JOURNAL OF VIROLOGY, Dec. 1994, p. 8466–8469
0022-538X/94/$04.00+0

Vol. 68, No. 12

# Effect of Template Size on Accumulation of Defective Interfering RNAs in Protoplasts

CHUNXIA ZHANG AND ANNE E. SIMON*

*Department of Biochemistry and Molecular Biology and Program in Molecular and Cellular Biology, University of Massachusetts, Amherst, Massachusetts 01003*

Received 1 August 1994/Accepted 14 September 1994

**A turnip protoplast system has been used to study the effects of template size and sequence on the replication and/or stability of a small defective interfering (DI) RNA associated with turnip crinkle virus. Our results indicated that as little as a single base difference in the size of the molecule in some regions, rather than the specific sequence, affected the level of DI RNA accumulating in protoplasts.**

Defective interfering (DI) RNAs are mutated versions of viral genomic RNAs that multiply in hosts only when associated with a helper virus (8, 16). DI RNAs are commonly much smaller than the corresponding genomic RNA and are therefore valuable for the analysis of *cis*-acting sequences and structures necessary for replication and encapsidation (5, 9, 11, 14, 17–19). Besides the presence of specific *cis*-acting sequences and structures, high-level accumulation of DI RNAs can also depend on preservation of an open reading frame (4, 6, 20), the length of the RNA in multiples of six nucleotides (1), and the size of the nucleocapsid (15).

A full-length cDNA of one DI RNA (DI RNA GA) associated with turnip crinkle virus (TCV) has been cloned, and transcripts synthesized in vitro are biologically active when coinoculated with TCV genomic RNA (13). DI RNA GA is similar to a natural DI RNA (DI RNA G) associated with TCV isolate TCV-B (12), except for the insertion of sequence corresponding to an *Ava*I linker at position 104. The 5′ region of DI RNA GA can tolerate deletions of up to 25 bases, while the biological activities of two more extensive deletion mutants were restored by replacing the deleted sequences with unrelated sequence of the same or greater size. This result suggested that the size of the molecule, along with *cis*-acting sequences, can affect multiplication in planta (13).

For the current study, the ability of mutated DI RNA GA to replicate in turnip protoplasts was tested to determine whether the lack of infectivity in planta was due to decreased replication competence and/or stability or failure to systematically invade the plant. To prepare protoplasts, turnip cv. Just Right leaves were stripped of epidermis and gently agitated in a solution containing 1% (wt/vol) cellulase (10,500 U/g; CalBiochem), 0.05% (wt/vol) macerase (3,500 U/g; CalBiochem), and 0.6 M mannitol for 2 h at 25°C. After filtration through a 0.53-μm-pore-size nylon mesh and centrifugation for 3 min at 10 × *g*, protoplasts were washed four or five times in 0.6 M mannitol and then combined with transcripts of DI RNA GA and TCV genomic RNA (27 μg [each]/3 × $10^6$ protoplasts) in 1.2 ml of 3 mM $CaCl_2$–40% polyethylene glycol 1500 (Boehringer Mannheim). In vitro-synthesized transcripts (3, 13) differed from natural (i.e., in planta synthesized) DI RNA GA by an additional 5′-terminal G residue and two additional residues at the 3′ end, while the TCV genomic RNA transcripts contained the natural ends. After 15 s at 25°C, 12 ml of 0.6 M mannitol–1 mM $CaCl_2$ was added, and the mixture was incubated on ice for 15 min and then centrifuged at 34 × *g* for 5 min. Protoplasts were washed several times in 0.6 M mannitol–1 mM $CaCl_2$ and then resuspended in 6 ml of the wash solution supplemented with 6 μg of nystatin (Sigma) per ml and 20 μg of carbenicillin (Sigma) per ml. Following incubation for various times at 25°C, protoplasts were centrifuged at

