Enhanced Competitiveness of Tomato Bushy Stunt Virus Defective Interfering RNAs by Segment Duplication or Nucleotide Insertion

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We have analyzed atypical tomato bushy stunt virus defective interfering (DI) RNA species which accumulated during a passage series in protoplasts. We present a rationale for the order of appearance of these molecules and show, using competition assays, that either segment duplication or single nucleotide insertion can enhance DI RNA competitiveness. Possible mechanisms for the introduction of the modifications observed in these DI RNAs are discussed.

Tombusviruses are small icosahedral plant viruses which contain single-stranded positive-sense RNA genomes with lengths of approximately 4.8 kb. Defective interfering (DI) RNAs (22), containing various small noncontiguous portions of the viral genome, have been found associated with several members of this group (1, 6, 8, 10). These molecules range in size from approximately 0.4 to 0.8 kb and contain four highly conserved regions (I to IV) which include segments derived from the 5' terminus (I), an internal region (II), and the 3' terminus (III and IV) of the viral genome. Regions I, II, and IV are all essential for tomato bushy stunt tombusvirus (TBSV) DI RNA accumulation, whereas molecules in which region III is deleted are still biologically active (3). The essential regions likely contain cis-acting elements important for RNA replication.

We have shown previously that the formation of tombusvirus DI RNAs likely involves replicase-mediated RNA recombination events, in which segments of the genome are deleted in a stepwise manner (24). It was observed that the accumulation of a particular DI RNA species in protoplasts correlated with its competitive ability and that the major factor influencing competitiveness was replication efficiency. It was proposed, therefore, that replication efficiency was likely a major factor influencing the evolution of these molecules (24). In the present study, we investigate DI RNA generation and evolution by analyzing additional structural features of TBSV DI RNAs which influence their competitiveness.

DI-73 evolves to both larger and smaller forms during serial passage in protoplasts. Previously, we have studied the evolution of TBSV DI RNAs by coinoculating in vitro-generated transcripts of various DI RNAs species with helper genome transcripts and subjecting the viral RNAs to serial passage in protoplasts (24). In these passage series, transcripts of the genome of cucumber necrosis tombusvirus (CNV [19], which shows 64% nucleotide identity to TBSV [7]) were used to differentiate between input TBSV DI RNAs and DI RNAs that might be generated de novo from the helper. Previously, DI-73 (Fig. 1) transcripts were coinoculated with CNV genomic transcripts into cucumber protoplasts and subjected to 10 serial passages by using a portion of the isolated total nucleic acid as inocula (24). Total nucleic acid prepared from

each passage 24 h postinoculation was examined by polyacrylamide gel electrophoresis under denaturing conditions. In five of six passage series analyzed, smaller DI RNAs with structures similar to that of DI-72 (containing small deletions in their 3' region; Fig. 1) eventually dominated (24). These DI-72-like molecules were shown to be derived from the input DI-73. In one passage series, however, a larger DI RNA, DI-R1 (containing a direct duplication of a 130-nucleotide [nt] segment of region II; Fig. 1), began to accumulate and became the dominant DI RNA species in the middle of the passage series (24). As shown in Fig. 2, DI-R1 accumulated to easily detectable levels in passage 6 but was no longer detectable by passage 9. The dominant DI RNA at passage 10, designated DI-B103 (Fig. 2), exhibited similar electrophoretic mobility to that of DI-72.

