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Host Effects and Sequences Essential for Accumulation of Defective Interfering RNAs of Cucumber Necrosis and Tomato Bushy Stunt Tombusviruses

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Passage of cucumber necrosis virus (CNV) containing defective interfering (DI) RNAs through cucumber plants decreased the accumulation of DI RNAs to undetectable levels. Subsequent passages in two *Nicotiana* species (*Nicotiana benthamiana* or *N. clevelandii*) resulted in the appearance of DI RNA species that were larger than the DI RNAs observed during exclusive serial passages of CNV through the *Nicotiana* species. Sequence analysis of cloned cDNAs corresponding to the two DI RNA populations indicated that the smaller CNV-DI RNAs contained the four conserved regions (I through IV) of the genome typical of tombusvirus DI RNAs, whereas the larger DI RNAs were of similar organization but had a direct repeat of the middle portion of the molecule. This result suggests that the host has an influence on the type of DI RNA that accumulates during consecutive high multiplicity of infection passages. A comparative analysis of deletions targeting the individual conserved regions in both CNV and tomato bushy stunt virus (TBSV) DI RNAs revealed that only region III was completely dispensable for accumulation of either DI RNA species. More refined deletion analyses in regions I and II indicated that smaller segments of 75 and 35 nucleotides (nt), respectively, could be deleted without abolishing infectivity. The dispensable sequences in region II of both TBSV and CNV DI RNAs mapped to the top portion of a putative stem–loop structure. These studies indicate that both essential and nonessential sequences are conserved in DI RNAs. The essential sequences in regions I, II, and IV likely contain important *cis*-acting elements, whereas nonessential regions such as region III may play secondary roles such as optimally spacing *cis*-acting elements or maintaining the DI RNA at an overall size that is stable.

INTRODUCTION

Defective interfering (DI) RNAs are deletion mutants that interfere with the replication of the parental virus from which they are derived. These entities have been reported for a wide variety of animal viruses and are thought to occur universally in animal virus infections (Perrault, 1981). In general, DI RNAs consist of portions of the parental virus genome that have lost functions essential for independent replication and hence have become dependent on the parental helper virus for *trans*-acting factors such as the viral replicase. Competition between DI RNAs and the helper virus for these factors leads to specific interference with normal replication of the helper virus (Schlesinger, 1988; Jones *et al.*, 1990). DI RNAs have now been reported in several positive-strand ssRNA plant virus groups (see Roux *et al.*, 1991, for review), including most of the characterized tombusviruses such as tomato bushy stunt virus (TBSV) (Hillman *et al.*, 1987; Morris and Hillman, 1989; Knorr *et al.*, 1991), cucumber necrosis virus (CNV) (Rochon, 1991; Rochon and Johnston, 1991), and cymbidium ringspot virus (CyRSV) (Burgyan *et al.*, 1989, 1991; Rubino *et al.*, 1990).

Generation of DI RNAs in a virus population is believed to involve two major events (Schlesinger, 1988). The first event involves the production of the defective viral genome via template "hopping" or "switching" of the viral replicase during RNA synthesis (Lazzarini *et al.*, 1981; Perrault, 1981). This may result in synthesis of an incomplete complementary strand on one template, followed by reinitiation of synthesis on a second template or a different portion of the same template (Lai, 1992). The second event in DI RNA genesis is amplification of the aberrant molecule. As a consequence, viable DI molecules must retain *cis*-acting elements required for viral replicase recognition as well as signal sequences necessary for packaging and/or movement (Li and Simon, 1991). Sequence comparisons of DI RNAs can thus be used as valuable tools to identify elements that may be involved in replication and encapsidation of the parental virus. For example, studies on Sindbis virus DI RNAs revealed that only 162 nucleotides (nt) at the 5′ terminus and 19 nt at the 3′ terminus are specifically required for replication and packaging (Levis *et al.*, 1986).

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Tombusviruses are small icosahedral plant viruses whose particles are composed of 180 copies of a 41-kDa coat protein subunit and a single-stranded, positive-sense RNA molecule of ca. 4.8 kb (Martelli *et al.,* 1988). All of the tombusvirus genomes that have been sequenced to date (Grieco *et al.,* 1989; Rochon and Tremaine, 1989; Hearne *et al.,* 1990) display the same coding organization consisting of five major open reading frames (ORFs). The two 5′ proximal ORFs encode proteins required for RNA replication (Dalmay *et al.,* 1993b; Scholthof *et al.,* 1995), the capsid protein gene resides internally, and two nested genes are located near the 3′ terminus of the genome (Grieco *et al.,* 1989; Rochon and Tremaine, 1989; Hearne *et al.,* 1990; Dalmay *et al.,* 1992). Although the function of the smaller of the two 3′ nested ORFs has not been elucidated, the larger ORF is required for systemic movement (Rochon and Johnston, 1991; Scholthof *et al.,* 1993; Dalmay *et al.,* 1993b). Comparison of sequences in the noncoding regions has revealed that the intergenic sequences are variable and that the 5′ and 3′ noncoding regions are almost identical (Heame *et al.,* 1990).

The presence of DI RNAs appears to be a common feature of the tombusviruses and different classes of DI RNAs have been generated during serial high multiplicity of infection (m.o.i.) passages of TBSV (Morris and Knorr, 1990), CyRSV (Burgyan *et al.,* 1989, 1991) and CNV (Finnen and Rochon, 1993) in *Nicotiana clevelandii* plants. In addition, CNV mutants defective for the smaller gene in the 3′-nested ORFs were reported to generate DI RNAs *de novo* without a requirement for serial high m.o.i. passage (Rochon, 1991). However, biologically active clones of CyRSV containing a similar mutation failed to exhibit accelerated generation of DI RNAs (Dalmay *et al.,* 1993b). Using a reverse transcriptase–polymerase chain reaction (RT–PCR), DI RNAs have also been detected in plants inoculated with TBSV transcripts without passage (Law and Morris, 1994). Recent studies have suggested that formation of tombusvirus DI RNAs likely involves a series of stepwise deletions of various segments of the viral genome (White and Morris, 1994a). Sequence analyses have revealed that tombusvirus DI RNAs do vary in size, but they all contain four distinct conserved regions of sequence derived from the 5′ terminus, an internal region and the 3′ terminus of the viral genome (Hillman *et al.,* 1987; Knorr *et al.,* 1991; Burgyan *et al.,* 1991; Rochon, 1991; White and Morris, 1994a; Law and Morris, 1994). These findings suggest that the conserved regions may contain *cis*-elements required for replication which are common to all tombusviruses. This may explain why DI RNAs derived from TBSV will replicate upon co-infection with other distantly related tombusviruses (Hillman, 1986; White and Morris, 1994a).

We have carried out these studies with CNV to assess the influence of the host plant on DI RNA formation and accumulation, and to identify CNV and TBSV DI RNAs sequences that are important for accumulation.