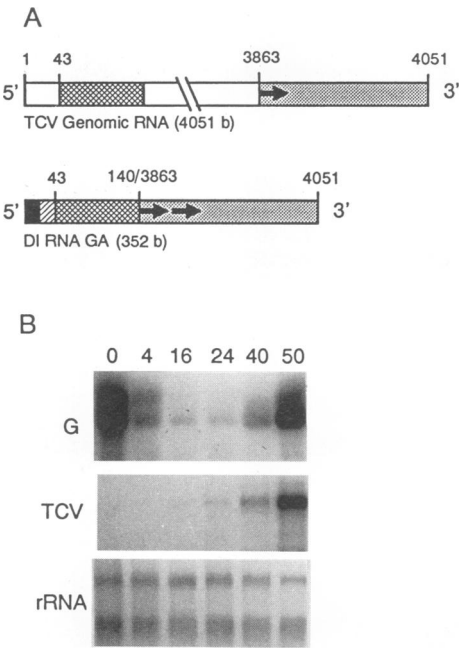


FIG. 1. (A) Schematic diagram of the relationship between TCV genomic RNA and DI RNA GA. The 5′ 10 bases are identical to bases in the same position of TCV satellite RNA D. The adjoining 10 bases are of unknown origin. Sequences in the genomic RNA and DI RNA GA that are similar are shaded similarly. The arrow represents a 36-base sequence that is duplicated in DI RNA GA. Numbers represent corresponding positions in the TCV genomic RNA. b, bases. (B) RNA gel blot indicating the levels of DI RNA GA and TCV genomic RNA accumulating in turnip protoplasts over time. Total RNA was isolated from protoplasts at the times (in hours) postinoculation indicated above the lanes. A single blot was hybridized in succession with probes specific for DI RNA GA (G), genomic RNA (TCV), and rRNA.

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, LGRT, University of Massachusetts, Amherst, MA 01003. Phone: (413) 545-0170. Fax: (413) 545-4529. Electronic mail address: simon@biochem.umass.edu.

