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## De Novo Generation of Defective Interfering RNAs of Tomato Bushy Stunt Virus by High Multiplicity Passage

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Defective interfering (DI) RNAs were generated *de novo* in each of 12 independent isolates of tomato bushy stunt virus (TBSV) upon serial passage at high multiplicities of infection (m.o.i.) in plants, but not in any of 4 additional isolates after 11 serial passages at low m.o.i. The DI RNAs were detected in RNA isolated from virus particles and in 2.3 M LiCl-soluble RNA fractions isolated from inoculated leaves. Symptom attenuation leading to persistent infections was closely correlated with the passage in which DIs first developed. Comparisons of nucleotide sequences of 10 cDNA clones from 2 DI RNA populations and with a previously characterized TBSV DI RNA revealed the same four regions of sequence from the TBSV genome were strictly conserved in each of the DI RNAs: the virus 5' leader sequence of 168 bases; a region of approximately 200-250 bases from the viral polymerase gene; approximately 70 bases from the 3' terminus of the viral p19 and p22 genes; and approximately 130 bases from the 3' terminal noncoding region. Conservation of the sequence motif present in all of the DIs suggests that there might be a common mechanism of DI formation as well as selection pressure to maintain sequences essential for replication and encapsidation. © 1991 Academic Press, Inc.

### INTRODUCTION

Defective interfering particles (DIs) are deletion mutants that interfere with replication of the virus from which they were derived (for reviews, see; Holland, 1990; Schlesinger, 1988; Barrett and Dimmock, 1986). DIs have been reported for virtually every family of animal viruses and are thought to occur universally (Perrault, 1981). The DIs associated with RNA viruses of animals have proved particularly useful for identifying sequence elements involved in viral functions such as encapsidation and replication (Levis *et al.*, 1986; Weiss *et al.*, 1989; Schlesinger, 1988) and they have also been implicated as important components in driving viral evolution (Steinhauer and Holland, 1987; De Polo *et al.*, 1987). Although most frequently studied in cell culture, there is increasing evidence that DIs play a role in natural infections in modulating disease symptoms and in establishing virus persistence (Barrett and Dimmock, 1986). However, due to complications presented by the immune and interferon systems in animal hosts, this role has been difficult to establish unambiguously (Huang, 1988).

In comparison to studies with animal viruses, relatively few plant viruses with defective genomes have been documented (Morris and Hillman, 1989). Among these, DI RNAs which meet the criteria of Huang and Baltimore (1970) have been described for only two related groups of plant viruses, the tombusviruses and carmoviruses (see Morris and Knorr, 1990, for a review). Biological and molecular characterization of a novel RNA species associated with the cherry strain of tomato bushy stunt virus (TBSV) provided the first definitive demonstration of DI RNAs associated with a plant virus infection (Hillman *et al.*, 1987). It was also shown that the lethal necrosis syndrome normally caused by TBSV in solanaceous hosts was prevented by the DI RNA and persistent infections resulted. The DI RNA (termed DI1) was shown to be a collinear deletion mutant of TBSV that was dependent on the parent virus for both replication and encapsidation. TBSV DI RNAs were shown to reduce virus accumulation in infected plants (Hillman *et al.*, 1987) and to inhibit genomic RNA replication in protoplasts (Jones *et al.*, 1990). Subsequently, DI RNAs were found associated with two other tombusviruses, cymbidium ringspot virus (Burgyan *et al.*, 1989) and cucumber necrosis virus (D. Rochon, personal communication; Y. Chang and J. Morris, unpublished). Among the carmoviruses, which are structurally related to tombusviruses (Morris and Carrington, 1988), turnip crinkle virus (TCV) has been

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. M59041-M59050.

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shown to support DI RNAs (Li *et al.*, 1989). It is interesting that in addition to DI RNAs, TCV also supports a number of small satellite RNAs, and at least one chimeric RNA with regions derived from both satellite and TCV genomic sequences.

The origin of DI RNAs has been attributed in some cases to template "hopping" or "switching" by the viral RNA polymerase which produces deletions in the viral genome (Lazzarini, 1981). In general, DIs can arise in cell cultures when a fully competent virus is passaged serially at high multiplicities of infection (m.o.i.). In such experiments, preexisting DIs would appear as species of uniform size after serial passage of independent isolates, while *de novo* generation of DIs from the helper virus genome would result in the appearance of DI species with different sizes (Holland, 1990). Accordingly, we investigated the *de novo* generation of TBSV DI RNAs by analogous high m.o.i. passage experiments performed *in planta*. Our results indicate that TBSV DI RNAs arise spontaneously upon passage, and that the *de novo* appearance of DI RNAs is closely associated with attenuation of symptoms induced by the virus. We have also characterized biologically active DI RNAs from two independent populations generated during passage. Comparisons of sequences revealed that DI RNAs retained four conserved regions from the TBSV genome.

