this concept is substantiated in an accompanying paper (25) where the CC mismatch in RII is shown (i) to be bound specifically by the p33 replicase protein in vitro and (ii) to be essential for TBSV genome replication in vivo (25).

RII activity in the TBSV genome. RII was also shown to function in the TBSV genome in a coding region. Interestingly, the presence of other cis-acting RNA elements in the readthrough portion of the p92 ORF has been reported pre-
viously (6, 13). However, these elements, located just 3' to RII, were shown to be involved specifically in activation of subgenomic mRNA transcription. In contrast, RII affects synthesis of all viral RNAs and thus represents a different class RNA element residing in this translationally active region. In terms of its position in a coding region, RII resembles the cis-acting RNA elements (i.e., CREs) found in picornaviruses (23). However, functionally, RII is likely more akin to the internal recruitment element found in RNA3 of Brome mosaic virus (33).

Identical S1 modifications in genomic and DI RNA contexts led to roughly proportional reductions in both positive- and negative-strand accumulation. However, a clear difference in the severity of the defect was seen when corresponding S1 mutants were tested in protoplasts at 22°C. The less detrimental effects of S1 disruption observed for the genome could have resulted from (i) other sequences in the genome partially compensating for the destabilization and/or (ii) lack of competition from another RNA replicon (i.e., the DI-72SXP S1 mutants had to compete against the wt genome present in the coinfections). This competition theory seems to be at least partially correct because, in coinoculations with wt DI-72SXP at 22°C, the genomic a and b mutants accumulated to ~30% the level of the c mutant and wt genome (H-X. Lin and K. A. White, unpublished data).

The viral genome is more complex than a DI RNA, and certain aspects of RII activity may differ in this multifunctional environment. In the genome, RII maps to a coding region, which is relevant because RII was shown to function in the positive strand. We also found that increased translation over the RII sequence inhibits the accumulation of DI-83-based viral replicons. This suggests that viral protein translation and RII function occur in the same cellular location, presumably the cytosol, and are required at similar time points during infections. For positive-stranded RNA viruses, this is, to the

FIG. 7. Model integrating translation and RII-mediated viral RNA replication. Both p33 and p92 are translated from the viral genome; however, p92 is expressed at much lower levels due to the inefficiency of readthrough of the p33 stop codon. Accordingly, one predicts that at certain times active translation of p92 will occur (A), while at other times, due to the inefficiency of translational readthrough, the downstream portion of the p92 ORF (and specifically RII) will be relatively free of ribosomes (B; for simplicity the readthrough portion of the p92 ORF is shown completely free of ribosomes). Active translation of the readthrough section would act to linearize and inactivate the RII element, as indicated in panel A. However, between these translational cycles, RII would be able to form its active helical structure, as shown in panel B. The dotted double-headed arrow is meant to represent the dynamic nature of RII structure and activity that is dictated by the translational status of the readthrough portion of the p92 ORF. (C) Once formed, active RII acts to facilitate an early step in genome replication, such as template recruitment and/or replicase complex assembly. (D) Later in the infection, RII function is not required for active viral RNA replication.
best of our knowledge, the first demonstration of an inhibitory effect of translation on (i) viral RNA replication in vivo and (ii) the activity of a viral cis-acting RNA replication element. This inhibition is most likely due to translating ribosomes that prevent the functional RII structure from forming and/or the process displacing a protein bound to it. Whatever the nature of the defect, these results clearly show that RII does not function well when it is located in an efficiently translated region.

Within the genome, RII is positioned in the readthrough portion of the p92 ORF, which encounters a lower level of ribosome traffic. In this context, RII may be able to form transiently and perform its function(s) in the time frame separating consecutively translating ribosomes (see Fig. 7). If this model is correct, there would be no need for complete shutdown of viral genomic translation prior to RII function. Indeed, our results with DI-83 do not support a translational shut-off mechanism for TBSV, because, if active, this system would have prevented translation-based inhibition of RII activity in DI-83 mutants containing extended ORFs (i.e., DI-83Y and DI-83Y2). Instead, a mechanism more akin to that proposed for Brome mosaic virus could operate in TBSV, where messages are ushered out of a translationally active cytosolic environment to a remote replication complex (4, 12, 32, 33). Considering the antagonistic effect that translation has on RII function, RII represent a likely cis-acting element for assisting the recruitment of viral RNAs out of translation and into replication.

The proposed function for RII in recruiting viral RNAs into replication is also consistent with its (i) positive-strand activity, (ii) early function in the infection process, and (iii) absence in subgenomic mRNAs. Both comparative sequence analysis and strand-preferential destabilization of secondary structure support RII functioning in the positive strand. Furthermore, the inhibitory effect of translation on RII also supports its activity in the positive strand. This positive-strand function of RII is in accordance with the predicted properties of a cis-acting RNA sequence involved in recruitment of a viral genome into replication.

When RII activity was down-regulated by a temperature shift at later stages of infection, robust genome replication still persisted. This finding is consistent with an early function for RII, such as template recruitment and/or replicase assembly (22). Furthermore, our results with DI-83 do not support a translational shut-off mechanism for TBSV, because, if active, this system would have prevented translation-based inhibition of RII activity in DI-83 mutants containing extended ORFs (i.e., DI-83Y and DI-83Y2). Instead, a mechanism more akin to that proposed for Brome mosaic virus could operate in TBSV, where messages are ushered out of a translationally active cytosolic environment to a remote replication complex (4, 12, 32, 33). Considering the antagonistic effect that translation has on RII function, RII represent a likely cis-acting element for assisting the recruitment of viral RNAs out of translation and into replication.

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