MATERIALS AND METHODS

Virus propagation and construction of cDNA clones

The initial stock of purified CNV was a gift from Dr. D. M. Rochon (Agriculture Canada Research Station, Virus Chemistry, Vancouver, British Columbia, Canada). This virus isolate had been maintained in *N. clevelandii* plants and contained DI RNAs (Rochon and Johnston, 1991). The isolates analyzed in our study were derived from purified virus of this stock inoculated initially to *N. clevelandii* at a concentration of 10 μg/ml. The resulting infected leaf tissue was used to infect individual cucumber (*Cucumis sativus* L. cv. Ashley) and two *Nicotiana* species (*N. benthamiana* and *N. clevelandii*) as illustrated in Fig. 1. A series of seven additional high m.o.i. passages in both of the *Nicotiana* species were conducted following the scheme in Fig. 1. Inocula for passages was prepared by grinding 4 cm^2 of systemically infected leaves in 1 ml of 100 m*M* potassium phosphate, pH 7.0, containing 1% Celite 545 (Fisher Scientific). LiCl-soluble RNA fractions isolated from selected passages were used for cloning of amplified cDNAs using the previously described RT–PCR procedure with primers complementary to the 5′ and 3′ termini of TBSV RNA (Knorr *et al.,* 1991). The resulting cDNAs contained a bacteriophage T7 RNA polymerase promoter and an *Apa*I site at the 5′ end and *Sma*I and *Sph*I restriction sites at the 3′ end (due to the incorporation of these sequences at the 5′ end of the primers used for RT–PCR). PCR-amplified cDNAs were digested with *Apa*I and *Sph*I and ligated into pDK41 (Knorr *et al.,* 1991). These constructs were then transformed into *Escherichia coli* strain DH5αF′ (BRL).

Plasmid DNA to be used for sequencing or for *in vitro* transcription was prepared by a miniprep method (Knorr *et al.,* 1991) or by isopycnic CsCl gradient centrifugation (Sambrook *et al.,* 1989). Dideoxynucleotide sequencing of the plasmids was performed with Sequenase Version 2.0 as recommended by the supplier (United States Biochemical).

Virus and DI RNA purification and analysis

CNV particles were purified by extraction in 200 m*M* sodium acetate (pH 5.2), concentrated by PEG precipitation and high-speed centrifugation (Hillman *et al.,* 1985), and analyzed in 1% agarose Tris–glycine gels (50 m*M* Tris, 50 m*M* glycine). For virion RNA analysis, two PEG precipitation steps were performed after which the viral RNA was extracted with phenol and then precipitated with ethanol (Hillman *et al.,* 1985).

DI RNAs were purified from fresh or frozen leaves (200 to 300 mg), ground with a mortar and pestle in 600 μl of

extraction buffer (200 m*M* NaCl, 100 m*M* Tris, pH 8.0; 2 m*M* EDTA, 1% SDS), 600 μl of phenol, and 25 μl of β-mercaptoethanol. Then, 600 μl of chloroform/isoamyl alcohol was added and the mixture was thoroughly emulsified. After two phenol extractions, the aqueous phase was precipitated with 2 vol of ethanol. The precipitated nucleic acids were concentrated by centrifugation and the pellet was resuspended in 400 μl of H_2O. A half volume of 7 *M* LiCl was then added and the mixture was incubated at 0° overnight. The samples were subsequently separated into LiCl-soluble and LiCl-insoluble fractions by centrifugation as described previously (Hillman *et al.,* 1987; Knorr *et al.,* 1991). The LiCl-soluble RNA fractions, which contain most of the DI RNAs and viral-specific dsRNAs, were treated with RNase-free DNase I, separated in 1.2 or 1.5% agarose gels containing TBE (45 m*M* Tris, 45 m*M* boric acid, 1 m*M* EDTA, pH 8.3) and stained with ethidium bromide (0.5 μg/ml).

Protoplast and plant inoculations with *in vitro* generated transcripts

In vitro generated transcripts of TBSV (pTBSV-100; Hearne *et al.,* 1990), CNV (pK2/M5, a gift from Dr. D. M. Rochon; Rochon and Johnston, 1991), TBSV-DI RNAs (B10-18; Knorr *et al.,* 1991) and CNV-DI RNAs were prepared essentially as described by Hearne *et al.* (1990). Purified plasmids (2 μg) were linearized with *Sma*I prior to their use as templates in 50 μl *in vitro* transcription reactions. RNA transcripts were used for inoculation of protoplasts and plants as previously described (Jones *et al.,* 1990; Scholthof *et al.,* 1993).

After 20 to 24 hr incubation, total nucleic acids from protoplasts were isolated with ammonium carbonate buffer containing SDS and bentonite (Donald and Jackson, 1994). Agarose gel electrophoresis, RNA blot analysis and the incorporation of [^{3}H]uridine into viral and host RNAs were carried out as previously described (Jones *et al.,* 1990). For fluorography, gels were immersed in 500 m*M* sodium salicylate for 20 min, rinsed with distilled water, blotted with 3MM Whatman paper, and dried under vacuum. Dried gels were exposed to X-ray film for 24 to 48 hr at −80°.

Mutagenesis of TBSV- and CNV-DI RNA cDNA clones

To permit flexibility in deletion mutagenesis, a unique *Hpa*I site was introduced into the prototypical CNV C2 DI RNA clone (Fig. 4), using oligonucleotide-directed mutagenesis (Kunkel *et al.,* 1987) to alter a T residue to a C at nt 314. Clones harboring the *Hpa*I site were identified by digestion of plasmid DNA, confirmed by DNA sequencing and one clone designated C2H was selected for further analysis. To identify the sequence motifs essential for DI RNA accumulation, four deletion mutants of C2H (Fig. 7) were constructed by excision of regions between the restriction enzyme sites *Sty*I and *Acc*I (nt 27 to 105; mutant C2ΔIa), *Acc*I and *Pfl*MI (nt 108 to 187; mutant C2ΔIb and IIa), *Pfl*MI and *Bst*BI (nt 185 to 258; mutant C2ΔIIb), and *Bst*BI and *Hin*fI (nt 257 to 311; mutant C2ΔIII). A transcript (C2ΔIV) that lacked the 3′ terminal 312 to 432 nt was obtained by using *Hpa*I to linearize the plasmid template before *in vitro* transcription.

Similar mutants were constructed from the TBSV-DI RNA cDNA clone B10-18 (a prototypical B10 clone described by Knorr *et al.,* 1991) by deleting regions between the *Acc*I and *Aat*II sites (nt 122 to 214; mutant B10ΔIb), *Aat*II and *Bst*XI (nt 211 to 266; mutant B10ΔIIa), and *Bst*XI and *Bst*BI (nt 263 to 384; mutant B10ΔIIb). The 3′ terminal truncated TBSV DI RNA transcripts (B10ΔIII and IV) were prepared by using *Bst*BI to linearize the B10 plasmid and filling the ends in with DNA polymerase (Klenow) before transcription. Another TBSV-DI RNA mutant (B10ΔIII) deleted between nucleotides 387 to 464 (according to the B10 sequence) was previously constructed by D. A. Knorr (unpublished results). This construction involved isolating the 5′ region from the *Apa*I–*Bst*BI fragment of the B10-323 clone (Knorr *et al.,* 1991), which has a CAA insertion between positions 198 and 199 of the B10 sequence. This 5′ fragment from B10-323 was then ligated to a 3′ fragment obtained by *Hin*fI–*Sph*I digestion of a second B-10 derivative with T and G residues substituted for the C and A residues at positions 542 and 557, respectively.