Duplication of a segment in region II can increase DI RNA competitiveness. The observed timing of appearance of the DI RNAs in the passage series shown in Fig. 2 suggested that the larger DI-R1 molecules were more competitive than their intermediate-sized DI-73 precursors and that the DI-72-sized molecules (i.e., DI-B103), which ultimately dominated, were more competitive than the DI-R1 molecules. Competition assays were performed in cucumber protoplasts, using CNV genomic RNA transcripts (CNV-K2/M5 [19]) as the helper. Coinoculations of the helper with equimolar amounts of transcripts from previously constructed cDNA clones of DI-R1 and DI-73 (24) resulted in the abundant accumulation of progeny of each of these DI RNAs (Fig. 3A, lanes 3 and 4, respectively). No DI RNAs were detected in mock and helper only inoculations (Fig. 3A, lanes 1 and 2, respectively) or in inoculations containing only the DI RNAs (data not shown), indicating that the DI RNAs observed in helper coinoculations were not residual inocula. In coinoculations of helper with equimolar amounts of both DI-R1 and DI-73, DI-R1 accumulated to levels similar to those with single DI-R1 inoculations (Fig. 3A, cf. lanes 6 and 3); however, the accumulation of DI-73 was greatly reduced compared with the accumulation when it was inoculated singly with the helper (Fig. 3A, cf. lanes 6 and 4). This result showed that DI-R1 was more competitive than DI-73. As previously observed (24), DI-72 was more competitive than DI-73 in coinoculations (Fig. 3A, lane 8), but interestingly, DI-R1 was also more competitive than DI-72 (Fig. 3A, lane 7). These results were reproducible in independent experiments, and the results were essentially identical to those observed when transcripts of the TBSV genome were

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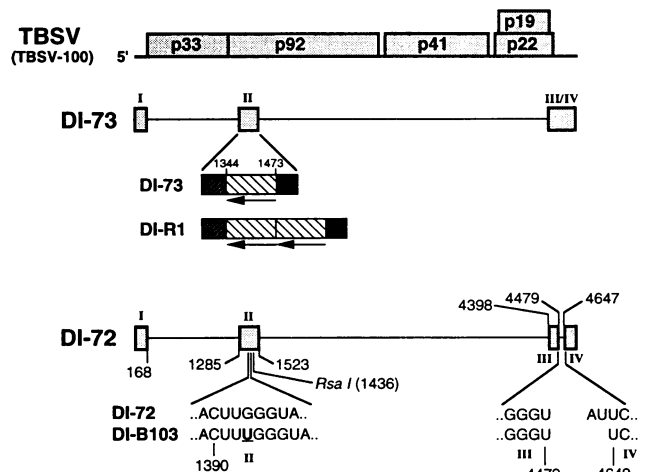
FIG. 1. Schematic representation of the genomic RNA of TBSV and of various TBSV DI RNAs. The organization of the coding regions in the 4.8-kb genomic RNA of TBSV is shown at the top, with the approximate molecular weight values (in thousands) of the encoded proteins (7). Regions from which the TBSV DI RNAs were derived are shown below as shaded boxes with the deleted intervening regions depicted as lines. The four regions which are conserved, to some degree, in all characterized, naturally occurring TBSV DI RNAs are indicated by roman numerals. Region III/IV represents a contiguous TBSV 3′ terminus which includes the segment between regions III and IV. Coordinates given correspond to those of the genomic RNA of TBSV (7) and represent junction sites, or internal regions, in the DI RNAs. An expanded version of region II is shown below DI-73, and region II of DI-R1 (containing a duplicated 130-nt segment) is also shown. Regions I and III/IV in DI-R1 are identical to those in DI-73. The differences in sequence between DI-72 and DI-B103 are shown below DI-72. DI-B103 contains an extra uracil residue in region II at position 1393 and has 2 fewer nt at the junction of regions III and IV. The position of a RsaI site in both DI-73 and DI-B103 is indicated. DI-73 and DI-72 are identical except that DI-72 contains a 167-nt deletion near its 3′ terminus. The data (see the text) suggest that the order of competitiveness of these DI RNAs is DI-B103 > DI-R1 > DI-72 > DI-73.