## MATERIALS AND METHODS

### Virus isolates, clones, and inoculations

Detailed studies of the biology, molecular cloning, and genome characterization of the cherry strain of TBSV used in this study have been previously reported (Hillman *et al.*, 1985, 1989; Hearne *et al.*, 1990). The DI RNA-free culture of the TBSV-cherry strain was isolated by passage of a single local lesion from *Nicotiana glutinosa* to the systemic host *N. clevelandii* from which a TBSV stock preparation was purified, stored in 50% glycerol at  $-20^{\circ}$ , and used for all subsequent inoculations. This stock was determined to be free of DI RNAs by both biological and Northern blot analysis as described by Hillman (1986).

A serial passage experiment was initiated from 16 independent local lesion isolates established from DI-free TBSV inoculated onto *N. glutinosa*. Eight isolates were passaged sequentially in *N. clevelandii* and eight in *N. benthamiana*. In each case, six of the independent isolates inoculated to each host species were passaged without dilution (high m.o.i.) and two were passaged at a sap dilution of 1/200 (low m.o.i.). The isolates passaged in *N. clevelandii* at high m.o.i. were designated C1–C6 and those passed at low m.o.i. were designated C13 and C14. Similarly, the isolates

passed in *N. benthamiana* at high m.o.i. were designated B7–B12 and those passed at low m.o.i. were designated B15 and B16. Inoculum for high m.o.i. passes was prepared by grinding 1.0 cm<sup>2</sup> of inoculated leaf tissue in 100  $\mu$ l of inoculation buffer (0.1 M potassium phosphate, pH 7.0, 1% Celite 545 [Fisher Scientific, Springfield, NJ]). For low m.o.i. passes, 10  $\mu$ l of sap was diluted to 2 ml with inoculation buffer. Plants were maintained at a constant temperature of 21 $^{\circ}$  and passages were performed at 7-day intervals for 11 weeks. Tissue was harvested from inoculated leaves at the time of passage and stored at  $-80^{\circ}$  until analyzed. Observations on symptom severity and plant survival were recorded at regular intervals throughout the passage experiment.

### DI RNA extraction and analysis

Total RNA was extracted from 0.3-g samples of inoculated leaves and fractionated in 2.3 M LiCl-soluble (contains DI RNA and viral-specific dsRNA) and -insoluble (total ssRNA) fractions as described previously (Hillman *et al.*, 1985, 1987). The LiCl-soluble RNA fractions were analyzed for the presence of DI RNAs by electrophoresis on nondenaturing 5% polyacrylamide gels in TBE buffer (0.04 M Tris-borate, 1 mM EDTA, pH 8.3) and stained with ethidium bromide. Virus particles were purified from separate 1.0-g samples of the same tissue by two cycles of precipitation in 8% polyethylene glycol, 0.2 M NaCl (Hillman *et al.*, 1985), and encapsidated RNAs then were extracted with phenol. Virion RNAs and LiCl-insoluble fractions were analyzed on 1.5% agarose gels or denaturing 4.5% polyacrylamide gels containing 7 M urea in TBE buffer, after denaturation of the samples in 50% formamide and 5% formaldehyde. RNA was transferred by electroblotting onto nylon membranes (Zeta-probe, Bio-Rad) as described by the manufacturer, and Northern analysis was performed as described (Hillman *et al.*, 1987).

### cDNA cloning and polymerase chain reaction amplification

Purified virion and DI RNAs, fractionated on sucrose density gradients, were used as templates for synthesizing double-stranded (ds) cDNAs essentially as described by Dawson *et al.* (1986). Approximately 5  $\mu$ g of DI RNA was denatured with methyl mercuric hydroxide, annealed with a synthetic oligonucleotide complementary to the 3' terminal 19 nucleotides of TBSV, and then treated with 20 units of AMV reverse transcriptase (Promega). Second-strand reactions of 100  $\mu$ l, containing the first-strand RNA:cDNA hybrids, 10 units of DNA polymerase I (BRL), 500  $\mu$ M each dNTP, and 20 ng of RNase A in 1 $\times$  DNA polymerase buffer (50 mM

potassium phosphate, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM β-mercaptoethanol), were incubated for 12 hr at 13°.

The polymerase chain reaction (PCR) was used to remove homopolymer tails from DI RNA clones made from virion RNAs, prepare cDNAs made from LiCl-soluble RNAs for cloning, and generate templates for *in vitro* RNA transcription. First-strand cDNA synthesis was initiated using a primer complementary to the 3' terminal 28 nt of TBSV. Samples of the RNA:cDNA hybrids then were used in PCR reactions containing the 5' oligonucleotide primer 5'GGGGCCCTAATACGACTC-ACTATAGGAAATTCTCCAGGATTTCTCGACC, which contains a bacteriophage T7 RNA polymerase promoter sequence fused to the 5' terminal 24 nt of TBSV, plus additional 3' primer. Alternatively, PCR amplifications were performed using a different 3' proximal primer, 5'GCATGCCCGGGCTGCATTTCTGC, which contained *Sma*I and *Sph*I restriction sites to facilitate cloning. Conditions for PCR were as follows: 90 μl of 1× *Taq* polymerase reaction buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl<sub>2</sub>; 0.1% gelatin; 200 μM each dNTP) containing approximately 10 ng of *Pst*I-digested plasmid DNA, and 50 pM of each oligonucleotide primer were overlaid with mineral oil, boiled for 5 min, quenched on ice, and then treated with *Taq* polymerase (Cetus/Perkin-Elmer, 2.5 U in 10 μl of 1× reaction buffer). Temperature cycling (25 sec at 95°, 50 sec at 50°, 2 min at 72°) was carried out for 25 cycles using an automatic thermal cycler (Cetus/Perkin-Elmer or MJ Research, Inc.). Yields were approximately 5 μg for each PCR amplification of cDNA.