To generate nested deletion mutants in the middle of region II of the TBSV- and CNV-DI clones, the plasmids B10 and C2H were digested with *Bst*XI and *Pfl*MI, respectively, and then treated with diluted *Bal*31 nuclease (1 U/μl, New England Biolabs) at 27°. The products were treated with DNA polymerase I Klenow fragment and T4 DNA polymerase to fill in the ends of the *Bal*31 nuclease-treated termini. The plasmid was then digested with *Alw*NI, which recognizes a unique restriction site in the plasmid vector and yields two populations of fragments. The shorter fragment population contained the 3′ part of the DI RNA insert with the progressive deletions and the longer fragments had most of the vector sequences and the 5′ part of the DI RNA insert with the progressive deletions. TBSV DI RNA clone B10 (which had not been treated with *Bal*31 nuclease) was digested with *Alw*NI and *Bst*XI, and the termini were rendered blunt by treatment with T4 DNA polymerase. The CNV C2H clone (which also had not been treated with *Bal*31 nuclease) was digested with *Alw*NI and *Pfl*MI, and the full-length long and short fragments were isolated from agarose gels. In order to generate clones with only progressive 3′ deletions, the large fragments from the *Bal*31-untreated plasmids were ligated to the small fragments of the *Bal*31-treated plasmids. Similarly, clones with progressive 5′ deletions were obtained by ligating the small fragments from the *Bal*31-untreated plasmids to the large fragments resulting from the *Bal*31 digestions. Double-deletion mutants such as C2ΔIa and III were created by selecting a C2ΔIa clone and deleting the sequences

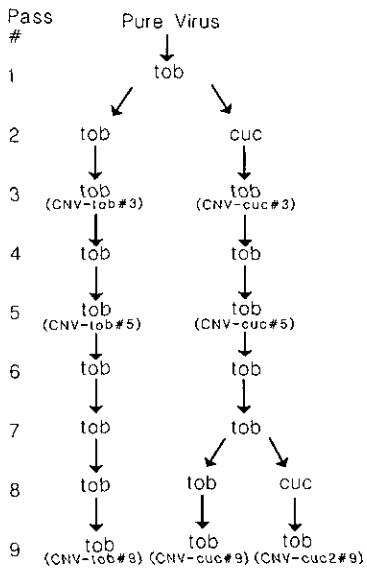

FIG. 1. Schematic representation of the serial passage experiments. Two independent passage experiments were conducted as outlined in the diagram using *Nicotiana clevelandii* and *Nicotiana benthamiana* as the tobacco hosts (labeled as tob in the diagram) and *Cucumis sativus* as the cucurbit host (labeled as cuc in the diagram). Sample names of individual plants referred to in the text and in the other figures are identified below the passage for which they apply (e.g., CNV-tob 3 refers to samples isolated from the tobacco host at passage 3).

between *Bsm*I and *Hpa*I sites. All mutants were confirmed by restriction mapping and DNA sequencing.

RESULTS

Passage of CNV through cucumber dramatically reduces the abundance of DI RNAs

Previous studies have shown that persistent, nonlethal infections normally result during passage of CNV in *N. clevelandii* plants (Rochon and Johnston, 1991; Rochon, 1991). We noted, however, that after passage of the virus through the natural host, cucumber, the disease severity increased upon subsequent inoculation to the tobacco hosts *N. benthamiana* and *N. clevelandii*. The experimental design is outlined in Fig. 1, which shows the high multiplicity passage (m.o.i.) experiment that was performed in plants. The virus stock was initially maintained in *N. benthamiana* or *N. clevelandii* (Fig. 1, passage 1) and was then subjected to 8 successive high m.o.i. passages in the same host (Fig. 1, CNV-tob series). The *Nicotiana* species exhibited progressively attenuated symptoms with each successive passage of the CNV-tob series as illustrated in *N. benthamiana* plants (Fig. 2, passages 3, 5, and 9). In both species the symptoms became progressively milder so that by passage 9 plants inoculated with the CNV-tob inocula developed only a moderate necrosis and were able to survive for more than a month (Fig. 2, CNV-tob passage 9). In marked contrast, when the same virus stock was passed through cucumber one time, followed by a subsequent series of passages in the tobacco hosts (Fig. 1, CNV-cuc passage series), the first plants in the series developed a severe necrosis that resulted in death within 14 days postinoculation. This severe syndrome persisted through the third successive inoculation to the tobacco hosts (Fig. 2, CNV-cuc passage 5). However, continued serial passage of CNV-cuc derived isolates in either tobacco host resulted in gradual attenuation of symptoms (Fig. 2, CNV-cuc passage 9). A second passage through cucumber following five successive tobacco passages (Fig. 2, CNV-cuc2 passage series) restored the lethal necrotic phenotype upon subsequent passage back to tobacco (Fig. 1, CNV-cuc2 passage 9). These results were analogous to those obtained in the high m.o.i. passage experiments conducted previously with TBSV, in which we observed increased virulence in our TBSV isolate after passage in *N. glutinosa* followed by a characteristic symptom attenuation after high m.o.i. passage in both *N. benthamiana*, and *N. clevelandii* (Knorr *et al.*, 1991).

To examine the nature of the DI RNA populations associated with plants expressing varied symptom phenotypes at different stages of passage, we analyzed the LiCl-soluble RNA extracts (dsRNAs and DI RNAs) from inoculated plants on agarose gels. The first two lanes in Fig. 3A clearly reveal the presence of a population of DI RNA-sized molecules in the attenuated isolate after passage in *N. benthamiana*. Separate Northern blot analyses confirmed that the DI RNA-sized species were derived from the CNV genome (data not shown). A complex heterogeneous pattern of DI-like RNAs was present after three successive passages in the tobacco hosts (Fig. 3A, CNV-tob 3), but after two additional passages, the abundance of one of the DI RNA species increased appreciably (Fig. 3A, CNV-tob 5). Continued passage in the tobacco hosts resulted in greater symptom attenuation, and shifts in abundance of the DI RNAs so that only one major DI RNA species of about 420 nt was prevalent by passage 9 (Fig. 3B, CNV-tob 9). The gel analysis also revealed that the DI RNAs were not detectable after passage through cucumber (Fig. 3A, CNV-cuc passage 5), nor were they present in uninfected tobacco extracts (not shown). However, continued transfers of the CNV-cuc isolate in tobacco resulted in increased attenuation, and analysis of the LiCl-soluble nucleic acids revealed a corresponding accumulation of distinctly larger DI RNA-like molecules than those observed in the CNV-tob series passages (Fig. 3B, CNV-cuc 9). When this same inoculum series was again interrupted by infection of cucumber (Fig. 1, passage 8), the symptom attenuation disappeared following inoculation of the tobacco hosts (Fig. 2, CNV-cuc2, passage 9) and DI RNA species were not evident in these plants (Fig. 3B, CNV-cuc2 9).