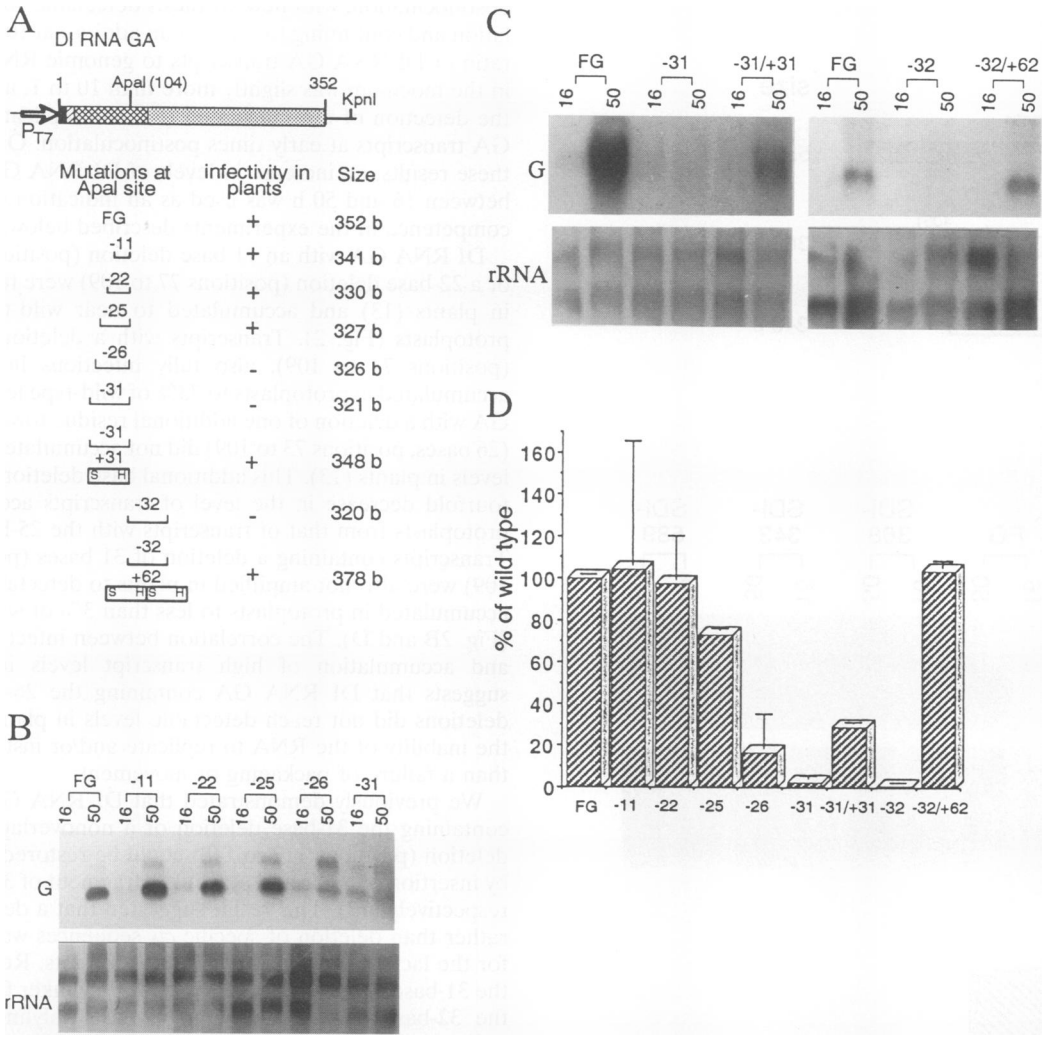


FIG. 2. Accumulation of DI RNA GA containing deletions and insertions in turnip protoplasts. (A) Positions of deletions (brackets) and insertions (open boxes) in a full-length cDNA of DI RNA GA (FG), located downstream of a T7 RNA polymerase promoter, are indicated. The orientation of the inserted *Sma*I-*Hin*dIII polylinker fragment is given. Numbers of bases (b) deleted or inserted, infectivity of transcripts in plants (13), and expected sizes of the mutant DI RNAs, are also indicated. (B) and (C) RNA gel blots indicating the levels of mutant and parental DI RNA GA accumulating in turnip protoplasts at the hours postinoculation (16 and 50) indicated above each lane. A single blot per duplicate experiment was hybridized in succession with probes specific for DI RNA GA (G) and rRNA. Horizontal numbers above the lanes denote the constructs from panel A that were assayed. The slower-migrating RNA species hybridizing to the DI RNA GA probe is of unknown origin and was not present in all experiments. (D) Autoradiograms from panels B and C (and the duplicate experiment) were scanned with a laser densitometer, and the amount of DI RNA in each lane was normalized to the amount of rRNA. The amount of DI RNA detected at 16 h was subtracted from the amount detected at 50 h and expressed as a percentage of DI RNA GA assayed in parallel. The bars denote standard errors from the two independent experiments. Because of the unknown origin of the slower-migrating species, this RNA was not included in the data tabulation presented in the histogram.

10 × g, combined with 0.2 ml of RNA extraction buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate), and phenol extracted twice, and the RNA was precipitated. Levels of DI RNA GA and TCV genomic RNA in protoplasts were analyzed by electrophoresis through 1.5% agarose gels containing 6% formaldehyde, followed by RNA gel blot analysis as previously described (2). DI RNA GA probes were oligonucleotides complementary to DI RNA GA positions 110 to 129 (specific for DI RNA GA) or 236 to 252 (also hybridizes to TCV genomic RNA and used for the detection of artificial DI RNAs). The TCV genomic RNA probe was an oligonucleotide complementary to positions 269 to 288 in the TCV genomic RNA. Oligonucleotides were labeled as previously described (12). Blots were also probed with random-primer-labeled cDNA of rRNAs (10) to normalize for differences in RNA loading and transfer.