Cloning the DI cDNAs was accomplished by adding polycytidine tails to non-PCR-amplified templates using terminal deoxynucleotidyltransferase (BRL) followed by ligation to oligo(dG)-tailed pUC9 DNA (Boehringer/Mannheim). Alternatively, PCR-amplified cDNAs were restricted with *Apa*I and *Sph*I and then inserted into pDK41 (Hearne *et al.*, 1990). DI cDNAs were used to transform *Escherichia coli* strain DH5α F' (BRL) (Hanahan, 1983).

#### Sequencing and *in vitro* transcription of PCR-amplified cDNAs

Templates for sequencing were prepared either by asymmetric PCR amplification of cloned cDNAs according to Gyllensten (1988) or by subcloning the inserts into pZF19U (Mead *et al.*, 1986) and preparing single-stranded DNA as outlined in Sambrook *et al.* (1989). Sequencing reactions were performed using *Taq* polymerase and reagents as recommended by the supplier (Stratagene). A series of standard sequencing primers and custom DI-complementary primers allowed determination of the sequence of each clone on

both strands. To test the biological activity of the cloned DI RNA sequences, PCR-amplified inserts were transcribed with T7 RNA polymerase as described by Janda *et al.* (1987) and used to inoculate plants together with DI-free TBSV RNA or virions as described previously (Hearne *et al.*, 1990).

## RESULTS

### *De novo* generation of TBSV DI RNAs

To test the hypothesis that TBSV DI RNAs arise spontaneously during virus infection, we performed a passage experiment similar to those reported using animal virus systems. Sixteen independent DI-free TBSV isolates, each derived from a single local lesion of the DI-free TBSV stock culture, were passaged serially on two systemic plant hosts, *N. clelandii* and *N. benthamiana*. On each host species, six isolates were passed at high m.o.i. and two control isolates were passed at low m.o.i. In these hosts, DI-free TBSV induces lethal necrosis within 7–10 days postinoculation (d.p.i.), except in the presence of DI RNAs which are associated with attenuation and the establishment of persistent infections (Hillman *et al.*, 1987). For each local lesion isolate, detection of DI RNAs and appearance of symptom attenuation were noted and the results are summarized in Table 1. None of the isolates passed at low m.o.i. developed attenuated symptoms, but by the eleventh passage, each of the isolates passed at high m.o.i. were inducing attenuated symptoms. The passage at which symptom attenuation appeared in each of the high m.o.i. isolates was not uniform, occurring as early as passage 3 for two isolates (C6 and B7), and as late as passage 11 for others (B11 and B12). As summarized in Table 1, in each case where symptom attenuation was apparent, low molecular weight (LMW) RNAs of approximately 400–600 bases were detected in the LiCl-soluble fraction of the cellular RNA extracts. Examples of these are presented for the different isolates at passage 11 in Figs. 1C and 1D. The small RNAs which developed in each of the high m.o.i. isolates hybridized to both 5'- and 3'-proximal TBSV genomic sequences, but generally were larger (~600 bases) than the previously characterized DI1 RNA species (~400 bases). In 9 of 12 high m.o.i. isolates, the passage at which DI RNAs were detected either coincided with or just preceded the passage at which symptom attenuation was first observed. However, in three isolates (C2, B8, and B9) DI RNAs were detected several passes prior to symptom attenuation, suggesting that all defective RNAs that were generated were not equally able to interfere with virus symptoms.

TABLE 1

RECORD OF SYMPTOM ATTENUATION AND DI INCIDENCE FOR EACH OF THE 16 TBSV LOCAL LESION ISOLATES USED IN THE PASSAGE EXPERIMENT

Isolate no. and host <sup>a</sup>	Inoculum concentration	Passage no. <sup>b</sup>	
		Symptoms attenuated	DI RNA detected
C1	high m.o.i.	9	9
C2	high m.o.i.	9	6
C3	high m.o.i.	8	7
C4	high m.o.i.	5	5
C5	high m.o.i.	8	7
C6	high m.o.i.	3	3
B7	high m.o.i.	3	3
B8	high m.o.i.	9	6
B9	high m.o.i.	8	6
B10	high m.o.i.	5	4
B11	high m.o.i.	11	11
B12	high m.o.i.	11	11
C13	low m.o.i.	N	N
C14	low m.o.i.	N	N
B15	low m.o.i.	N	N
B16	low m.o.i.	N	N

<sup>a</sup> Each of the single lesion isolates is numbered sequentially with the host in which the serial passages were made designated C for *N. clevelandii* and B for *N. benthamiana*.