To further evaluate the patterns of DI expression in *N. benthamiana, N. clevelandii,* and cucumber, single- and double-stranded RNAs from passage 9 were extracted

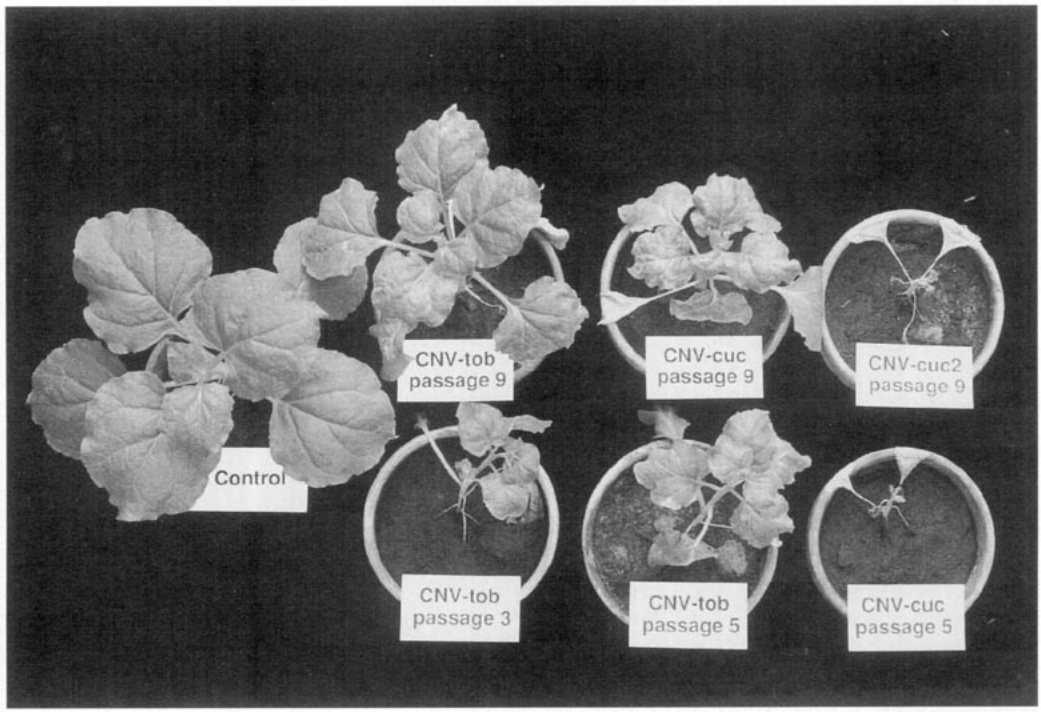

FIG. 2. Symptoms on *N. benthamiana* plants 19 days after inoculation at high multiplicity of infection with the series shown in Fig. 1. The plants illustrated show the typical progressive modulation of symptoms elicited by CNV-tob passages 3, 5, and 9 for the experimental transfers in *N. benthamiana*. Very similar results were observed for the passage series performed in *N. clevelandii*. The severe symptoms evident on the plants labeled CNV-cuc passage 5 and CNV-cuc2 passage 9 illustrate the typical effects observed on the *Nicotiana* species after passages through cucumber.

from the three hosts and subjected to electrophoresis in agarose gels. The results of ethidium bromide staining indicated that the patterns of LiCl-insoluble extracts (ssRNAs and DI RNAs) from the two tobacco hosts were similar (Fig. 3C). These two hosts also exhibited very similar patterns of dsRNAs and DI RNAs when LiCl-soluble extracts were examined (data not shown). Comparisons of RNAs from inoculated tobacco plants revealed a high-molecular-weight ssRNA species with an electrophoretic mobility characteristic of the viral RNA (Fig. 3C). This band was particularly pronounced in the tobacco plants after passage through cucumber, but was much less intense in infected cucumber, irrespective of the source of the inoculum (Fig. 3C). Intense DI-like bands that differed slightly in size were also present in the tobacco hosts inoculated with the cuc 9 and tob 9 passages. These low-molecular-weight bands were not evident in the cuc2 9-infected tobacco, or in any of the inoculated cucumber plants (Fig. 3C).

From these experiments, we conclude that the patterns of viral expression in the two *Nicotiana* species are essentially identical. Moreover, the DI RNAs accumulate to very high levels in tobacco plants inoculated with the isolates that elicit highly attenuated symptoms, in contrast to their low abundance in tobacco infected with the virulent inoculum derived from cucumber. Taken together, these observations provide a correlation between the level of symptom attenuation and the abundance of the DI RNA species. They also indicate that the host has a substantial effect on maintenance of the DIs because passage of DI containing isolates through cucumber plants effectively "filtered out" the DI RNAs that had accumulated during passage through tobacco. The DI RNAs accumulating after passage through cucumber also differed in size from those maintained in the tobacco hosts (Fig. 3C), suggesting that the history of host passage has a role in the evolution of the DI RNAs.

Cloning and sequence analysis of CNV-DI RNAs

We next investigated the structure of the DI RNAs present in the plants at passage 9 by isolating recombinant cDNA clones from LiCl-soluble nucleic acid fractions as previously described for the TBSV DI RNAs appearing after high m.o.i. passages (Knorr *et al.*, 1991). A total of 26 CNV-DI RNA clones from the CNV-tob and CNV-cuc lines were obtained which included: (i) 11 small (ca. 420 nt) DI RNA clones derived from nine uninterrupted passages through *N. benthamiana* and one clone from nine passages through *N. clevelandii* (Fig. 1, CNV-tob 9), and (ii) 15 clones of two distinct sizes that evolved following a single passage through cucumber followed by serial passages through the tobacco hosts (Fig. 1, CNV-cuc 9). These included seven small clones and one large clone (ca. 595 nt) from the *N. benthamiana* passages and five

