Virology 256, 162–171 (1999)
Article ID viro.1999.9630, available online at http://www.idealibrary.com on IDEAL®

Enhancer-like Properties of an RNA Element That Modulates Tombusvirus RNA Accumulation

Debashish Ray and K. Andrew White[1]

Department of Biology, York University, Toronto, Ontario, Canada M3J 1P3

Received December 1, 1998; returned to author for revision January 4, 1999; accepted February 2, 1999

Prototypical defective interfering (DI) RNAs of the plus-strand RNA virus tomato bushy stunt virus contain four noncontiguous segments (regions I–IV) derived from the viral genome. Region I corresponds to 5′-noncoding sequence, regions II and III are derived from internal positions, and region IV represents a 3′-terminal segment. We analyzed the internally located region III in a prototypical DI RNA to understand better its role in DI RNA accumulation. Our results indicate that (1) region III is not essential for DI RNA accumulation, but molecules that lack it accumulate at significantly reduced levels (~10-fold lower), (2) region III is able to function at different positions and in opposite orientations, (3) a single copy of region III is favored over multiple copies, (4) the stimulatory effect observed on DI RNA accumulation is not due to region III-mediated RNA stabilization, (5) DI RNAs lacking region III permit the efficient accumulation of head-to-tail dimers and are less effective at suppressing helper RNA accumulation, and (6) negative-strand accumulation is also significantly depressed for DI RNAs lacking region III. Collectively, these results support a role for region III as an enhancer-like element that facilitates DI RNA replication. A scanning-type mutagenesis strategy was used to define portions of region III important for its stimulatory effect on DI RNA accumulation. Interestingly, the results revealed several differences in the requirements for activity when region III was in the forward versus the reverse orientation. In the context of the viral genome, region III was found to be essential for biological activity. This latter finding defines a critical role for this element in the reproductive cycle of the virus.

Key Words: RNA structure, RNA synthesis, RNA polymerase, plus-strand RNA virus, plant virus, TBSV.

INTRODUCTION

A key step in the reproductive cycle of plus-sense RNA viruses is the replication of their genomes. This process is carried out by a virus-specific RNA-dependent RNA polymerase that recognizes signal sequences and/or higher-order structures in viral RNA templates. For many viruses, the promoter elements for negative-strand synthesis reside at the 3′ terminus of the plus-sense genome (Dreher and Hall, 1988; Havelda and Burgyan, 1995; Lin *et al.,* 1994; Song and Simon, 1995; van Rossum *et al.,* 1997); however, more distal regions can also be involved (Meyer *et al.,* 1981). The negative strands that are synthesized are then used as templates for the production of progeny genomes. The promoters for plus-strand synthesis are less well understood; however, for certain viruses, potential elements have been localized in the 5′-noncoding regions of their genomes (Andino *et al.,* 1993; Guan *et al.,* 1997; Kim and Hemenway, 1996; Pogue *et al.,* 1990; Wu and White, 1998).

Tomato bushy stunt virus (TBSV) is the prototype member of the genus *Tombusvirus*. This small spherical virus contains a 4.8-kb plus-sense RNA genome (Fig. 1; Hearne *et al.,* 1990) that encodes five functional open reading frames, the products of which are involved in genome replication (p33, p92; Oster *et al.,* 1998; Scholthof *et al.,* 1995a), encapsidation (p41; Hillman *et al.,* 1989); and movement (p19, p22; Scholthof *et al.,* 1995b). The proteins involved in the latter two activities are translated from two subgenomic (sg) mRNAs that are synthesized during infections. On serial passage, at a high multiplicity of infection, small replicable deletion mutants of the viral genome accumulate (Hillman *et al.,* 1987; Knorr *et al.,* 1991). These amplifiable molecules, or defective interfering (DI) RNAs, suppress the accumulation of helper viral RNAs and ameliorate symptoms in host plants (Hillman *et al.,* 1987; Knorr *et al.,* 1991). A prototypical *Tombusvirus* DI RNA contains four noncontiguous segments (regions I–IV): two of which correspond to 5′- and 3′-terminal segments (regions I and IV, respectively) and two that are derived from internal sections of the genome (regions II and III; Fig. 1). Their maintenance of *cis*-acting elements involved in virus-dependent amplification has made these molecules useful for studying viral RNA replication (Chang *et al.,* 1995; Havelda *et al.,* 1995; Havelda and Burgyan, 1995). Previously, deletion analyses of prototypical TBSV and cucumber necrosis *Tombusvirus* (CNV) DI RNAs suggested an absolute requirement for regions II and IV; however, region III could be removed while maintaining essentially wild-type levels of accumulation (Chang *et al.,* 1995). Recently, it was shown that TBSV DI RNAs lacking all of region I are replicable, albeit at significantly reduced levels (Wu and White, 1998). Studies on a prototypical DI RNA from the closely related *Tombusvirus* cymbidium

[1] To whom reprint requests should be addressed at 4700 Keele Street. Fax: (416) 736-5698. E-mail: kawhite@yorku.ca.

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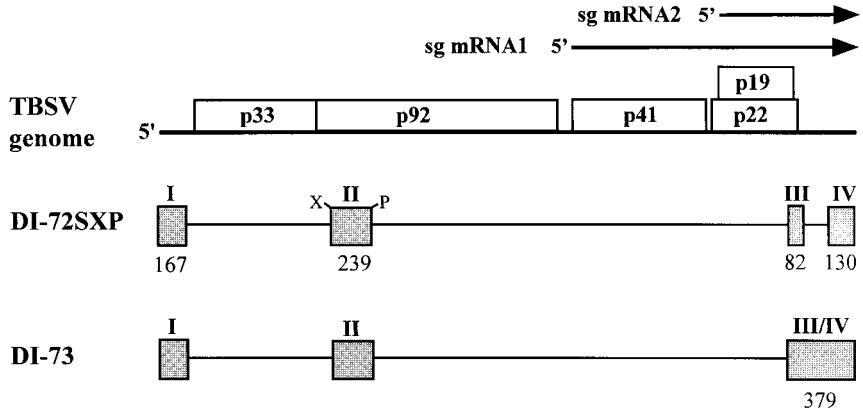