<sup>b</sup> Passage number identifies the number of serial passages on the designated host plants after the initial local lesion transfer at which attenuated symptoms were observed or DIs were detected. N designates inoculations that resulted in lethal necrosis at 7 d.p.i. and in which DI RNAs could not be detected.

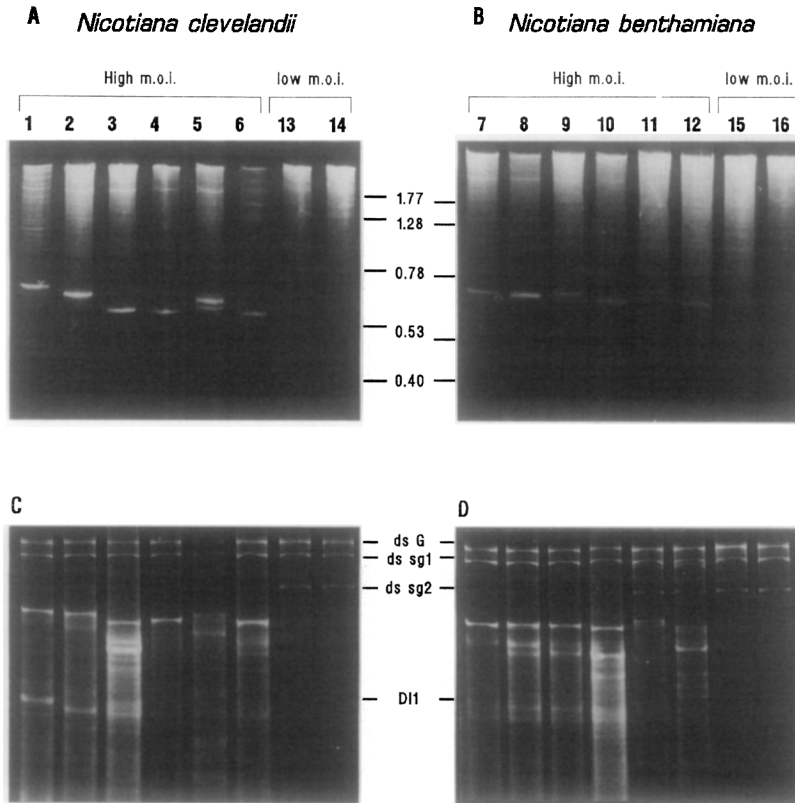
The experiment was terminated after passage 11 at which time symptoms in each of the high m.o.i. isolates had become attenuated. Gel analysis of ssRNAs isolated from virus purified from plants at passage 11 and of the LiCl-soluble RNA fraction from each of the high and low m.o.i. isolates passaged in *N. clevelandii* (Figs. 1A and 1C) or *N. benthamiana* (Figs. 1B and 1D) then was performed. It is evident from the analysis of virion RNAs on the high-resolution polyacrylamide gels (Figs. 1A and 1B) that each of the 12 high m.o.i. isolates contained 1 or 2 major DI RNA species of a discrete size that were not detectable in any of the 4 low m.o.i. isolates. Additional analyses of the LiCl-soluble fraction isolated from the same infected plants of each isolate (Figs. 1C and 1D) showed a complex population of low molecular weight RNAs in each of the high m.o.i. lines in addition to the prominent DI species corresponding in size to the DI RNA present the virion RNA fraction. Northern analyses were performed on all of these samples to confirm the TBSV-specific nature of the low molecular weight DI-like species. An analysis of LiCl-fractionated RNAs from tissue and virion RNAs which is typical and representative for 1 high m.o.i. DI RNA-containing isolate (B10, passage 11) and 1 DI-free low m.o.i. isolate (B15, passage 11) is given in Fig.

2. The results clearly demonstrate that the low m.o.i. line is completely free of detectable DI RNA. Furthermore, the DI RNA species purified from virions in the high m.o.i. line is the same size as the major DI RNA species present in the 2.3 M LiCl-soluble fraction isolated from the same tissue, suggesting both that the RNA is highly structured and that it is encapsidated. These results are consistent with the properties of previously characterized DI RNAs found to be associated with this strain of TBSV (Hillman *et al.*, 1987).

#### Molecular characterization of the *de novo* generated DI RNAs

The DI RNAs from two representative isolates passed at high m.o.i. (C6, passage 11, and B10, passage 11) were sequenced to compare them with each other and with the smaller DI1 species characterized previously (Hillman *et al.*, 1987). The encapsidated DI RNAs from isolates C6 and B10 migrated during electrophoresis as apparently homogeneous populations with an estimated size of approximately 600 bases. Complete sequences for four cDNA clones generated from C6 LiCl-soluble RNAs and six cDNA clones generated from B10 virion RNAs were determined and are presented in Fig. 3. Although derived independently, the sequence alignments show that the DI RNAs from the C6 and B10 TBSV isolates are strikingly similar. Each of the DI RNAs appears to have been formed by deletion of three large internal regions from the TBSV genome, leaving four regions of sequence with highly conserved boundaries: (region I) the TBSV 5' leader sequence including the initiator methionine codon; (region II) a block of approximately 200 to 250 bases from the putative polymerase gene; (region III) approximately 70 bases from the 3' terminus of the viral P19 and P22 open reading frames (ORFs); and (region IV) the terminus of the 3' nontranslated region (see Fig. 3). In each of the clones, patterns of small deletions and nucleotide replacements were identified, indicating that each sequence represented an independent DI RNA. Nevertheless, the entire sequence of each of the DI RNA clones clearly was derived from the TBSV genome without rearrangement.