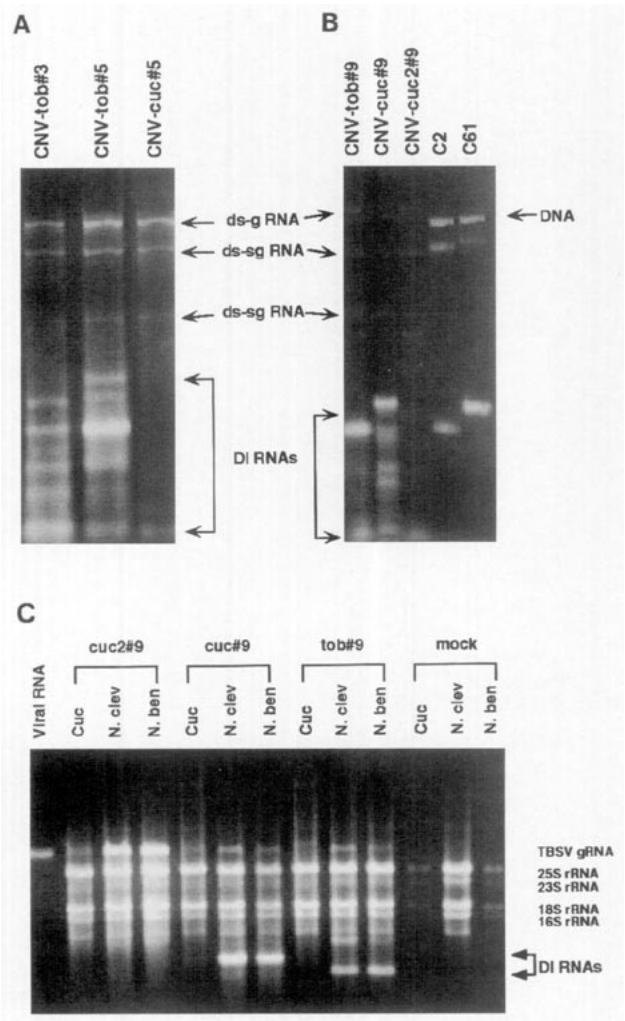

FIG. 3. Analysis of RNAs isolated from infected leaves of plants inoculated with sap from plants at different passages. (A) An ethidium bromide-stained 1.2% agarose gel showing the LiCl-soluble nucleic acids isolated from *N. benthamiana* plants infected with the CNV-tob line at passages 3 and 5, respectively, and the CNV-cuc series at passage 5. (B) Electrophoresis of LiCl-soluble nucleic acids isolated from CNV-tob 9, CNV-cuc 9, and CNV-cuc2 9-infected *N. benthamiana* plants in a 1.5% agarose gel. Lanes C2 and C61 show RNAs transcribed from the smaller (C2) and the larger (C61) clones that were derived from the CNV-tob 9 and CNV-cuc 9 DI RNAs, respectively. Double-stranded genomic RNA (ds-g RNA) and subgenomic RNAs (ds-sg RNA), defective interfering RNA species (DI RNAs), and the C2 and C61 plasmid template DNAs are indicated. The DI RNAs labeled on the figure were confirmed to be genome-related by independent RNA hybridization analysis (data not shown). (C) A 1.2% agarose gel of LiCl-soluble RNAs isolated from infected *N. benthamiana*, *N. clevelandii*, and *C. sativus* leaves 6 days after inoculation. The CNV-cuc2 9, CNV-cuc 9, and CNV-tob 9 passages, and extracts from mock-infected plants are compared. The migration of the TBSV genomic RNA (TBSV gRNA), the host 25S, 23S, 18S, and 16S ribosomal RNAs (rRNAs), and the defective interfering RNA species (DI RNAs) are indicated along the side of the gel.

small clones and two large clones from *N. clevelandii* passages. Three of the CNV-tob clones designated C1 and C2 (from *N. benthamiana* and C66 from *N. clevelandii*) and three of the larger CNV-cuc clones (C39 and C61 from *N. benthamiana* and C62 from *N. clevelandii*) were characterized more extensively.

The nine representative clones were sequenced completely on both strands (GenBank Accession Nos. U16698 to U16706). Analysis of the sequences demonstrated that both the large and small DI RNAs represent collinear deletion derivatives of the CNV genome (Fig. 4) similar to the previously described TBSV DI RNAs obtained in high m.o.i. passage experiments (Knorr *et al.*, 1991), and other previously characterized DI RNAs associated with CNV infections (Rochon, 1991; Finnen and Rochon, 1993). These CNV-DI RNAs all contained the same four conserved regions identified in other tombusvirus DI RNAs. Region I (152 to 153 nt) consisted of the entire 5′ leader sequence including the G residue after the first AUG codon. Region II (90 to 111 nt), which was derived from a portion of the putative polymerase gene (p92), could be divided into two parts (IIa and IIb) because different DI RNA clones contained deletions in the central portion of region II (Fig. 4). The deletions within region II were similar to deletions observed in region II of TBSV DI RNAs (Hillman *et al.*, 1987). The size of region III was variable, but included 18 nucleotides derived from the carboxyl terminus of the p21 protein gene and the first 30 nucleotides of the 3′-noncoding region. Region IV contained the last 120 nucleotides at the 3′ end of the CNV genome. Deletions between regions III and IV were typical of the more mature DI RNAs that accumulate after extensive serial passages (Knorr *et al.*, 1991). The CNV-DI RNAs contain regions with conserved motifs similar to those found previously in CNV-DI RNAs generated from mutant CNV RNA transcripts containing an untranslatable 20-kDa gene (Rochon, 1991) and DI RNAs from CyRSV tombusvirus (Rubino *et al.*, 1990; Burgyan *et al.*, 1991).

A unique duplicated region, not found in previous studies, was also present in the three large CNV-DI clones (C39, C61, and C69). These DI RNA species had essentially the same organization as the smaller DI RNAs except that the central portion of the DI genome contained a direct sequence repeat of regions II, III, and part of IV (Fig. 4). These sequence repeats suggest that the larger DI RNAs may have arisen as a result of DI RNA rearrangement or recombination between two DI RNAs rather than being generated directly from the parental viral genome. A similar phenomenon of segment duplication has also been observed recently in TBSV DI RNA passage experiments in protoplasts (White and Morris, 1994b).

Infectivity and interference capability of CNV-DI RNAs in protoplasts

In order to gain some insight into the mechanism whereby the DI RNAs are excluded upon infection of cucumber, *in vitro* generated transcripts of each of the

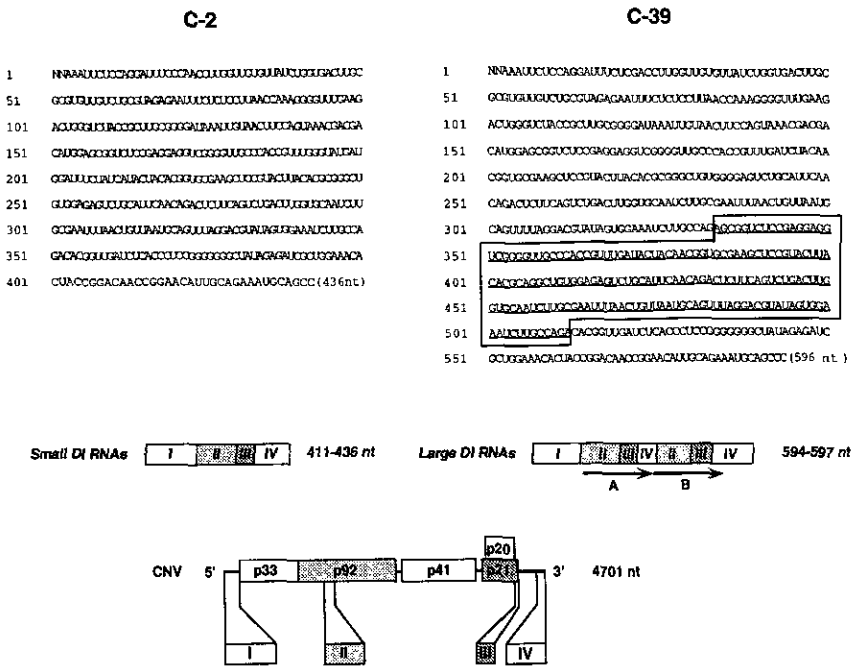

FIG. 4. RNA sequences determined from the cDNA clones of two representative CNV DI RNAs isolated from RNA extracts of infected plants at passage 9. The sequence of one of the small DI RNA clones (C2) is compared with the sequence of one of the large clones (C-39). The boxed region defining the underlined sequence in C39 encompasses the repeated sequence motif observed in this clone and the other larger DI clones. Diagrams illustrating the DI regions derived from the CNV genome are shown below the sequences. A diagram of the CNV genome is presented at the bottom of the figure with the five coding regions (p33, p92, 41, and p21/20) illustrated. The four conserved regions that were found in the small (C1, C2, C12, C40, C46, and C66) and the large (C39, C61, and C62) DI RNA clones are represented by blocks with different shading patterns designated I, II, III, and IV. Note that only minor base differences were present in the different DI clones and that the complete sequence of each clone is available through GenBank (Accession Nos. U16698 to U16706).

clones were synthesized that were of the same size as the predominant species present in plant extracts (cf. Fig. 3B, lanes C2 and C61 with lanes CNV-tob 9 and CNV-cuc 9). The biological activities of transcripts from each representative class of DI RNA cloned (e.g., C1, C2, C12, C39, C40, C46, C61, C62, and C66) were then assessed in protoplasts from both the tobacco species and from cucumber by co-inoculation with helper CNV transcripts (K2/M5). In contrast to the results with whole plants, RNA transcripts from all nine of the sequenced DI RNA clones were amplified to a similar extent in both tobacco and cucumber protoplasts (data not shown). These results demonstrated that the lack of accumulation of CNV-DI RNAs in cucumber plants was not due to their inability to replicate in cucumber cells.