FIG. 1. Schematic representation of the TBSV genome and DI RNAs. The wild-type TBSV genome is shown at the top as a thick horizontal line with coding regions depicted as open boxes with the approximate molecular mass values (in thousands) of the encoded proteins indicated. The regions corresponding to the two sg mRNAs are shown as arrows above the genome. Below, DI-72SXP, a prototypical DI RNA containing engineered restriction enzyme sites (*Xba*I, X, *Pst*I, P), is depicted. Shaded boxes represent regions of the genome retained in the molecule, whereas lines correspond to genomic segments that are absent. DI-72SXP is composed of four highly conserved noncontiguous regions (I–IV), the lengths of which are indicated (in nt). DI-73 contains a contiguous 3′ segment that includes the sequence deleted between regions III and IV in DI-72SXP. Regions I and II are identical in the two DI RNAs shown (except for the engineered restriction enzyme sites).

ringspot virus (CyRSV) showed that each of its corresponding regions (i.e., I–IV) is absolutely required for detectable accumulation of progeny (Havelda *et al.,* 1995). Further analysis of region IV revealed that the base-pairing potential of certain computer-predicted structures was required for viability (Havelda and Burgyan, 1995).

The conflicting results reported (Chang *et al.,* 1995; Havelda *et al.,* 1995) on the necessity for region III in *Tombusvirus* DI RNAs (i.e., it is essential in CyRSV DI RNAs but not in TBSV and CNV DI RNAs) prompted us to investigate further the role of this segment in the accumulation of TBSV DI RNAs. In contrast to previous results (Chang *et al.,* 1995), we observed a substantial reduction in progeny accumulation levels when region III was deleted from a prototypical TBSV DI RNA. Further analyses indicated that region III possesses enhancer-like properties and suggested that its positive effect on DI RNA accumulation is related to stimulation of RNA replication.

RESULTS

Region III facilitates accumulation of a prototypical DI RNA

In an effort to understand better the role of region III in DI RNA accumulation, this highly conserved region was deleted from a prototypical TBSV DI RNA. DI-72SXP is a derivative of DI-72, a naturally occurring DI RNA molecule, into which restriction enzyme sites were introduced (Fig. 1; White and Morris, 1994b; Wu and White, 1998). The mutant DI-72SXPΔIII was generated from DI-72SXP by deleting its 82-nucleotide (nt)-long region III. This mutant was tested for biological activity by coinoculating it with helper TBSV genome transcripts (T100) into cucumber protoplasts. The accumulation of viral RNAs was then assessed by Northern blot analysis of total nucleic acids isolated from the infection. The absence of region III in DI-72SXPΔIII led to greatly reduced levels of progeny DI RNA accumulation (~10-fold, Fig. 2B), as well as readily detectable accumulation of a dimer-sized product (Fig. 2A). The accumulation of head-to-tail DI RNA dimers in *Tombusvirus* infections has been described previously

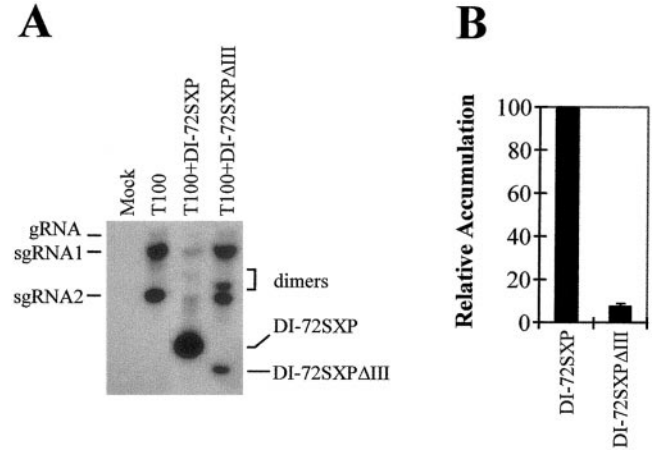

FIG. 2. Analysis of a prototypical DI RNA lacking region III. (A) Northern blot analysis of a prototypical DI RNA lacking region III after infection of protoplasts. The RNA transcripts used in the inoculations are indicated at the top, and the positions of progeny viral RNAs are shown at the left and right. Total nucleic acids were isolated from ~4×10^5 cucumber coytledon protoplasts after a 24-h incubation, separated in a 4.5% polyacrylamide gel in the presence of 8 M urea, transferred to nylon membrane, and hybridized with a ^{32}P-end-labeled oligonucleotide probe (P9) complementary to the 3′-terminal 23 nt of the TBSV genome. (B) Relative accumulation levels of DI RNAs. The relative accumulation levels, which represents monomeric DI RNA levels standardized to the level of monomeric DI-72SXP, were quantified by radioanalytical scanning of Northern blots. The values shown represent mean values (with standard errors) from two separate protoplast infections.

(Dalmay *et al.,* 1995; Finnen and Rochon, 1995). Interference with the accumulation of helper viral RNAs was also significantly reduced in the coinoculation containing DI-72SXPΔIII (Fig. 2A).

Stability and accumulation kinetics of DI RNAs containing and lacking region III

To address the possibility that the different accumulation levels of DI-72SXP and DI-72SXPΔIII were related to altered stability, the relative *in vivo* decay rates of the two molecules were examined. Protoplasts were inoculated with transcripts of DI-72SXP or DI-72SXPΔIII (without helper), and the chemical decay of the two RNAs was monitored by quantifying the amounts of transcripts remaining at different times postinoculation (Fig. 3). The protoplasts were treated with RNase A after inoculations to ensure that only RNA internalized would be analyzed. In addition, control inoculations that excluded the use of polyethylene glycol (PEG)-$CaCl_2$ solution (which facilitates transfection) were performed to assess inoculation efficiency. At 1, 5, and 10 h postinoculation, aliquots of total nucleic acids were isolated and analyzed by Northern blotting (Fig. 3A). The relative levels of radioactivity corresponding to the abundance of the DI RNAs were then quantified, and the values are presented graphically (Fig. 3B). The results indicated that the two RNAs possessed comparable decay rates, with DI-72SXPΔIII showing slightly greater stability than DI-72SXP.