Biological activity of the cloned DI cDNAs was assessed by coinoculating plants with *in vitro* RNA transcripts and DI-free virus. For each sample, a DI RNA of expected size accumulated to high titer in the presence of TBSV, but not when transcripts were inoculated without virus (Fig. 4). As shown previously with cloned TBSV, a dinucleotide cap was not required for infectivity of the *in vitro* transcripts of cloned DI RNAs. The individual cloned DI RNAs attenuated symptoms to the same degree as the parental, noncloned B10 DI RNA population. Interestingly, the homogeneous DI RNA inoculum derived from cloned cDNAs (Fig. 4, lane 2) gave rise to a complex pattern of smaller RNA spe-



**FIG. 1.** Identification of DI RNAs in 16 independent TBSV local lesion isolates after 11 serial passes in *Nicotiana clevelandii* or *Nicotiana benthamiana*. Virions were purified from inoculated leaves 1 week after infection and the viral RNAs were phenol extracted, denatured, and electrophoresed in 4.5% polyacrylamide, 7 M urea gels and the gels were stained with ethidium bromide [(A) and (B)]. Lanes 1–6 in (A) and 7–12 in (B) are isolates passed at high m.o.i. in which DI RNAs appeared. Lanes 7 and 8 in (A) and 13 and 14 in (B) were isolates passed at low m.o.i. in which DI RNAs were not detected. The numbers between (A) and (B) identify the positions of single-stranded RNA molecular weight markers with sizes in kilobases. (C) and (D) contain LiCl-soluble fractions of total RNA isolated from the same leaves used for the virion preparations analyzed above. The positions of dsRNAs normally found in TBSV-infected plants are shown between (C) and (D) [ds G, genomic RNA; ds sg1, subgenomic RNA 1 (2.2 kilobases); ds sg2, subgenomic 2 (0.9 kilobases)]. The bands appearing below ds sg2 RNA are DI RNAs that hybridized with TBSV-specific probes in Northern blots (not shown). The position of the 395-base DI1 RNA is indicated.

cies in the infected plants, suggesting that DI RNAs may be capable of sequence evolution upon reintroduction into plants. *In vitro* RNA transcripts derived from cDNA clones of C6 and B10 DI RNAs have also been found to replicate in protoplasts in the presence of DI-free TBSV (Y. Chang and J. Morris, unpublished).

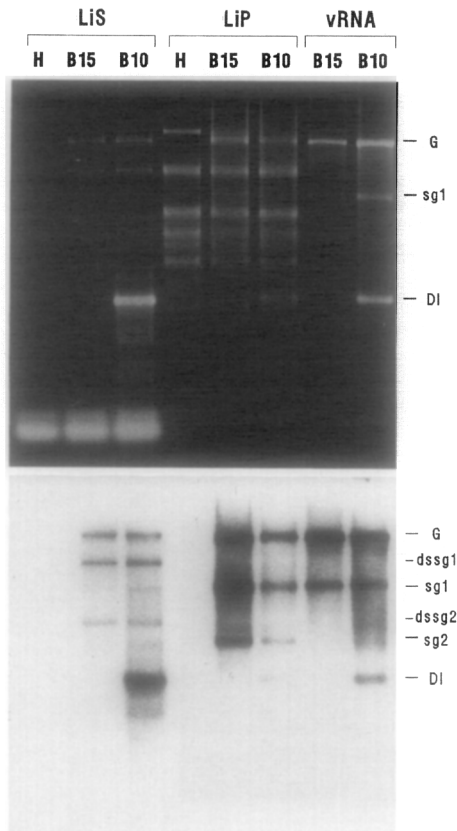
It should be noted that the strategy used to clone the TBSV DI RNAs selected molecules capable of hybridizing to the 5' and 3' termini of the virus genome. Although this strategy precluded cloning possible DI RNAs that did not contain these termini, this was not of great concern since each of the cloned DI RNAs corresponded precisely in size to the predominant species of DI RNA encapsidated in the population and each was also biologically active when reinoculated to plants as RNA transcript.

#### DISCUSSION

In this study, DI RNAs were generated in all TBSV isolates passed at high m.o.i., but in none of the iso-

lates passed at low m.o.i. We interpret these results as strong evidence that each DI RNA population arose spontaneously from the viral genome during passage and not from a preexisting template. This conclusion is supported by the demonstration that DI RNAs appeared in each virus isolate at different times during serial passage and were also of different sizes. These observations are consistent with the generally accepted proof for *de novo* generation of DIs in many well-studied animal virus infections (Holland, 1990). Subsequent to completing these experiments, we were able to corroborate this conclusion by observing that DI RNAs similar in size to those reported here appeared after high m.o.i. passage of TBSV inocula derived from *in vitro* transcripts of TBSV cDNA clones. A thorough characterization of those DI RNAs and conditions conducive to their generation will be published elsewhere.