We next investigated the ability of the CNV-DI RNAs to interfere with CNV helper virus accumulation in cucumber protoplasts. Although accumulation of the genomic and DI RNAs was readily evident in ethidium bromide-stained agarose gels, incorporation of [^{3}H]uridine and subsequent fluorography provided a better visualization of the *de novo* RNA synthesis of both the helper virus and DI RNAs during the course of infection (Fig. 5A). Co-inoculation of protoplasts with a mixture of each of the DI RNAs and CNV transcripts significantly decreased the synthesis of CNV genomic RNA. The results presented in Fig. 5B illustrate the relative incorporation of [^{3}H]uridine into viral and cellular RNAs at 22 hr postinoculation in CNV infections either lacking or containing DI RNAs. Infections containing DI RNAs generally showed similar levels of interference (>50%) of helper genome accumulation. An exception was the C40 DI RNA, in which the parental CNV RNA consistently accumulated to more than 70% of the amounts observed in the DI RNA-free inoculum. Whole plant infections were also performed to determine whether the reduced interference observed by C40 in protoplasts could be correlated with differences in symptom attenuation. To investigate this question, *N. benthamiana* and *N. clevelandii* plants were inoculated with CNV RNA alone or with CNV RNA inocula containing the C40 or C61 DI transcripts. Surprisingly, similar levels of symptom attenuation and DI amplification were observed in both cases (data not shown), suggesting that only a small diminution of the level of parental genomic RNA synthesis is sufficient to ameroliate the normal lethal syndrome observed in the two *Nicotiana* species.

Similar sequences affect accumulation of TBSV- and CNV-DI RNAs in protoplasts

The structural similarities between CNV- and TBSV-DI RNAs prompted an analysis of the conserved regions

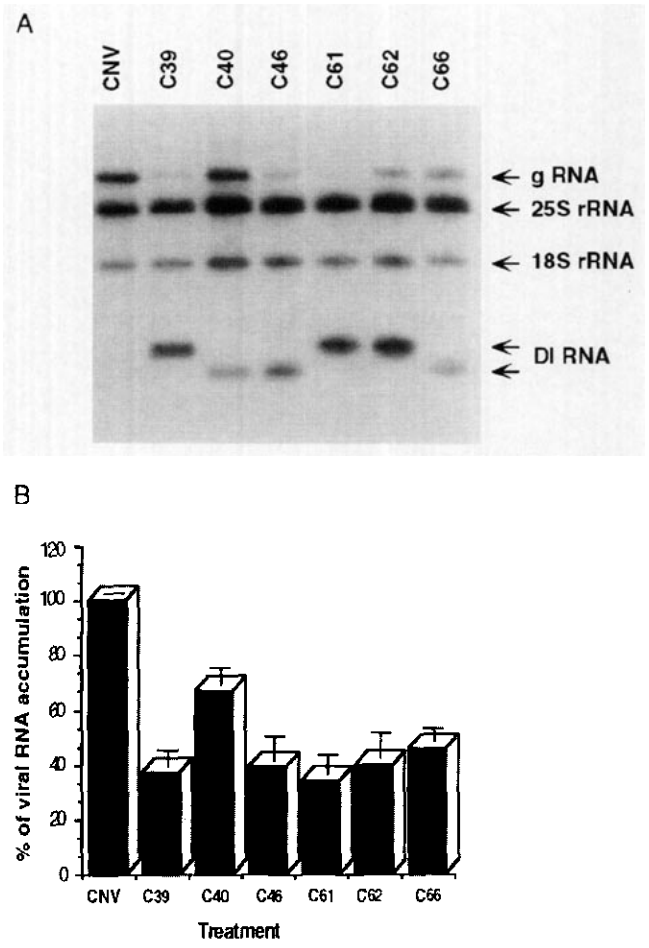

FIG. 5. Total RNA extracts isolated from cucumber protoplasts transfected with cloned CNV transcripts with and without the indicated CNV DI transcript in the inoculum. Protoplasts were transfected with transcripts and total RNA was extracted from protoplasts at 22 hr post-transfection and electrophoresed in 1% agarose TBE gels. (A) Fluorographic analysis of [^{3}H]uridine incorporation into total RNAs. The positions of the CNV genomic (g) RNA, 25 and 18 S host ribosomal (r) RNAs, and the DI RNA species are indicated along the side of the gel. (B) Quantitation of CNV genomic RNA accumulation in protoplasts. The RNA bands were excised, radioactivity was determined, and the relative accumulation of the CNV genomic RNAs was calculated as a percentage of the genomic RNA accumulating in control transfections lacking DI RNAs. The results represent an average of three independent experiments.

present in both types of molecules. A series of large deletions was introduced into the different regions identified in the prototypical TBSV-B10 DI RNA (Knorr *et al.*, 1991; Fig. 6A). Infectivity assays in protoplasts revealed that deletion mutants lacking major portions of regions I (B10ΔIb), II (B10ΔIIa, B10ΔIIb) and III to IV (B10ΔIII and IV) were not amplified when co-inoculated with TBSV genomic transcripts in *N. benthamiana* protoplasts (Fig. 6B). Interestingly, the TBSV DI RNA mutant with region III completely deleted (B10ΔIII) accumulated to the same extent as the wild-type DI RNA (Fig. 6B). This result demonstrated that region III of the TBSV DI RNAs is dispensable for replication, but that the 5′- and 3′-noncoding sequences comprising regions I and IV, and a portion of the replicase encoding sequences comprising region II contain elements necessary for TBSV-DI RNA accumulation.

We then performed an analogous series of experiments (Fig. 7) using the representative CNV-DI RNA clone C2 and its derivative C2H (containing an intro-