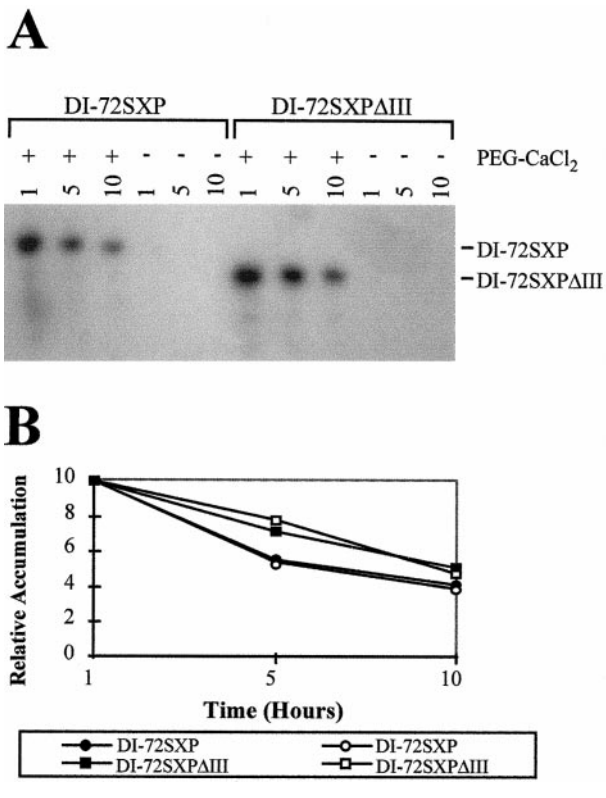

FIG. 3. Stability of DI-72SXP and DI-72SXPΔIII in protoplasts. (A) Northern blot analysis of total nucleic acids extracted from protoplasts inoculated with DI-72SXP or DI-72SXPΔIII only, in the presence (+) or absence (−) of PEG-$CaCl_2$. The time (in h) postinoculation at which nucleic acids were prepared is indicated above the lanes. Total nucleic acids were isolated at the time points indicated and analyzed as described in the legend to Fig. 2. (B) The data shown in panel A were quantified by radioanalytical scanning of the membrane, and a graph of relative RNA concentration as a function of time is presented. The open and filled symbols represent data from two independent experiments.

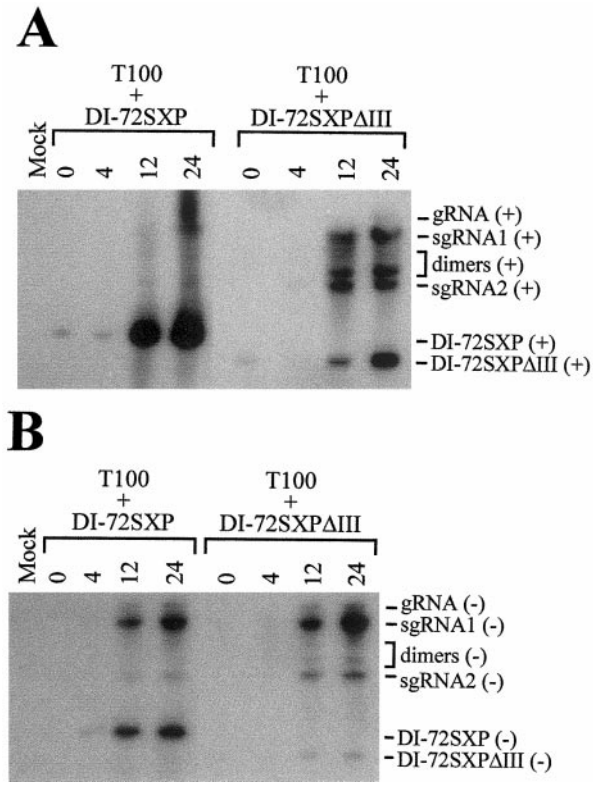

FIG. 4. Northern blot analyses of progeny plus- and minus-strand viral RNAs isolated from protoplasts. (A) Accumulation profile of viral plus-sense strands. (B) Accumulation profile of DI RNA minus-sense RNAs. For A and B, the RNA transcripts used in the inoculations and the times (in h) of harvest postinoculation are indicated at the top. The positions of progeny viral RNAs are shown at the right. Total nucleic acids were isolated and analyzed as described in the legend to Fig. 2, except for B, where ^{32}P-labeled oligonucleotide P50, which detects negative-sense viral RNAs, was used.

To determine whether there were any discernible differences in the kinetics and/or levels of progeny accumulation during the progress of infections, a time course experiment examining the accumulation of plus and minus strands of DI-72SXP and DI-72SXPΔIII was carried out. A clear increase in progeny plus-strand levels was detected for both DI RNAs at the 12-h time point and these levels were increased further at 24 h (Fig. 4A). Although no significant difference in the onset of detectable accumulation of plus strands was observed, a clear contrast in relative accumulation levels was evident. DI-72SXPΔIII minus strands were detectable at the 12- and 24-h time points, but the levels were significantly less than those for DI-72SXP (Fig. 4B). Interestingly, the dif-

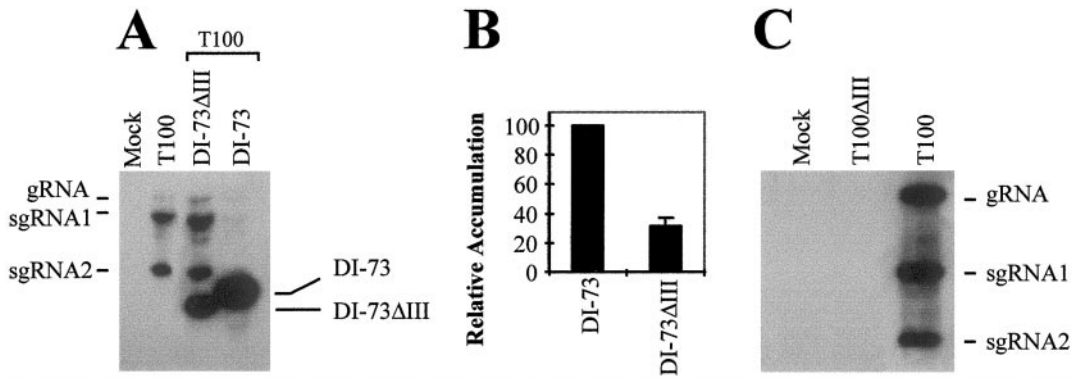

FIG. 5. Analysis of region III in different viral RNA contexts. (A) Northern blot analysis of accumulation of DI-73 and DI-73ΔIII. (B) Relative accumulation levels of DI RNAs. The relative accumulation levels, which represent monomeric DI RNA levels standardized to the levels of monomeric DI-73, were quantified by radioanalytical scanning of Northern blots. The values shown represent mean values (with standard errors) from two separate protoplast infections. (C) Northern blot analysis showing accumulation of T100ΔIII. For A and C, the RNA transcripts used in the inoculations are indicated at the top, and the positions of progeny viral RNAs are shown at the left and/or right. Total nucleic acids were isolated and analyzed as described in the legend to Fig. 2, except that in C, the samples were separated in a 1% agarose gel.