FIG. 3. Accumulation of various DI RNA progeny in cucumber protoplast infections with either CNV-K2/M5 or TBSV-100 transcripts as helper. Approximately 3×10^5 protoplasts were inoculated with helper and various equimolar amounts of DI RNA transcripts. Total nucleic acid was isolated 24 h postinoculation, aliquots were separated in 4.5% polyacrylamide gels containing 8 M urea, and the gels were stained with ethidium bromide. The viral RNAs present in the inoculum are indicated above the lanes. (A) Lanes: 1, mock infection; 2 to 8, CNV-K2/M5 (2 μg) with or without DI RNA transcripts, DI-R1 (0.39 μg), DI-73 (0.34 μg), and DI-72 (0.27 μg), as indicated. The positions of DI-R1, DI-72, and DI-73 in the gel are indicated. (B) Lanes: 1, mock infection; 2 to 9, 2 μg of either CNV-K2/M5 or TBSV-100 alone or with DI RNA transcript DI-R1 (0.39 μg) or DI-B103 (0.27 μg) or both, as indicated above the lanes. The positions of DI-R1 and DI-B103 in the gel are indicated.

used as the helper (data not shown). These data establish that DI-R1 (containing a segment duplication) is more competitive than either of the previously characterized smaller DI RNAs (DI-73 and DI-72).
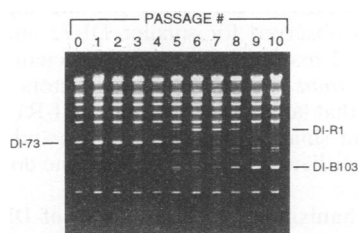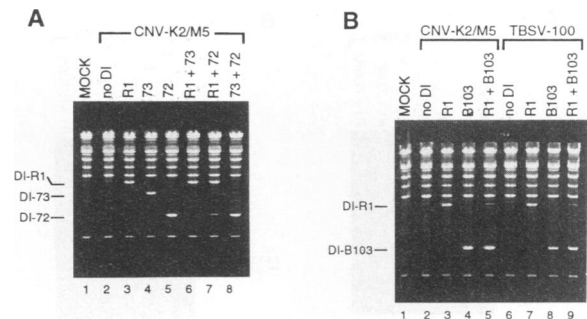

FIG. 2. Transition of DI-73 to both larger (DI-R1) and smaller (DI-B103) forms during 10 serial passages in cucumber protoplasts. The protoplasts were initially inoculated with CNV-K2/M5 (2 μg) and DI-73 (0.0034 μg). Passages were carried out by inoculating a new set of protoplasts with an aliquot (one-fourth) of the total nucleic acid isolated from the previous infection. Aliquots of total nucleic acid isolated 24 h postinoculation from the initial infection (lane 0) and from 10 serial passages (lanes 1 to 10, respectively) were separated in a 4.5% polyacrylamide gel containing 8 M urea, and the gel was stained with ethidium bromide. The positions of DI-73, DI-R1, and DI-B103 in the gel are indicated.

Direct segment duplication has been reported in DI RNAs of various RNA viruses (9, 12, 23); however, the significance of such duplications has not been determined. We have demonstrated that a duplicated segment in region II is able to confer enhanced competitiveness and that the fitness of the molecule is consistent with its observed order of appearance in the passage series (i.e., DI-R1 replaced DI-73). Duplication of segments in other naturally occurring TBSV DI RNAs in whole plants have been reported (3, 8), and, similar to DI-R1, they may also be more competitive than their counterparts which do not contain the duplications.

The role of region II in DI RNA accumulation is unclear, but it has been shown to be essential for the biological activity of TBSV DI RNAs, since deletion of the entire region, or of certain portions of it, render the molecules nonviable (3). In turnip crinkle virus DI RNAs, it was shown that replacement of deleted segments in nonviable DI RNAs with nonviral sequences of similar length resulted in restored viability (13). We have found that transcripts generated from a modified pDI-72 construct, in which region II was inverted, do not accumulate to detectable levels (26), suggesting that this region is not simply a spacer element. If cis-acting elements do reside in the region duplicated in DI-R1, this may increase the odds of trans-acting factors interacting with DI-R1 and may help to explain the enhanced competitive ability of this molecule. It is unclear, however, whether such an element would function in the plus or minus strand. We cannot, however, preclude the possibility that the duplication may be acting as a spacer element which is optimally positioning other cis-acting elements.