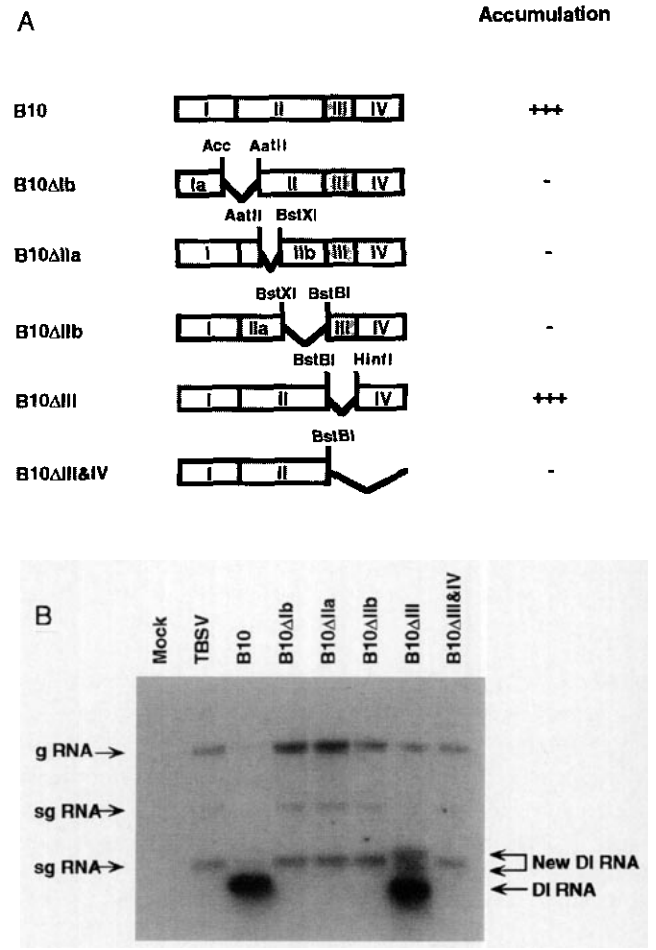

FIG. 6. Identification of conserved regions required for accumulation of TBSV-DI RNAs in *N. benthamiana* protoplasts. (A) Map of the genome of TBSV-DI RNA (B10) and its deletion derivatives. Blocks with shading patterns similar to those in Fig. 4 represent the conserved regions derived from different parts of the TBSV genome. The mutant clone designations are shown on the left, diagrams of the mutants with restriction enzyme sites and deletions indicated as bent lines are illustrated in the center, and relative accumulation of the mutant transcript DI RNAs are shown on the right. (B) Hybridization analysis of total nucleic acid preparations from *N. benthamiana* protoplasts inoculated with TBSV transcripts plus the indicated DI RNA transcripts (from left to right): no transcript (mock), TBSV transcripts only (TBSV), and TBSV transcript mixed with DI transcript from B10, B10ΔIb, B10ΔIIa, B10ΔIIb, B10ΔIII, and B10ΔIII and IV. The blot was hybridized with TBSV probes obtained by nick translation. The diffuse DI-like species above the major band in B10ΔIII are thought to represent newly generated DI species (new DI RNAs) that appear in protoplasts after transfection. The TBSV genomic RNA (g RNA) and subgenomic RNAs (sg RNA) are indicated along the left side of the figure and the positions of the DI RNAs are shown on the right.

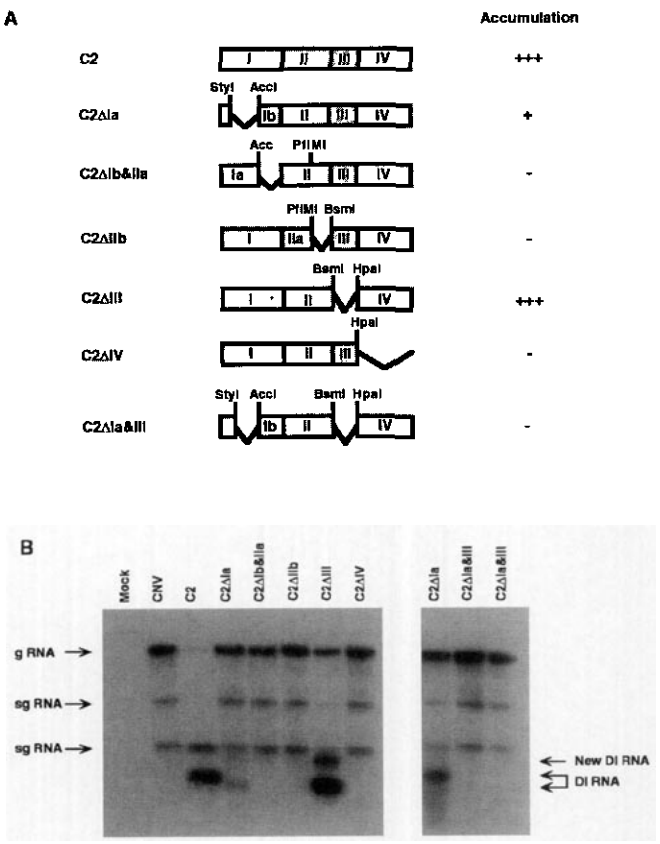

FIG. 7. Identification of conserved regions required for accumulation of CNV-DI RNA in *N. benthamiana* protoplasts. (A) Map of the genome of CNV-DI RNA (C2) and its deletion derivatives. Blocks with different shading patterns represent the conserved regions derived from different parts of the CNV genome as described in Fig. 6. (B) Blot hybridization of total nucleic acid preparations from *N. benthamiana* protoplasts inoculated with CNV transcripts plus the indicated DI RNA transcripts (from left to right): no transcript (mock), CNV transcript alone (CNV), and CNV transcripts mixed with DI RNA transcripts from C2, C2ΔIa, C2ΔIb and IIa, C2ΔIIb, C2ΔIII, and C2ΔIV plasmids. The right panel shows the results of a similar experiment using a different C2ΔIa clone and two independent double mutant clones (C2ΔIa and III). Hybridization was carried out with CNV-specific probes generated by nick translation of CNV clones. CNV genomic RNAs (g RNA) and subgenomic RNAs (sg RNA) are indicated on the left of the gel. The intense band above the major band in C2ΔIII is thought to be a newly generated *DI-like RNA (new DI RNA). Designations along the side of the gel* correspond to those shown in Fig. 6.

duced *Hpa*I site), both of which were found to accumulate to approximately the same levels in protoplasts (data not shown). The infectivity assays showed that deletion mutants lacking portions of regions I and II (C2ΔIb and IIa, C2ΔIIb) and region IV (C2ΔIV) were unable to replicate (Fig. 7), as was previously observed with analogous TBSV DI RNA deletion mutants (Fig. 6). Convenient restriction sites in the CNV clones permitted a partial deletion of the central portion of region I of C2H, and the resultant mutant, C2ΔIa, displayed markedly reduced accumulation in *N. benthamiana* protoplasts (Fig. 7). Deletion of region III in the CNV DI RNA clone (C2ΔIII) also *failed to eliminate replication (Fig. 7), as was also true* for the similar TBSV mutant (Fig. 6). Although both of the region III deletion mutants (B10ΔIII and C2ΔIII) accumulated as well as the wild-type parental DI RNAs (B10 and C2), the infections were somewhat abnormal in that a population of larger RNAs appeared in the protoplast infections (see Figs. 6B, lane B10ΔIII and Fig. 7B, lane C2ΔIII). These larger RNA species appear to be dimers of the mutants. Direct sequence analysis of the RNAs indicated that the region III deletions were duplicated in *the clones because the sequencing primers appeared* to bind within both motifs and the resulting sequence appearing at the 5′ boundary of the RNA appeared to represent a mixture of the duplicated sequence and the sequences at the 5′ terminus of region III (data not shown). Moreover, only monomers identical in size to the input inoculum were recovered in RT–PCR cloning experiments (not shown). However, despite the ability of the region III deletion mutants to accumulate to wild-type levels in *N. benthamiana* protoplasts, the level of interference with helper virus accumulation was lower than that of the wild-type DI RNAs (Chang *et al.*, unpublished data). In addition, a double deletion mutant, C2ΔIa and III, containing two deletions which individually allowed replication, was unable to accumulate to detectable levels in protoplasts (Fig. 7B). Taken together, these results suggest that both the Ia and III regions are independently dispensable for replication, but that each motif contains sequences that promote DI RNA competitiveness. Deletion of both motifs appears to have a more pronounced effect which eliminates DI RNA accumulation.

Sequences in region II are required for efficient accumulation of DI RNAs

The comparable results obtained with the larger deletions of region II in both the TBSV and CNV DI RNA suggested that a more refined analysis might help define essential sequences in this motif. We were able to construct a series of nested deletions in region II using *Bal*31 nuclease digestion beginning at comparable sites in TBSV (*Bst*XI in B10) and CNV (*Pfl*MI in C2H) DI RNA clones. The deletions introduced into mutant TBSV and CNV DI RNA clones, and the ability of the mutants to accumulate in *N. benthamiana* protoplasts are shown in Figs. 8A and 8B, respectively. The results clearly demonstrate that comparable segments in region II of the TBSV and CNV DI RNAs (Fig. 8, shaded region) can be deleted without affecting accumulation of either DI RNA. Mutants containing deletions adjacent to the borders of the shaded region failed to accumulate. These results are consistent with the observation that B10ΔIIa is replication defective (Fig. 6), and they further delineate the 3′ border of the essential element for TBSV (Fig. 8A, B103D16 and B103D27).