ference in accumulation levels of the minus strands of helper RNAs in infections containing the strongly interfering DI-72SXP and the weakly interfering DI-72SXPΔIII were less pronounced (Fig. 4B) compared with their plus-strand counterparts (Fig. 4A). This observation suggests that DI RNA-mediated inhibition may function via a mechanism that preferentially suppresses accumulation of plus-sense strands. Additional studies are currently addressing this possibility.

Role of region III in other viral RNA contexts

The preceding results suggested a significant role for region III in the accumulation of prototypical DI-72SXP. To determine whether this conserved segment could also influence the accumulation of other amplifiable viral RNAs, the 82-nt-long region III was deleted from a larger TBSV DI RNA, DI-73 (Fig. 1; White and Morris, 1994b), and from the TBSV genome. In DI-73, region III is separated from region IV by a 167-nt-long segment designated region 3.5 (Oster *et al.,* 1998); therefore, regions III and IV are not directly contiguous in this molecule. For DI-73ΔIII, the absence of region III led to significantly reduced levels of progeny DI RNA (Figs. 5, A and B); however, the relative reduction was not as dramatic as that observed for DI-72SXPΔIII (Fig. 2B). As observed for DI-72SXP, deletion of region III from DI-73 appreciably decreased its ability to suppress helper viral RNA accumulation (Fig. 5A). When region III was deleted from T100, no genomic or sg mRNAs were detected (Fig. 5C), suggesting an essential role for this region within the viral genome.

Effect of region III orientation and copy number on DI RNA accumulation

Orientation and dosage effects of region III on DI RNA accumulation were assessed using a series of mutants containing inversions and direct repeats of the region III sequence. Mutant IIIF was created by reintroducing region III, in its forward orientation, into DI-72SXPΔIII. In generating this mutant, a slightly smaller segment (70 nt) of region III was used because other studies had shown that this segment performed comparably to the longer 82-nt-long region III (D. Ray and K. A. White, unpublished data). The smaller size of the functionally equivalent region III offered the advantage of reducing the amount of sequence requiring analysis. Mutant IIIF contains a region III that is 12 nt shorter at its 3′ extremity than that present in DI-72SXP; however, it showed accumulation levels essentially identical to those of DI-72SXP (Figs. 6A and B). Interestingly, the levels of DI RNA progeny accumulation observed for mutant IIIR, in which the 70-nt segment was introduced in the opposite orientation, were similar to those for IIIF (Figs. 6A and B). In contrast to IIIF, IIIR was unable to efficiently suppress helper RNAs and generated significantly more dimer (Fig. 6A). When direct repeats of this region, as dimers or trimers (i.e., 2III, or 3III, respectively), were inserted in either the forward or reverse orientation (F or R, respectively), greater levels of accumulation of DI RNA progeny were observed for those molecules with forwardly-oriented region IIIs (Figs. 6A and B). For both 2IIIF and 3IIIF, a small amount of progeny was observed with electrophoretic mobilities consistent with those of molecules containing a single region III (Fig. 6A), suggesting that excision of copies of region III had occurred. When 3IIIF-containing infections were passaged further, the relative levels of the smaller DI RNA increased concomitantly with decreased levels of the larger (Fig. 6C), indicating that molecules with a single copy of region III are more competitive. This notion is further supported by the reduced levels of accumulation observed for 2IIIF and 3IIIF (Fig. 6B).