Our results with TBSV parallel reports demonstrating *de novo* generation of DIs in animal viruses (Holland,



**FIG. 2.** Northern analysis of LiCl-fractionated RNAs and virion RNAs isolated from inoculated leaves of plants infected with a representative high m.o.i. (B10) and low m.o.i. (B15) isolate of TBSV after 11 serial passages. Total RNAs were purified from inoculated and healthy (H) leaves and fractionated in 2.3 M LiCl as described at 7 days postinoculation. Virion RNAs (vRNA) were extracted from virions purified from the same inoculated leaves. Samples were electrophoresed in a 1.5% agarose-TBE gel (top), stained with ethidium bromide, electroblotted onto a nylon membrane, and probed with pTBSV-28 (Hearne *et al.*, 1990). Locations of single-stranded genomic RNA (G) and the two subgenomic RNAs 1 (sg1) and 2 (sg2) along with their replicative forms (dssg1 and dssg2) are indicated at the right.

1990). An important difference, however, is that our studies utilized the intact virus host species rather than cultured cells. The difficulties of examining the role of DIs in modulating virus disease and in establishing persistent infections are well known (Huang, 1988; Barrett

and Dimmock, 1986). A plausible alternative would be to pursue such studies using plant virus systems. The strong correlation between symptom attenuation and appearance of DIs supports our previous findings that inhibition of virus replication by DI RNAs also lessens disease severity (Jones *et al.*, 1990; Hillman *et al.*, 1987). However, we also observed that in three isolates (C2, B8, and B9) symptom attenuation was delayed by several passes after the initial detection of DIs, and in many of the isolates the dominant DI RNA species became smaller after its initial appearance. Although the overall effects of individual DI RNA species upon virus replication was not determined, it seems likely that some DIs may differ in their ability to interfere with TBSV. A more thorough characterization of the evolution and biological activities of different DI RNA species will be reported elsewhere (D. Knorr and J. Morris, in preparation).

The sequences retained by the DI RNAs suggest possible mechanisms for their formation. Both the processivity of the viral replicase and local structure of the viral template have been implicated in the formation of DI RNAs in other viruses. Makino *et al.* (1988) have suggested that DIssE of the coronavirus MHV A59 was formed by viral polymerase "hopping" within regions of secondary structure in the genomic RNA. Earlier work by Lazzarini *et al.* (1981) identified a hexanucleotide sequence on the vesicular stomatitis virus RNA at which initiation by the viral polymerase led to formation of DI RNAs. In the TBSV, the hexanucleotide motif 5'-APuAGAA-3' occurs at or near the endpoints of regions II and III in the DI RNAs. It is possible that polymerase dissociation and reinitiation take place in these regions and may account for the large deletions generated between these regions. In cloning TBSV genomic sequences we encountered several regions of "strong stop" signals. These polymerase-destabilizing regions in the RNA could also contribute to the formation of DI RNAs. Interestingly, the most highly conserved deletion in the DI RNAs, occurring between regions III and IV, does not contain a sequence duplication, but rather an inverted repeat of the sequence AGGTTA.

The schematic diagram in Fig. 5 shows the relationships between the TBSV genome and the DI RNAs

**FIG. 3.** Alignment of the TBSV DI RNA sequences with the four conserved regions of the TBSV genome. DI RNAs present in the B10 and C6 isolates of TBSV were cloned after the eleventh passage in *Nicotiana benthamiana* and *Nicotiana clevelandii*, respectively. Sequences of six independent DI RNA clones from the B10 isolate and four from the C6 DI RNA were aligned with the consensus sequence from DI1 and the four conserved regions from the TBSV genome. A dash (-) indicates the nucleotide at that position is the same as the TBSV genomic sequence shown below and only nucleotides which differ from the genome are shown, except that the start and stop codons for different viral genes are indicated. A diamond (◆) or a space indicates no nucleotide is present at the corresponding position. The endpoints of cDNA clones made by tailing, prior to PCR amplification to modify the termini, are indicated by brackets [], and the size of each DI is given in parentheses. Conserved regions I-IV, as discussed in the text, are identified below each block of sequence. The APuAGAA hexamer sequences and the AGGTTA inverted repeats are underlined.