A single nucleotide insertion in region II can increase DI RNA competitiveness. The observation that DI-R1 was more competitive than DI-72 suggested that DI-B103, the molecule which ultimately dominated in the passage and which was predicted to be DI-72-like, must somehow be structurally different from DI-72. We used reverse transcription-PCR to amplify cDNAs to DI-B103, using the protocol described
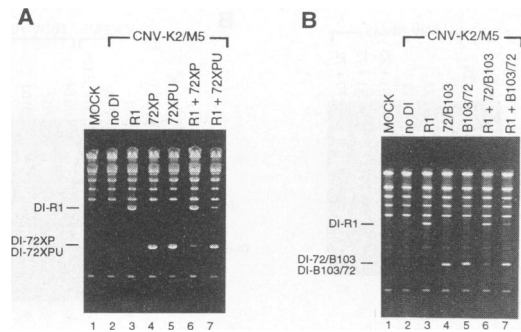
FIG. 4. Accumulation of DI RNA progeny in cucumber protoplast infections with CNV-K2/M5 transcripts as helper. Approximately 3 × 10⁵ protoplasts were inoculated with helper and various equimolar amounts of DI RNA transcripts. Total nucleic acid was isolated 24 h postinoculation, aliquots were separated in a 4.5% polyacrylamide gel containing 8 M urea, and the gels were stained with ethidium bromide. The viral RNAs present in the inoculum are indicated above the lanes. (A) Lanes: 1, mock infection; 2 to 7 CNV-K2/M5 (2 μg) with or without DI RNA transcripts, DI-R1 (0.39 μg), DI-72XP (0.27 μg), and DI-72XPU (0.27 μg). The positions of DI-R1, DI-72XP, and DI-72XPU in the gel are indicated. (B) Lanes: 1, mock infection; 2 to 7, CNV-K2/M5 (2 μg) with or without the DI RNA transcripts, DI-R1 (0.39 μg), DI-72/B103 (0.27 μg), and DI-B103/72 (0.27 μg). The positions of DI-R1, DI-72/B103, and DI-B103/72 are indicated.

previously (24), and the amplified molecules were cloned and sequenced. The DI-B103 molecules differed from DI-72 at only three nucleotide positions; an additional U residue was present at position 1393 and 2 nt (A and U) were deleted at the junction of regions III and IV (Fig. 1). Transcripts of DI-B103 were synthesized in vitro and used in competition experiments with DI-R1 (Fig. 3B). When coinoculated individually with CNV-K2/M5 helper, progeny of each DI RNA accumulated to similar levels (Fig. 3B, lanes 3 and 4). When both DI-R1 and DI-B103 were coinoculated with CNV-K2/M5 helper, DI-B103 progeny accumulated to levels higher than those of DI-R1 progeny (Fig. 3B, lane 5). DI-B103 was also more competitive than DI-R1 when TBSV helper (TBSV-100) was used (Fig. 3B, lane 9). This suggested that one of the three single nucleotide modifications in DI-B103, not present in DI-72, must be responsible for the observed enhanced competitiveness.

To test whether the extra U residue in region II could, on its own, cause enhanced competitiveness, region II of the DI-B103 clone was amplified with primers which introduced restriction sites XbaI and PstI at the 5' and 3' ends, respectively, of the PCR product. The DI-B103 region II was then used to replace the comparable region in DI-72XP (a DI-72 derivative containing XbaI and PstI sites introduced between regions I and II and regions II and III, respectively). The resulting construct, DI-72XPU, was identical to DI-72XP, except that the former contained an extra U residue at coordinate 1393 (as confirmed by sequencing). Both DI-72XP and DI-72XPU accumulated to similar levels when singly coinoculated with CNV-K2/M5 helper (Fig. 4A, lanes 4 and 5), but in competition experiments with DI-R1, only DI-72XPU was able to compete more effectively than DI-R1 (Fig. 4A, cf. lanes 6 and 7). Results essentially identical to these were observed when TBSV-100 was used as helper (data not shown). These data indicated that the additional U residue in region II could, on its own, confer enhanced competitive ability.