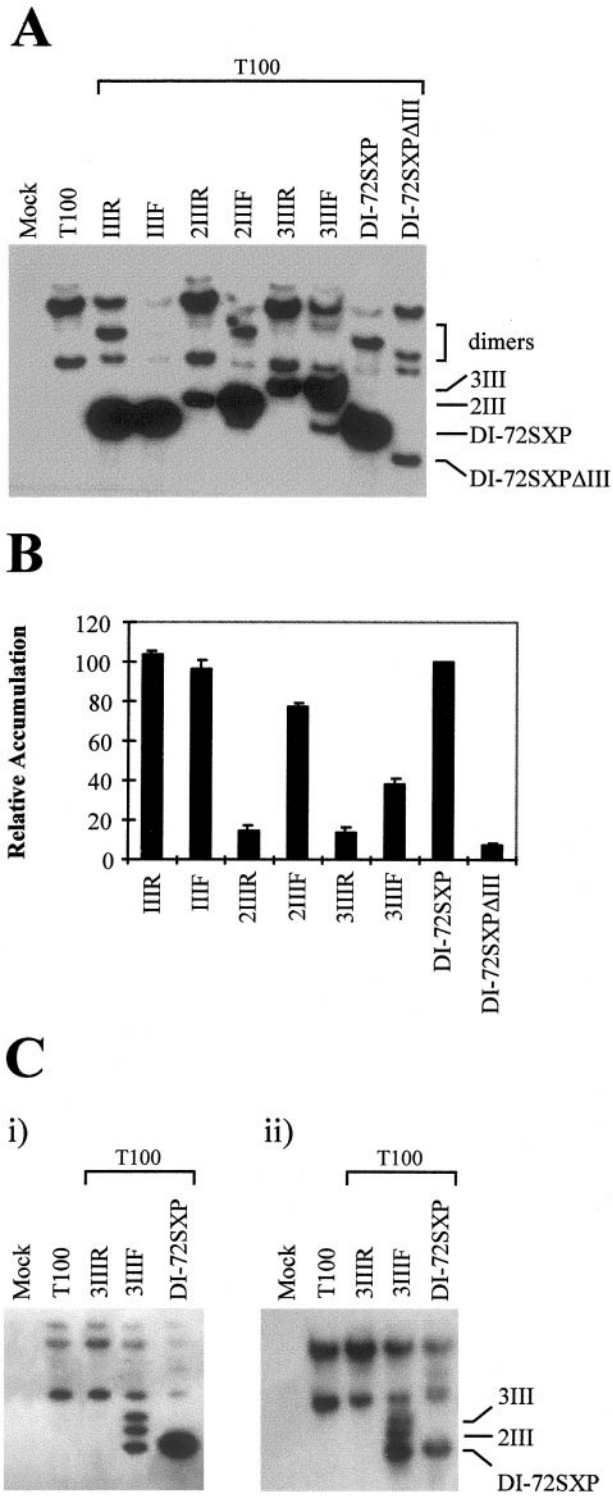

FIG. 6. Analysis of mutant DI RNAs containing inversions and direct repeats of region III. (A) Northern blot analysis of progeny viral RNA accumulation for DI RNAs containing single, multiple, and oppositely oriented copies of region III. Region III (III) was reintroduced in the forward (F) or reverse (R) orientations. DI RNAs containing two or three direct repeat copies of region III have 2 or 3, respectively, as prefixes. (B) Relative accumulation levels of DI RNAs. The relative accumulation levels, which represent monomeric DI RNA levels standardized to the levels of monomeric DI-72SXP, were quantified by radioanalytical scanning of Northern blots. The values shown represent mean values (with standard errors) from two separate protoplast infections. (C) Analysis of the first (i) and second (ii) passage of an infection initiated with DI RNAs containing three copies of region III in the forward or reverse orientations. For A and C, the RNA transcripts used in the initial inoculations are indicated at the top, and the positions of progeny viral RNAs are shown at the right. Total nucleic acids were isolated and analyzed as described in the legend to Fig. 2.

Scanning mutagenesis of region III

Our results, thus far, have implicated region III as being important for DI RNA accumulation. To gain further insight into what portions of region III contribute to its activity, we analyzed this sequence via scanning substitution mutagenesis. This strategy was chosen over deletion analysis because it allows for modification of local elements while maintaining overall size. The overall molecule size can influence DI RNA accumulation as was demonstrated previously for TCV where size reductions caused by deletions resulted in significantly reduced DI RNA accumulation (Li and Simon, 1991; Zhang and Simon, 1994). Efficient accumulation was restored when the deleted regions were replaced by similarly sized nonviral sequences, thereby implicating size rather than sequence as the crucial property.

Seven different modifications, each composed of four- or three-nucleotide substitutions, were introduced independently at successive positions within the 70-nt region III (Fig. 7A). Single copies of each of the resulting modified region IIIs were introduced into DI RNAs in both forward and reverse orientations. When coinoculated with T100, several of the modifications had a significant negative impact on DI RNA accumulation levels (Fig. 7B). In general, substitutions in the 3′ half of the region appeared to be less detrimental than those in the 5′ half (smIII-6F and smIII-6R being the exceptions) (Fig. 7C). In addition, differences were observed between the constituents of oppositely oriented mutant pairs. For example, modification smIII-4 had little effect on DI RNA accumulation in the forward orientation (i.e., smIII-4F, Fig. 7C) but showed reduced accumulation in the reverse orientation (i.e., smIII-4R, Fig. 7C). Conversely, for modification smIII-2, its presence in the forward orientation was lethal (i.e., smIII2F, Fig. 7C), whereas low, but detectable, levels were observed for the reverse orientation (i.e., smIII-2R, Fig. 7C). These results indicate that various sequences and/or secondary structures within region III are important for DI RNA accumulation and that some of the effects of these elements are sense specific.

DISCUSSION

By studying the structure and biological activity of DI RNAs, it may be possible to gain insight into the roles of various *cis*-acting RNA elements in genome replication. In this study, we analyzed the structure and function of region