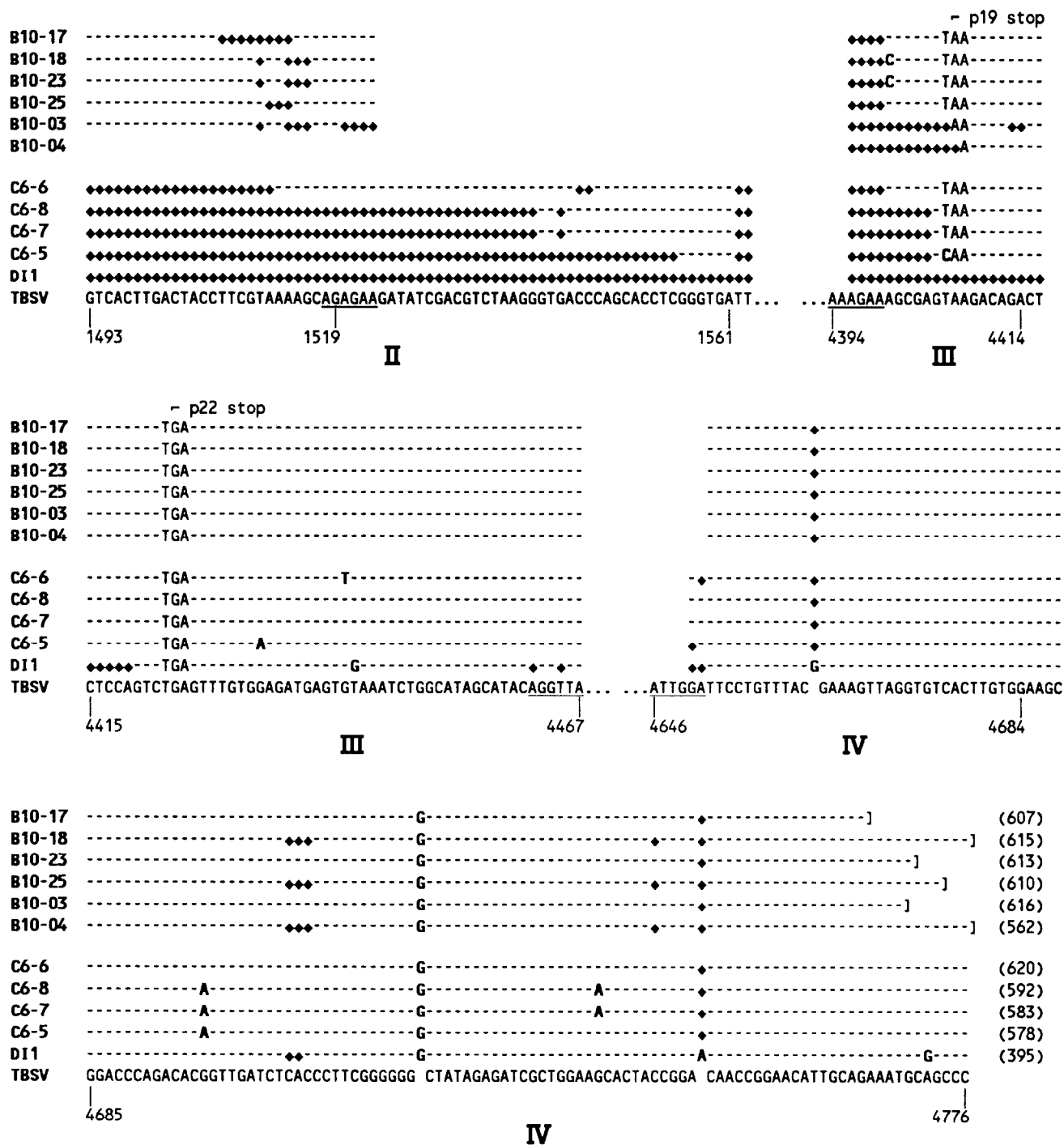
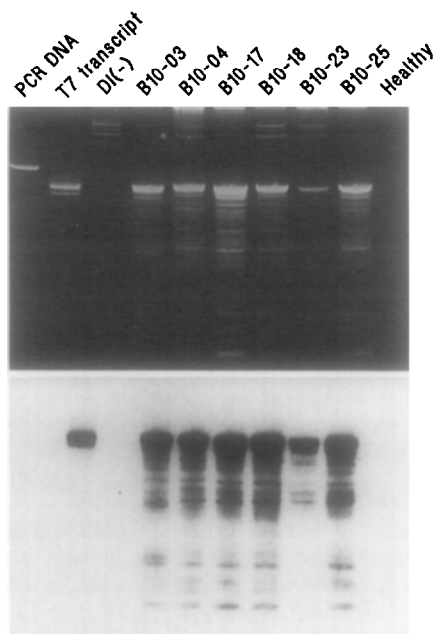


FIG. 3—Continued

sequenced to date. Conservation of the same four regions of TBSV sequences in the DI RNA clones suggests that the retained elements are required for RNA replication and possibly encapsidation. It is not surprising that DIs maintain viral 5' and 3' termini because these regions are likely to be involved in polymerase binding and the initiation of replication. Possible functions for the two internal domains (regions II and III) are less obvious; nevertheless, internal regions are com-