Artificial DI RNAs containing various lengths of 5′- and 3′-terminal sequence were constructed by digesting pTCV-T1d1, a plasmid containing a full-length cDNA copy of TCV-B genomic RNA (7) with *Ava*I (Bethesda Research Laboratories). The fragment containing the plasmid backbone along with 283 bases at the 5′ end and 417 bases at the 3′ end was treated with *Bal*31 (IBI), according to the supplier's suggested procedures, followed by ligation of the ends. The deletion endpoints were identified by sequencing.

TCV genomic RNA was detectable in protoplasts by 16 h

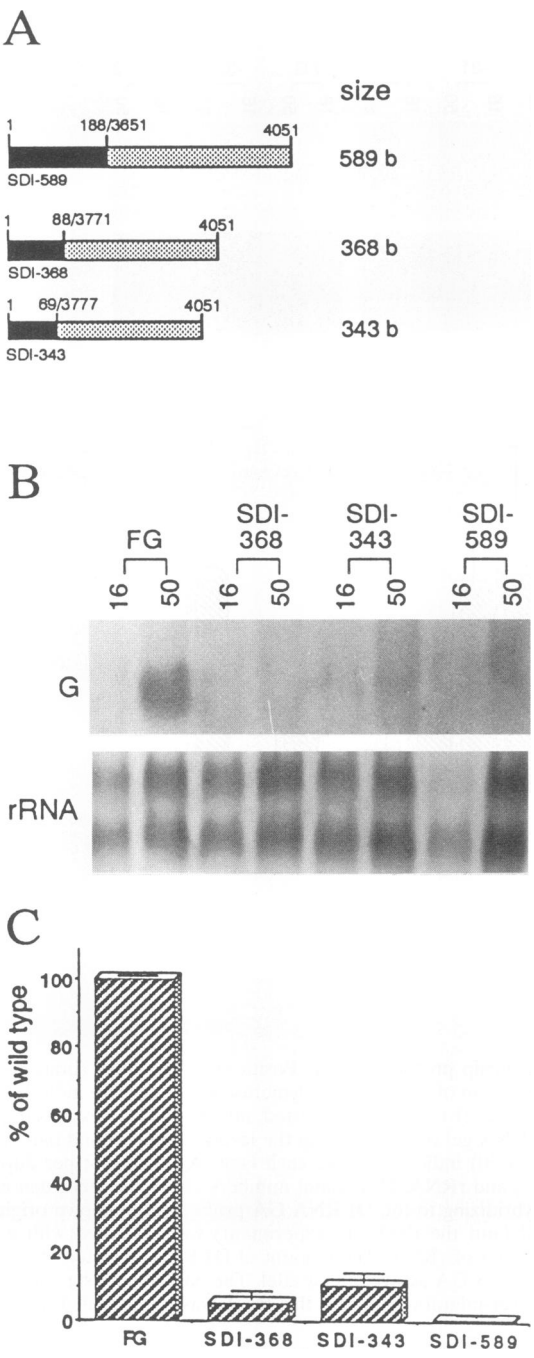


FIG. 3. Accumulation of in vitro-generated DI RNAs in protoplasts. (A) Sizes and sequences of the artificial DI RNAs. Numbering is from the corresponding sequence of TCV genomic RNA. (B) RNA gel blot indicating the levels of DI RNA GA and artificial DI RNAs at the hours postinoculation (16 and 50) of turnip protoplasts indicated above each lane. For details, see the legend to Fig. 2B. The DI RNA GA probe was an oligonucleotide complementary to positions 236 to 288. (C) Quantitation of the results in panel B. See the legend to Fig. 2D for details.

postinoculation, with levels increasing until at least 50 h postinoculation (Fig. 1). DI RNA GA transcript levels in protoplasts coinoculated with TCV genomic RNA decreased from high initial levels to minimal levels between 16 and 24 h postinoculation, with new synthesis detectable 40 h postinoculation and continuing for at least an additional 10 h. The molar ratio of DI RNA GA transcripts to genomic RNA transcripts in the inoculum was slightly more than 10 to 1, accounting for the detection of the inoculated (i.e., nonreplicated) DI RNA GA transcripts at early times postinoculation. On the basis of these results, an increase in levels of DI RNA GA transcripts between 16 and 50 h was used as an indication of replication competence in the experiments described below.