To determine whether the 2-nt deletion at the junction between regions III and IV in DI-B103 was also able to

independently enhance competitive ability, a chimeric molecule of DI-72 and DI-B103 was constructed by using a RsaI site present in the 3' portion of region II (coordinate 1436) (Fig. 1). DI-72/B103 contained the DI-72 sequence 5' to the RsaI site and the DI-B103 sequence 3' to the RsaI site and thus contained the 2-nt deletion between regions III and IV but not the U residue insertion in region II. DI-72/B103 was unable to effectively compete against DI-R1 in coinfections (Fig. 4B, lane 6), indicating that the 2-nucleotide deletion, on its own, could not confer enhanced competitiveness. The reciprocal chimera, DI-B103/72, containing only the extra U residue in region II, was more competitive than DI-R1 (Fig. 4B, lane 7), confirming earlier results with DI-72XP (Fig. 4A). Both the DI-72/B103 and DI-B103/72 constructs were sequenced completely to confirm that they contained only the predicted modifications, and all of these results were reproducible in independent experiments. Taken together, these data indicate that a single nucleotide insertion of a U residue at position 1393 is able to enhance TBSV DI RNA competitiveness.

It is interesting to note that region II in CNV DI RNAs contains the additional U residue at the corresponding position (6, 18), as does the CNV genome (20). The additional U residue in region II of DI-B103 therefore makes it more similar to the region II of CNV DI RNAs. If this region does represent a cis-acting element, making it more CNV-like might enhance its ability to interact with CNV-encoded trans-acting factors. This is relevant in light of the fact that CNV was the helper in the passage series in which DI-B103 was formed. Our competition assays indicated, however, that both DI-B103 and DI-72XPU (both of which contain the extra U residue) showed approximately the same level of competitiveness over DI-R1, with either TBSV or CNV as the helper. This suggests that the higher level of competitiveness of DI RNAs containing the extra U residue is not helper specific.

A previous study of sequences essential for TBSV DI RNA accumulation indicated that a portion of region II containing the extra U residue can be deleted without eliminating the biological activity of the DI RNA (3). Our results show that although this region may be dispensable for accumulation, it can have a marked effect on DI RNA competitiveness. This result illustrates that nonessential regions of DI RNAs may play important auxiliary roles in DI RNA fitness. Furthermore, the observation that both of the modifications which enhanced competitiveness mapped to region II suggests that this region can play a significant role in DI RNA fitness and evolution.

Previously, we have shown that the smaller DI-72 is more competitive than the larger DI-73 (24). We suggested that size alone was not entirely responsible for the higher level of competitiveness observed for smaller DI-72 and that the 3' deletion in DI-72 may make it more proficient at recruiting and/or utilizing trans-acting replication factors. Our present results indicate that larger DI RNAs (e.g., DI-R1) can be more competitive than smaller ones (e.g., DI-73 and DI-72), thus confirming our earlier proposal that size alone does not dictate competitiveness.