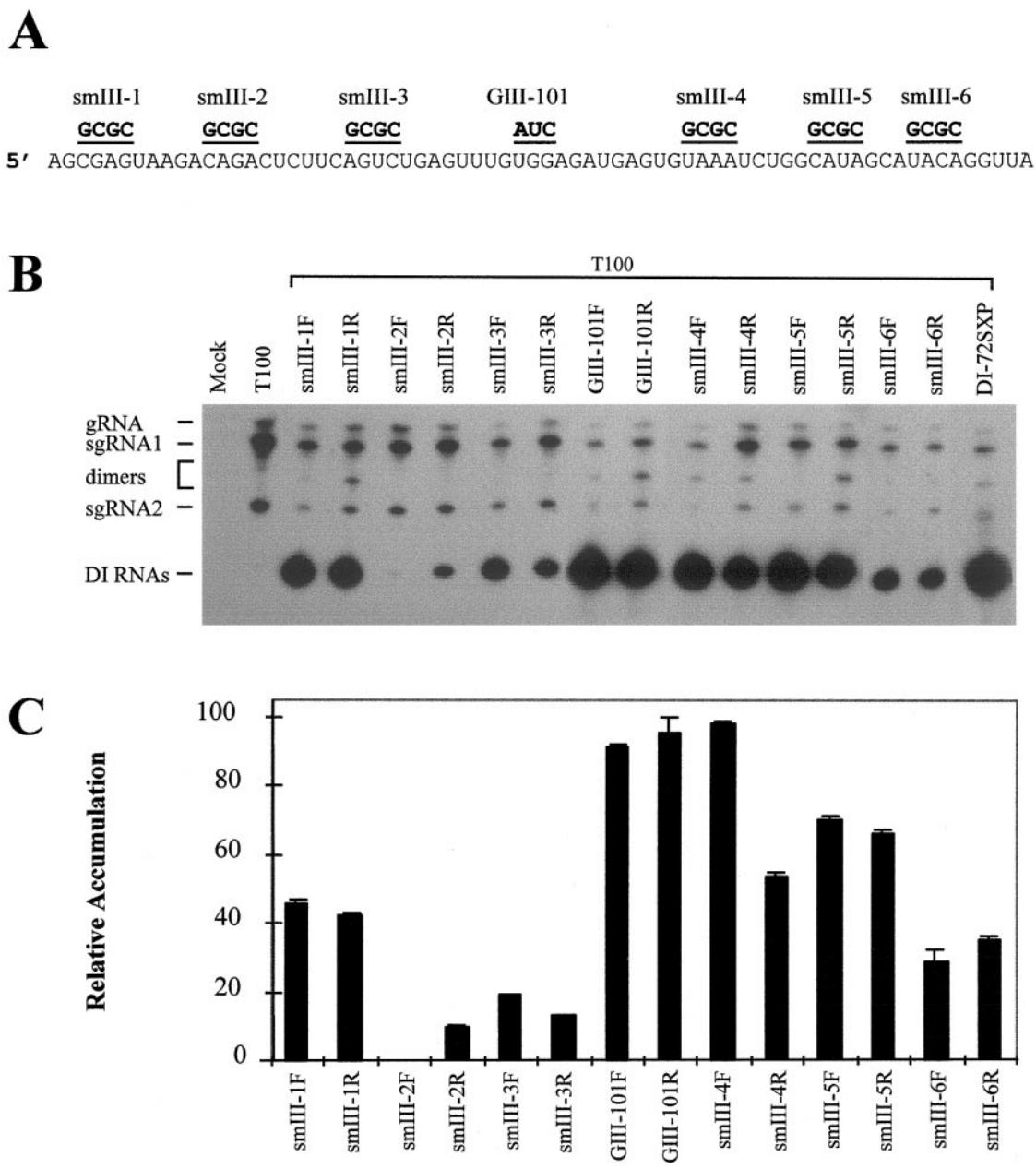

FIG. 7. Analysis of DI RNAs containing scanning-type mutations in region III. (A) Region III sequence showing the scanning mutations introduced. The individual four-base substitutions are indicated on top of the region III sequence along with the name of the corresponding mutant DI RNA. (B) Northern blot analysis of DI RNAs containing scanning-type mutations in region III. (C) Relative accumulation levels of DI RNAs, which represent monomeric DI RNA levels standardized to the levels of monomeric DI-72SXP, were quantified by radioanalytical scanning of Northern blots. The values shown represent mean values (with standard errors) from two separate protoplast infections.

III, a conserved segment in naturally occurring *Tombusvirus* DI RNAs. Our results indicate that this region possesses enhancer-like properties and represents a noncritical but important *cis*-acting element within the context of prototypical TBSV DI RNAs. However, in the TBSV genome, this element appears to play an essential role.

Enhancer-like properties of region III

The following characteristics of region III are similar to those of DNA transcriptional enhancers (Hertel *et al.*, 1997): (1) deletion of the element significantly reduces, but does not abolish, DI RNA accumulation; (2) the element is able to function at different positions; and (3) the element works efficiently in both forward and reverse orientations. The latter feature suggests that either both senses of the sequence maintain the relevant *cis*-acting activity or only one sense maintains the activity and is able to exert its effect either in *cis* or in *trans*. The ability to differentially affect activity in the forward and reverse orientations with the same modification suggests that

both senses may be functional; however, each would exert its activity via somewhat different structural features. The maintenance of the activity in both orientations could also be interpreted as a nonspecific effect; however, molecules in which region III was replaced with various similarly sized nonviral sequences were found to be nonviable (data not shown). Results similar to these were observed when region III in a CyRSV DI RNA was replaced with nonviral sequences (Havelda *et al.,* 1995). Region III must therefore possess some distinct properties that confer its activity. For TBSV DI RNAs, duplication of region III did not appear to confer increased competitiveness, and molecules containing multiple copies of it evolved to forms containing a single copy, suggesting that the latter structure is optimal. This is in contrast to results observed previously for region II, where duplication led to enhanced DI RNA fitness (White and Morris, 1994a).

Despite the fact that region III is highly conserved in TBSV DI RNAs, it appears to represent a nonessential element in the context of both DI-72SXP and DI-73. These molecules did, however, show significantly decreased levels of accumulation when region III was deleted. In contrast, other studies on a different prototypical TBSV DI RNA (B-10) showed that progeny accumulation levels were similar either with or without region III (Chang *et al.,* 1995). A comparison of this B-10 DI RNA with DI-72SXP revealed several sequence differences (all of which mapped in region II) that could potentially account for the apparent inconsistency of the results. The concept of a compensatory effect by modifications in region II is supported by the previous observation that a single nucleotide insertion in this region can significantly enhance DI RNA competitiveness (White and Morris, 1994a). Other studies on a prototypical CyRSV DI RNA showed that deletion of its region III produced nonviable molecules (Havelda *et al.,* 1995). However, it was found that the reintroduction of either half of the deleted region III restored viability. Taken together, these results suggest that the activity of, and necessity for, region III is variable and very sensitive to context.

The influence of region III on the ability of the DI RNA to suppress helper viral RNA accumulation is of interest. Both the absence of region III (e.g., DI-72SXPΔIII) and its presence in the reverse orientation (e.g., IIIR) resulted in reduced helper RNA suppression. However, for DI-72SXPΔIII, the accumulation levels of monomeric DI RNA was greatly decreased, whereas for IIIR, normal levels of monomeric DI RNA accumulation were observed (i.e., levels similar to those of IIIF). This result indicates that efficient DI RNA accumulation does not always correlate with efficient helper RNA supression and that other, yet unidentified, factors are involved. Region III was also able to influence the levels of accumulation of the dimeric form of the DI RNA. Again, both the absence and reverse orientation of region III facilitated the accumulation of dimers. Previous studies have suggested that the size of *Tombusvirus* DI RNAs modulates dimer accumulation levels, with smaller molecules promoting efficient dimer accumulation (Dalmay *et al.,* 1995). Our results with IIIR clearly indicate that factors other than size are able to influence the efficiency of dimer accumulation.

Possible functions for region III

The maintenance of some form of region III in all naturally occurring DI RNAs characterized to date suggests that its presence provides a selective advantage. An alternative explanation is that a mechanism to efficiently excise this particular segment from DI RNAs does not exist. The former of these two concepts is supported by the clear decrease in fitness of DI RNAs lacking region III. Thus even if such molecules were to form *in vivo*, they would likely be rapidly outcompeted and would not accumulate to detectable levels.