DI RNA GA with an 11-base deletion (positions 98 to 109) or a 22-base deletion (positions 77 to 109) were fully infectious in plants (13) and accumulated to near wild-type levels in protoplasts (Fig. 2). Transcripts with a deletion of 25 bases (positions 74 to 109), also fully infectious in planta (13), accumulated in protoplasts to 73% of wild-type levels. DI RNA GA with a deletion of one additional residue toward the 5′ end (26 bases, positions 73 to 109) did not accumulate to detectable levels in plants (13). This additional base deletion resulted in a fourfold decrease in the level of transcripts accumulating in protoplasts from that of transcripts with the 25-base deletion. Transcripts containing a deletion of 31 bases (positions 68 to 109) were also not amplified in plants to detectable levels and accumulated in protoplasts to less than 3% of wild-type levels (Fig. 2B and D). The correlation between infectivity in planta and accumulation of high transcript levels in protoplasts suggests that DI RNA GA containing the 26- and 31-base deletions did not reach detectable levels in plants because of the inability of the RNA to replicate and/or instability, rather than a failure of packaging or movement.

We previously demonstrated that DI RNA GA transcripts containing the 31-base deletion or a nonoverlapping 32-base deletion (positions 117 to 148) could be restored to infectivity by insertion of a plasmid polylinker fragment of 31 or 62 bases, respectively (13). This result suggested that a decrease in size rather than deletion of specific *cis* sequences was responsible for the lack of infectivity of these transcripts. Replacement of the 31-base deletion with the 31-base polylinker fragment or of the 32-base deletion with the 62-base polylinker fragment increased the level of accumulation of DI RNA in protoplasts from undetectable levels to 32 and 104% of the DI RNA GA level, respectively (Fig. 2C and D). This result correlates well with the in planta studies and again suggests that loss of activity of these deleted transcripts in planta is manifested at the level of replication and/or stability of the RNA.

The abilities of artificially constructed DI RNAs containing various lengths of 5′- and 3′-end genomic RNA sequence to accumulate in protoplasts were also determined (Fig. 3). None of the constructs accumulated to >10% of wild-type levels. All constructs contained at least the minimal amounts of 3′- and 5′-end sequence required for infectivity of DI RNA GA in planta (67 and 188 bases, respectively [13]), and the sizes of two artificial DI RNAs (SDI-343 [343 bases] and SDI-368 [368 bases]) were within the range of infectious DI RNA GA molecules (327 to at least 379 bases). However, DI RNA GA differed from these constructs by containing non-TCV genomic RNA sequence at the 5′ end consisting of a segment from a TCV satellite RNA joined to a segment of unknown origin. In addition, DI RNA GA contained a repeat of a 36-base region at the crossover site between genomic RNA segments (Fig. 1). These results suggest that the simple presence of 3′- and 5′-terminal genomic sequence in a molecule of a size competent for accumulation is insufficient to ensure biological activity of TCV DI RNAs and that additional features are required for efficient accumulation in protoplasts.

We are grateful to C. D. Carpenter for critical reading of the manuscript.

This work was supported by grants MCB-9105890 and MCB-9315948 from the National Science Foundation to A.E.S.