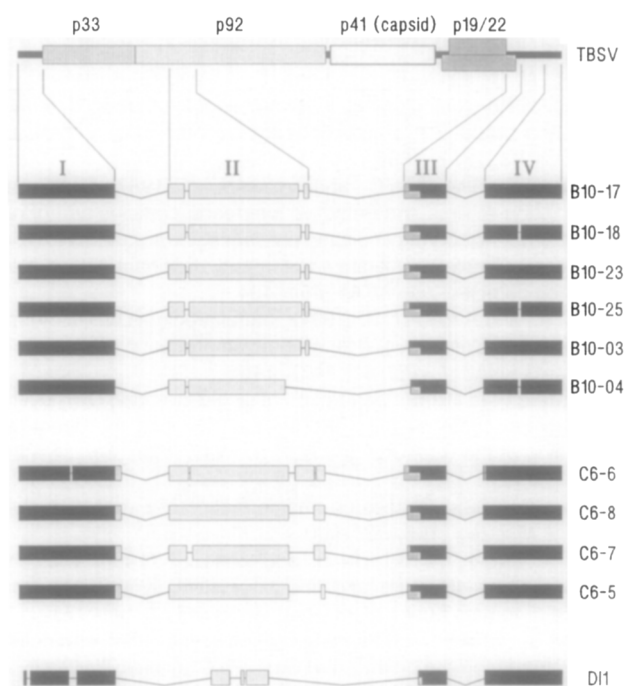
monly found in other DIs that have been characterized. It is possible that internal regions function nonspecifically, such as to augment packaging or to separate different functional domains, but if this is the case we would not expect the consistency present in the DI RNA sequences. It is more likely that internal regions function more specifically, perhaps as sites of recognition for *trans*-acting factors important for the initiation or regulation of replication. The replicase of bacterio-



**Fig. 4.** Infectivity of RNA transcripts from cloned TBSV B10 DI RNAs. The DI sequences from independent cDNA clones of the B10 DI RNAs were amplified by PCR, transcribed *in vitro* and inoculated onto *Nicotiana clevelandii* plants together with TBSV DI-free genomic RNA. RNA was isolated from inoculated leaves at 7 d.p.i. and the 2.3 M LiCl-soluble fraction was separated in a 5% polyacrylamide gel under nondenaturing conditions (top), together with DI-free TBSV (DI-), a sample product of the T7 transcriptions, a PCR-amplified product, and the LiCl-soluble RNA from a noninoculated plant (Healthy). Below is a Northern blot of the same RNAs probed with a TBSV DI-specific sequence. Because no denaturant was employed, only single-stranded TBSV-specific RNAs hybridized with the probe.

phage Q $\beta$  initiates replication at the 3' terminus of the plus-strand RNA after first binding to an internal region (Blumenthal, 1979) and an internal region is also required for maintenance of brome mosaic virus RNA 3 (French and Ahlquist, 1987). Internal sequences in DIs associated with vesicular stomatitis virus appear to provide stability and may contribute to the overall persistence of viral infection (O'Hara *et al.*, 1984). Another possible function for the internal domains is in promoting the initiation of encapsidation. It has been suggested for TCV that several internal regions may be involved in nucleating virus assembly (Wei *et al.*, 1990). Of significance in that study was the identification of a primary binding site near the amber readthrough codon of the viral replicase, a location similar to that of region II in the TBSV DI RNAs. Judging by relative levels of DI versus genomic RNAs in purified virion preparations (see, Fig. 2), DI1 is packaged less efficiently than B10, and also contains a smaller, more dissected, region II. It is possible that biological properties such as infection persistence are related to the ability of TBSV DI RNAs to become encapsidated, hence the strong conservation of these sequences in TBSV DIs.

The mechanism of DI-mediated symptom attenuation in TBSV infections is not understood, although recent experiments using protoplasts clearly show that DI RNAs replicate at the expense of the helper virus (Jones *et al.*, 1990). It seems unlikely that competition for available coat protein during encapsidation is an important factor because proportionally less DI than genomic RNA is encapsidated. It is also unlikely that a specific DI-encoded protein is responsible for attenuation because there are no conserved ORFs of significant size which could be translated without internal initiation and all previous attempts to translate DI RNAs *in vitro* have been negative (B. Hillman, personal communication). Consequently, the most likely mechanism leading to symptom attenuation is reduced viral replication resulting from competition between the viral genome and DI RNAs for replicase. It seems possible that selection pressure resulting in symptom attenuation would favor replicative fitness of the DI RNAs through



**Fig. 5.** Diagram of the relationship between the TBSV genome, regions present in the sequenced TBSV B10 and C6 DI RNA clones, and DI1. A map of the TBSV genome is presented at the top with the five gene coding regions identified (p33, p92, p41, and p19/22) and represented as different shaded blocks. The same shading pattern is used below to represent each DI sequence in expanded form, with lines to indicate the derivation of each of four conserved regions from the TBSV genome (I–IV). Major deletions forming the four regions are shown as bent lines and straight lines connect the smaller deleted sequences occurring within a region. Each DI RNA contained the entire 5' leader sequence as well as the 3' terminal ~130 nucleotides (regions I and IV). The largest difference between the different sequences were the size and internal deletions within region II.

selection of optimal polymerase binding sequences. In addition, the small size and structural stability of DI RNAs might also contribute to their ability to be maintained. Whatever mechanisms are involved, it is clear that selection pressures strongly favor maintenance of DI RNAs that function to down-regulate virus replication and thereby allow host survival and virus persistence.

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