Because no significant differences were observed between the relative decay rates of DI RNAs containing and lacking region III, it is unlikely that the dramatic difference in accumulation levels observed is related to altered RNA stability. Also, because these molecules are not efficiently packaged (Hillman *et al.,* 1987), a role for region III as an encapsidation signal allowing for preferential packaging (leading to stabilization) seems improbable. A more likely possibility is that this element facilitates some aspect of DI RNA replication. For alfalfa mosaic virus RNA 3, a 21-nt sequence that enhances promoter activity has been identified just 5′ to the 3′-terminal core promoter for minus-strand synthesis (van Rossum *et al.,* 1997). Similarly, region III is positioned just 5′ to the 3′ terminus of DI RNAs, which has been implicated in minus-strand synthesis (Havelda and Burgyan, 1995). In addition, the clear decrease in accumulation of minus strands of DI-72SXPΔIII is consistent with the concept that region III promotes synthesis of negative strands. However, the similar relative accumulation profiles for the plus and minus strands suggest that their synthesis is tightly coupled temporally; therefore, further studies will be necessary to address this possibility.

In terms of mechanism of action, it is possible that region III may interact with one or more specific *trans*-acting factor involved in RNA synthesis (Lai, 1998). An alternative, more general mode of action may be to reduce inhibitory effects within and/or between the other three regions. For instance, when present between regions II and IV, region III may not interfere with critical sequence and structure within these regions and/or may prevent inhibitory interactions between them. Removal of region III, replacement of it with nonviral sequences, or substitutions of small sections of it with inappropriate sequences would prevent such beneficial effects. The scanning mutagenesis has helped to identify positions within the region that influence DI RNA accumulation.

TABLE 1

Oligonucleotides Used in the Study

Oligonucleotide	Position[a]	RE[b] site	Sequence[c]	Sense[d]
P2	4684–4663	*Pst*I	5′-CGC*TCTAGA*GCTTCCACAAGTGACACCTAAC	−
P24	3805–3826	*Xba*I	5′-GGCCC*TCTAGA*TTTCTCTAATTTAGTGTGTCCT	+
P50	4754–4776		5′-GGAACATTGCAGAAATGCAGCCC	+
P59	4647–4670	*Pst*I	5′-CGGCG*CTGCAG*ATTCCTGTTTACGAAAGTTAGGTG	+
P60	4467–4448	*Pst*I	5′-CCGG*CTGCAG*TAACCTGTATGCTATGCCAG	−
P101	4398–4452	*Pst*I	5′-GCCGCGG*CTGCAG*AGCGAGTAAGACAGACTC-TTCAGTCTGAGTTTGATCAGATGAGTGTAAATCTGGC	+
PF3	149–168	*Xba*I	5′-CGCG*TCTAGA*CATGTCGCTTGTTTGTTGGA	−
PF4	1285–1304	*Xba*I	5′-GCGC*TCTAGA*AGAAACGGGAAGCTCGCTCG	+
PF5	1504–1523	*Pst*I	5′-CGCGC*CTGCAG*TTCTCTGCTTTTACGAAGGT	−
PF6	4398–4417	*Pst*I	5′-GCGCG*CTGCAG*AGCGAGTAAGACAGACTCTT	+
PF7	1–20	*Sac*I	5′-GGCC*GAGCTCT*AATACGACTCACTATAGGAAATTCTCCAGGATTTCTC	+
PR2	4398–4374	*Pst*I	5′-GCGCG*CTGCAG*TTCTTTTTCGAAGGTCTCAGTACC	−
PR7	4480–4495	*Pst*I	5′-GCGCC*CTGCAG*TCTGGATGTTAGGATGACG	+
PR22	4398–4415	*Pst*I	5′-CCGC*CTGCAG*AGGCGCTAAGACAGACTC	+
PR23	4398–4420	*Pst*I	5′-CGCCG*CTGCAG*AGCGAGTAAGAGCGCCTCTTCAG	+
PR24	4398–4433	*Pst*I	5′-GGCGC*CTGCAG*AGCGAGTAAGACAGACTCTTCGCGCTGAGTTTGTGG	+
PR25	4432–4467	*Pst*I	5′-GCGCG*CTGCAG*TAACCTGTATGCTATGTCAGAGCGCCACTCATCTCC	−
PR26	4440–4467	*Pst*I	5′-CCGCGCTGCAGTAACCTGTATGCGCGCTCAGATTTACAC	−
PR27	4448–4467	*Pst*I	5′-CGCCG*CTGCAG*TAACCGCGCTGCTATGTCAG	−

[a] Coordinates refer to those of the TBSV genome (Hearne *et al.,* 1990).

[b] Restriction enzyme.

[c] Viral sequences are underlined and correspond to the coordinates shown. Italicized sequences represent restriction site(s), the identities of which are indicated.

[d] Refers to the sense of the oligonucleotide in reference to the plus-sense viral RNA.

Some of these modifications are predicted to disrupt base-pairing in computer-generated secondary structure models (data not shown). This information will be integrated with ongoing RNA structural studies of this region to determine whether such interactions are relevant. The finding that region III is essential within the context of the viral genome underscores the importance of this *cis*-element in viral reproduction and justifies further investigation of this unusual element.

MATERIALS AND METHODS

Viral constructs

Plasmid construct T100 corresponding to a full-length genome of TBSV and TBSV DI RNA constructs DI-72 and DI-73 have been described previously (Hearne *et al.,* 1990; White and Morris, 1994b). DI-72SXP is a derivative of DI-72, which contains *Xba*I and *Pst*I restriction sites introduced at the 5′ and 3′ ends of region II, respectively. DI-72SXP was assembled with PCR products generated using the DI-72 construct as template. The primers (refer to Table 1 for a list and descriptions of oligonucleotides used in this study) were designed so as to introduce the desired restriction enzyme sites at terminal regions in the products. Subsequent restriction enzyme digestion of the PCR products with the appropriate enzymes, followed by ligation into pUC19, generated DI-72SXP. The segments amplified, the primer pairs used, and the subsequent restriction enzyme digestions, respectively, were PF7, PF3, *Sac*I, and *Xba*I for region I; PF4, PF5, *Xba*I, and *Pst*I for region II; and PF6, P9, *Pst*I, and *Sph*I for region III–IV. After digestion, the products were gel-purified and ligated into an *Sac*I–*Sph*I-digested pUC19 vector.