Possible mechanisms for the formation of DI-R1 and DI-B103. Previously, we proposed a replicase-mediated copy choice-type mechanism (11, 17) to explain the formation of tombusvirus DI RNAs (24), and a similar mechanism may be responsible for the generation of the direct duplication in DI-R1. The most stable computer-generated structure (4) of the 130-nt segment which was duplicated in DI-R1 is shown in Fig. 5A. The sequence is predicted to form a hairpin structure (Fig. 5A), the majority of which is also present when the entire DI-73 molecule is folded (data not shown). The formation of a hairpin such as that shown in Fig. 5A could potentially assist
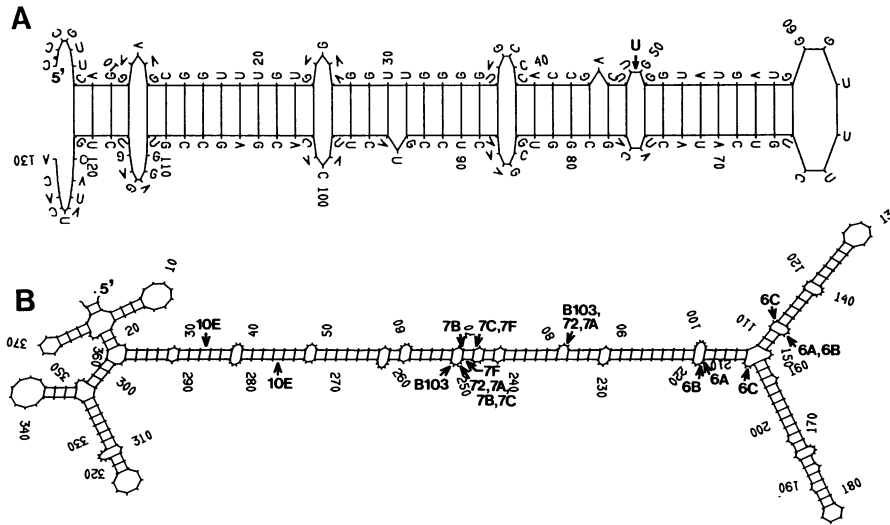
FIG. 5. Computer predicted secondary structures for 130-nt segment which was duplicated in DI-R1 (A) and region III/IV of DI-73 (B). The structures were generated by using the program FOLD (4), and the calculated free energies were −37.8 and −98.4 (A and B, respectively). Position 1 in panel A corresponds to coordinate 1344 of the TBSV genome (7), and position 1 in panel B corresponds to coordinate 4398. In panel A, the position of the extra U residue identified in DI-B103 is indicated. In panel B, the junction sites observed between regions III and IV in DI-B103 (B103) and previously characterized TBSV DI RNAs (72, 6A to 6C, 7A to 7C and 7F, and 10E [24]) are indicated by arrows.

the replicase (during negative-strand synthesis) in transferring to a 3′ position on the template after copying the segment (i.e., by placing the sites of dissociation and reinitiation in close proximity). Evidence suggests that intramolecular recombination (24) and recombination during negative-strand synthesis (25) can occur in tombusvirus infections.

Two separate events were likely involved in the formation of DI-B103: deletion of a 169-nt segment between regions III and IV and insertion of a U residue in region II. We reported previously the possible involvement of guide sequences in directing sites of recombination and also the potential for base-paired heteroduplex structures to form between same-sense sequences at the upstream and downstream junction sites (24). In the computer-predicted structure of region III/IV, the junction sites of the deletion between regions III and IV in DI-B103 map close to each other, as do previously identified junctions of region III and IV of other DI-72-like molecules (Fig. 5B). The close proximity of the junction sites on the predicted structure suggests that an intramolecular secondary structure may be involved in the generation of the deletions by (i) providing an obstacle for the replicase which may promote its stalling and dissociation and (ii) positioning the dissociation and reinitiation sites close to each other. A similar proposal has been made for the formation of DI RNAs of broad bean mottle virus (21), and an analogous mechanism has been shown to function in intermolecular nonhomologous RNA recombination in brome mosaic virus (16). A structure similar to that in Fig. 5B could also theoretically form between two regions III/IV, suggesting that intermolecular recombination events similar to those described for brome mosaic virus (16) may also be possible. Intermolecular recombination has previously been demonstrated in tombusvirus infections of both protoplasts and whole plants (25). Secondary structures have also been implicated in the formation of DI RNAs in corona-viruses (15) and flock house nodavirus (14). For TBSV DI RNA formation, both secondary structure in the template(s) and guide sequence(s) in the nascent strands may function either together or independently to direct the deletion of sequences.

The introduction of the U residue into region II of DI-B103 may have also been the result of a recombination event involving DI RNAs. Recombinant tombusvirus RNAs in which nontemplated U and A residues were identified at junctions have been described previously (24, 25). Alternatively, we cannot preclude the possibility that polymerase error (5) may be responsible for the addition of the U residue. The presence of other U residues at the site of the addition suggests that a replicase stuttering-type mechanism (2) may have been responsible for the addition.