## REFERENCES

1. **Calain, P., and L. Roux.** 1993. The rule of six, a basic feature for efficient replication of Sendai virus defective interfering RNA. J. Virol. **67**:4822–4830.
2. **Carpenter, C. D., and A. E. Simon.** 1994. Recombination between plus and minus strands of turnip crinkle virus. Virology **201**:419–423.
3. **Carpenter, C. D., J.-W. Oh, C. Zhang, and A. E. Simon.** Involvement of a stem-loop structure in the location of junction sites in viral RNA recombination. J. Mol. Biol., in press.
4. **De Groot, R. J., R. G. Van der Most, and W. J. M. Spaan.** 1992. The fitness of defective interfering murine coronavirus DI-a and its derivatives is decreased by nonsense and frameshift mutations. J. Virol. **66**:5898–5905.
5. **Fosmire, J. A., K. Hwang, and S. Makino.** 1992. Identification and characterization of a coronavirus packaging signal. J. Virol. **66**: 3522–3530.
6. **Hagino-Yamagishi, K., and A. Nomoto.** 1989. In vitro construction of poliovirus defective interfering particles. J. Virol. **64**:4108–4114.
7. **Heaton, L. A., J. C. Carrington, and T. J. Morris.** 1989. Turnip crinkle virus infection from RNA synthesized in vitro. Virology **170**:214–218.
8. **Huang, A. S., and D. Baltimore.** 1970. Defective interfering particles and viral disease processes. Nature (London) **226**:325–327.
9. **Jeong, Y. S., and S. Makino.** 1994. Evidence for coronavirus discontinuous transcription. J. Virol. **68**:2615–2623.
10. **Jorgensen, R. A., R. E. Cuellar, W. F. Thompson, and T. A. Kavanagh.** 1987. Structure and variation in ribosomal RNA genes of pea. Plant Mol. Biol. **8**:3–12.
11. **Levis, R., B. G. Weiss, M. Tsiang, H. Huang, and S. Schlesinger.** 1986. Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. Cell **44**:137–145.
12. **Li, X. H., L. A. Heaton, T. J. Morris, and A. E. Simon.** 1989. Turnip crinkle virus defective interfering RNAs intensify viral symptoms and are generated de novo. Proc. Natl. Acad. Sci. USA **86**:9173–9177.
13. **Li, X. H., and A. E. Simon.** 1991. In vivo accumulation of a turnip crinkle virus defective interfering RNA is affected by alterations in size and sequence. J. Virol. **65**:4582–4590.
14. **Makino, S., K. Yokomori, and M. M. C. Lai.** 1990. Analysis of efficiently packaged defective interfering RNAs of murine coronavirus: localization of a possible RNA-packaging signal. J. Virol. **64**:6045–6053.
15. **Re, G. G., and D. W. Kingsbury.** 1988. Paradoxical effects of Sendai virus DI RNA size on survival: inefficient envelopment of small nucleocapsids. Virology **165**:331–337.
16. **Roux, L., A. E. Simon, and J. J. Holland.** 1991. Effects of defective interfering viruses on virus replication and pathogenesis in vitro and in vivo. Adv. Virus Res. **40**:181–211.
17. **Tsiang, M., B. G. Weiss, and S. Schlesinger.** 1988. Effects of 5′-terminal modifications on the biological activity of defective interfering RNAs of Sindbis virus. J. Virol. **62**:47–53.
18. **Van der Most, R. G., P. J. Bredenbeek, and W. J. M. Spaan.** 1991. A domain at the 3′ end of the polymerase gene is essential for encapsidation of coronavirus defective interfering RNAs. J. Virol. **65**:3219–3226.
19. **Van der Most, R. G., R. J. de Groot, and W. J. M. Spaan.** 1994. Subgenomic RNA synthesis directed by a synthetic defective interfering RNA of mouse hepatitis virus: a study of coronavirus transcription initiation. J. Virol. **68**:3650–3666.
20. **White, K. A., J. B. Bancroft, and G. A. Mackie.** 1992. Coding capacity determines in vivo accumulation of a defective interfering RNA of clover yellow mosaic virus. J. Virol. **66**:3069–3076.