DI-72SXPΔIII was constructed by digesting a region IV PCR product (generated with primer pair P59–P9 and the DI-72 construct as template) with *Pst*I–*Sph*I and using it to replace the corresponding region III–IV fragment in DI-72SXP. T100ΔIII was constructed by digesting T100 with *Eco*RI–*Sph*I, gel-purifying the larger fragment, and then ligating it with two PCR products. The first PCR product was generated using primer pair P24–PR2 and the T100 construct as template, and the second PCR product was generated using primer pair PR7–P9 and the DI-73 construct as template. The two products were digested with *Eco*RI–*Pst*I and *Pst*I–*Sph*I, respectively, gel-purified, and used in the ligation described above. DI-73ΔIII was generated using primer pairs PR7–P9 and DI-73 as template. The PCR product generated was then digested with *Pst*I–*Sph*I and used to replace the smaller *Pst*I–*Sph*I fragment in DI-72SXP.

Constructs containing forward- or reverse-oriented region III monomers (IIIF and IIIR), dimers (2IIIF and 2IIIR), or trimers (3IIIF and 3IIIR) were generated by ligation of *Pst*I-digested DI-72SXPΔIII with a *Pst*I-digested PCR product corresponding to a 70-nt segment of region III.

The PCR product was generated using primer pair P60–P59 and the DI-72 construct as template.

Forward- and reverse-oriented scanning mutants were constructed by digesting PCR products, generated using DI-72 as template, with *Pst*I and ligating the respective gel-purified fragments into a *Pst*I linearized DI-72SXPΔIII vector. The primer pairs used for PCR were P2–PR22 for smIII-1, P2–PR23 for smIII-2, P2–PR24 for smIII-3, P2–P101 for GIII-101, PF6–PR25 for smIII-4, PF6–PR26 for smIII-5, and PF6–PR27 for smIII-6. All constructs used in this study, with the exception of the 2III and 3III mutants, which were verified via PCR and restriction enzyme analyses, were sequenced to verify the accuracy of the modifications introduced.

In vitro transcription

Viral transcripts were generated *in vitro* via transcription of *Sma*I-linearized constructs using the Ampliscribe T7 RNA polymerase transcription kit (Epicentre Technologies). After the transcription reaction, DNA templates were hydrolyzed by treatment with DNase I (Epicentre Technologies), and unincorporated nucleotides were removed using a Sephadex G-25 spin column (Pharmacia). Ammonium acetate was added to the flowthrough to a final concentration of 2 M, and the transcripts were extracted twice with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and then precipitated with ethanol. Subsequently, the transcripts were quantified spectrophotometrically, and an aliquot was analyzed by agarose gel electrophoresis to verify integrity.

Isolation and inoculation of protoplasts

Protoplasts were prepared from 6- to 8-day-old cucumber cotyledons (var. Straight 8) as described previously (White and Morris, 1994b). Briefly, the lower epidermis of the cotyledons were peeled off using forceps, and the cotyledons were digested in 20 ml of an enzyme mix containing of 0.25 g of cellulase (Calbiochem), 0.025 g of pectinase (ICN), and 0.025 g of BSA (ICN) for 3–5 h with gentle shaking (40 rpm) in the dark. The protoplasts were then washed in 10% mannitol and purified by banding twice on a 20% sucrose cushion. Quantification was carried out by bright field microscopy using a hemacytometer. Purified protoplasts ($\sim 4 \times 10^5$) were inoculated as described (White and Morris, 1994b) with viral RNA transcripts (1 μg for DI RNA transcripts and 5 μg for genomic transcripts) and were incubated in a growth chamber under fluorescent lighting at 22°C for the periods of time specified.

Analysis of viral RNAs

Total nucleic acids were harvested from protoplasts by resuspension in 300 μl of a buffer containing 2× STE (100 mM Tris, 2 mM EDTA, 200 mM NaCl) and 1% SDS. After two extractions with phenol/chloroform/isoamyl alcohol, 100 μl of 8 M NH_4 acetate was added to the aqueous phase, and the mixture was precipitated with ethanol. Aliquots of the total nucleic acid preparation (a fifth) were separated in denaturing 4.5% polyacrylamide gels containing 8 M urea or in 1% agarose gels. The gels were then stained with ethidium bromide, and the integrity and quantity of ribosomal RNAs were assessed under ultraviolet light to ensure that the samples were not degraded and were loaded evenly. Plus-strand viral RNAs were detected by electrophoretic transfer to nylon (Hybond-N; Amersham) followed by Northern blot analysis using a ^{32}P-end-labeled oligonucleotide probe (i.e., P9; White and Morris, 1994a) complementary to the 3′-terminal 23 nucleotides of the TBSV genome. Quantification of the bound viral RNAs was performed by radioanalytical scanning of the blot using an InstantImager (Packard Instrument Co.).

For analysis of minus strands, an RNase treatment (Ishikawa *et al.,* 1991) was performed on isolated nucleic acids before Northern blotting. Briefly, aliquots of total nucleic acid (a fifth) were sequentially incubated at 85°C for 5 min, at 60°C for 10 min, and at 37°C for 10 min in 30 μl of annealing buffer (300 mM KCl, 50 mM Tris–HCl, 1 mM EDTA, pH 8.0) to promote annealing of plus- and minus-strand viral RNAs. After the annealing step, the mixture was treated with RNase A (1 μg/ml) at 30°C for 30 min to remove single-stranded RNAs. Subsequent to inactivation of RNase A with 15 μl of 1% SDS and 1.5 μl of Proteinase K (20 mg/ml) for 30 min at 30°C, double-stranded RNAs were recovered after phenol/chloroform extraction and ethanol precipitation. Samples were analyzed by Northern blotting using a ^{32}P-end-labeled oligonucleotide (P50) probe identical to the last 23 nt at the 3′ terminus of the TBSV genome.

Determination of RNA stabilities

Protoplasts were inoculated, as described above, with 5 μg of DI-72SXP or DI-72SXPΔIII transcripts. The efficiency of transfections was monitored by carrying out additional sets of inoculations as described above, except that no PEG-$CaCl_2$ solution was used. The inoculated protoplasts were incubated in media containing 10 μg/ml of RNase A to remove transcripts that were not taken up by the protoplasts (Kim and Hemenway, 1996). Before total nucleic acids extraction, the incubation medium was removed, and protoplasts were washed with 1 ml of 10% mannitol to remove excess RNase A. Further isolation and analysis of viral RNAs by Northern blotting were performed as described above.

ACKNOWLEDGMENTS

We thank members of our laboratory for reviewing the manuscript. This work was supported by grants to K.A.W. from the Natural Sciences and Engineering Research Council of Canada.

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