REFERENCES

1. **Burgyan, J., F. Grieco, and M. Russo.** 1989. A defective interfering RNA molecule in cymbidium ringspot virus infections. J. Gen. Virol. **70:**235–239.
2. **Carpenter, C. D., P. J. Cascone, and A. E. Simon.** 1991. Mutations in a satellite RNA of turnip crinkle virus result in addition of poly(U) in vivo. Virology **183:**595–601.
3. **Chang, Y. C., M. Borja, H. B. Scholthof, A. O. Jackson, and T. J. Morris.** Unpublished data.
4. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
5. **Drake, J. W.** 1993. Rates of spontaneous mutation among RNA viruses. Proc. Natl. Acad. Sci. USA **90:**4171–4175.
6. **Finnen, R. L., and D. A. Rochon.** 1993. Sequence and structure of defective interfering RNAs associated with cucumber necrosis virus infections. J. Gen. Virol. **74:**1715–1720.
7. **Hearne, P. Q., D. A. Knorr, B. I. Hillman, and T. J. Morris.** 1990. The complete genome structure and synthesis of infectious RNA from clones of tomato bushy stunt virus. Virology **177:**141–151.
8. **Hillman, B. I., J. C. Carrington, and T. J. Morris.** 1987. A

defective interfering RNA that contains a mosaic of a plant virus genome. Cell **51:**427–433.

9. **Jalanko, A., and H. Soderlund.** 1985. The repeated regions of semliki forest virus defective-interfering RNA interferes with the encapsidation process of the standard virus. Virology **141:**257–266.

10. **Knorr, D. A., R. H. Mullin, P. Q. Hearne, and T. J. Morris.** 1991. *De novo* generation of defective interfering RNAs of tomato bushy stunt virus by high multiplicity passage. Virology **181:**193–202.

11. **Lazzarini, R. A., J. D. Keene, and M. Schubert.** 1981. The origins of defective interfering particles of the negative-strand RNA viruses. Cell **26:**145–154.

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. **Li, Y., and L. A. Ball.** 1993. Nonhomologous RNA recombination during negative-strand synthesis of flock house virus RNA. J. Virol. **67:**3854–3860.

15. **Makino, S., C.-K. Shies, L. H. Soe, S. C. Baker, and M. M. C. Lai.** 1988. Primary structure and translation of a defective interfering RNA of murine coronavirus. Virology **166:**550–560.

16. **Nagy, P. D., and J. J. Bujarski.** 1993. Targeting the site of RNA-RNA recombination in brome mosaic virus with antisense sequences. Proc. Natl. Acad. Sci. USA **90:**6390–6394.

17. **Perrault, J.** 1981. Origin and replication of defective interfering particles. Curr. Top. Microbiol. Immunol. **93:**151–207.

18. **Rochon, D. M.** 1991. Rapid *de novo* generation of defective interfering RNA by cucumber necrosis virus mutants that do not express the 20-kDa nonstructural protein. Proc. Natl. Acad. Sci. USA **88:**11153–11157.

19. **Rochon, D. M., and J. C. Johnston.** 1991. Infectious transcripts from cloned cucumber necrosis virus cDNA: evidence for a bifunctional subgenomic promoter. Virology **181:**656–665.

20. **Rochon, D. M., and J. H. Tremaine.** 1989. Complete nucleotide sequence of the cucumber necrosis virus genome. Virology **169:**251–259.

21. **Romero, J., Q. Huang, J. Pogany, and J. J. Bujarski.** 1993. Characterization of a defective interfering RNA components that increase symptom severity of broad bean mottle virus infections. Virology **194:**576–584.

22. **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.

23. **White, K. A., J. B. Bancroft, and G. A. Mackie.** 1991. Defective RNAs of clover yellow mosaic virus encode nonstructural/coat protein fusion products. Virology **183:**479–486.

24. **White, K. A., and T. J. Morris.** 1994. Nonhomologous RNA recombination in tombusviruses: generation and evolution of defective interfering RNAs by stepwise deletions. J. Virol. **68:**14–24.

25. **White, K. A., and T. J. Morris.** 1994. Recombination between defective tombusvirus RNAs generates functional hybrid genomes. Proc. Natl. Acad. Sci. USA **91:**3642–3646.

26. **White, K. A., and T. J. Morris.** Unpublished data.