The similarity in the sequence requirements of region

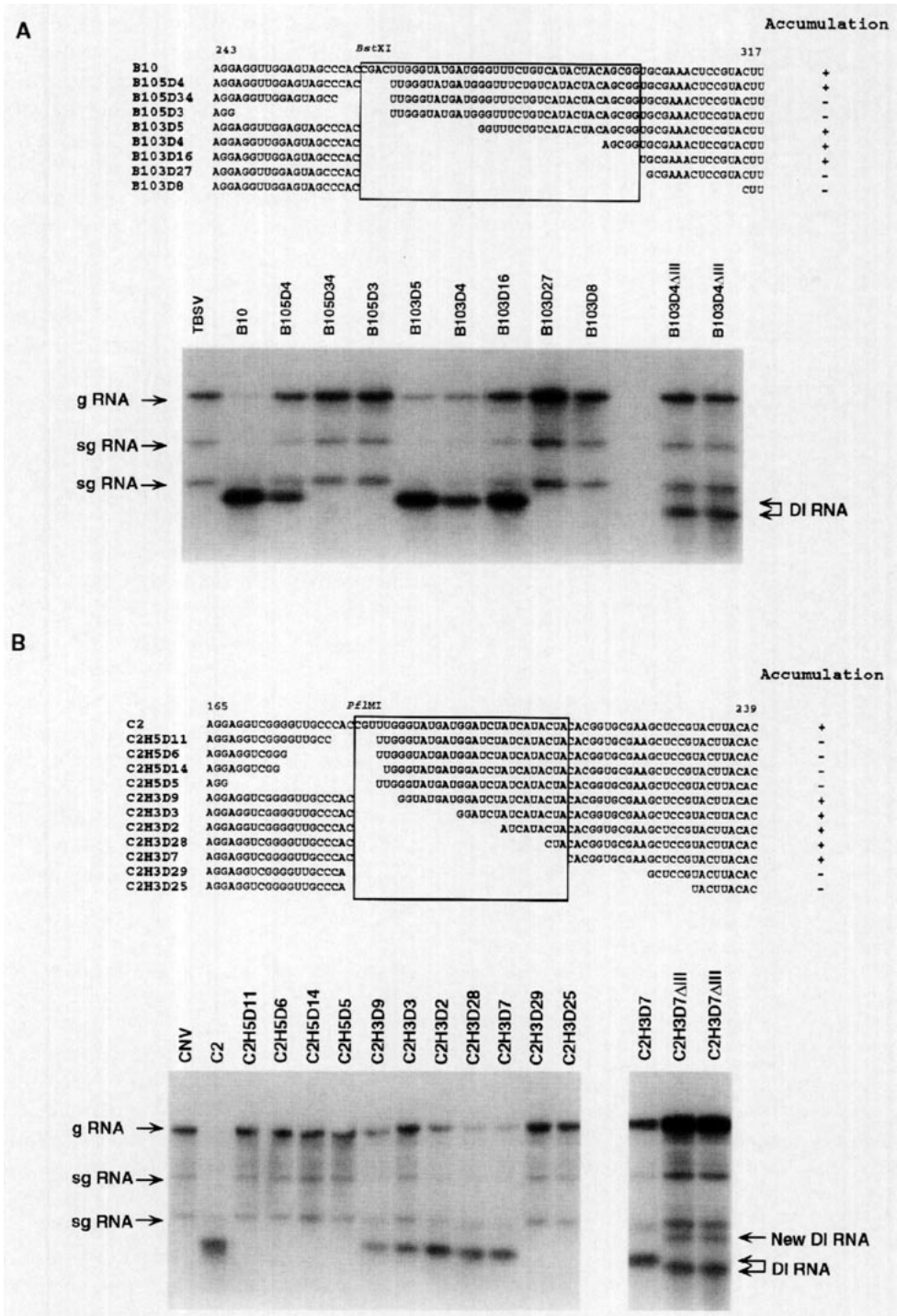

FIG. 8. Infectivity of TBSV- and CNV-DI RNAs after Bal31 deletion mutagenesis of region II. The region at the top of the gels shows the sequence in the mutagenized region and the deleted sequences of each mutant are indicated by gaps. The sequences inside the boxes identify dispensable regions that have little effect on infectivity in *N. benthamiana* protoplasts. (A) RNA from *N. benthamiana* protoplasts transfected with transcripts of TBSV and B10 DI RNA or the region II deletion derivatives illustrated above. The blots were hybridized with radioactive probes specific for TBSV RNAs. Designations of the viral and DI-like RNAs are indicated along the side of the gel. (B) Hybridization of RNA from *N. benthamiana* protoplasts transfected with transcripts of CNV and C2 DI RNA or the region II deletion mutants illustrated above. The blots were hybridized with radioactive probes derived from CNV RNA.

II for accumulation of both the TBSV and CNV deletion mutants prompted a computer-aided secondary structure analysis of these regions. A portion of the secondary structure with the lowest free energy predicted by the program MFold (Zuker, 1989) in the Sequence Analysis Software Package (Version 7.2-UNIX) supplied by GCG (Genetics Computer Group, Inc., Madison, WI) is illustrated in Fig. 9. The computer-generated structures sug-

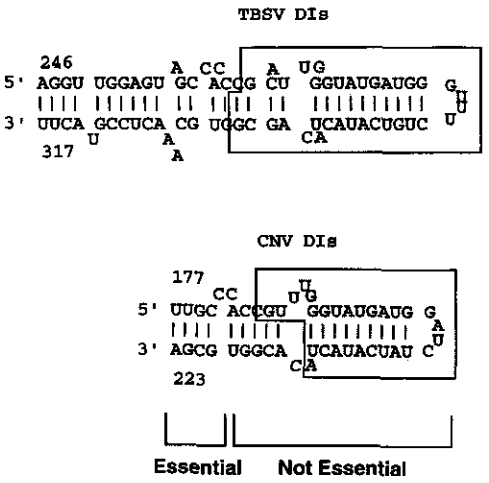

FIG. 9. Predicted secondary structure of the border of the essential and nonessential sequences within region II of TBSV- and CNV-DI RNAs. Sequences within the shaded boxes were dispensable for efficient accumulation of the DI RNAs in *N. benthamiana* protoplasts. The unshaded sequences to the left and at the border of the shaded boxes are conserved among the DIs and constitute an essential *cis*-element required for DI replication.

gest that similar stable hairpin structures can form in the central portion of region II in both DI RNAs and that the border of the essential and nonessential sequences in both DI RNAs occurs at about the same site. In each case, the nonessential sequences map to the top of the predicted hairpin (shaded in Fig. 9). These results suggest that some of the sequences in region II which are necessary for DI RNA amplification may be part of a conserved secondary structure.

Finally, to more extensively define the minimal sequences required for DI RNA accumulation, we constructed double-deletion derivatives by removing region III of the minimal region II mutants, B103D4 (Fig. 8A) and C2H3D7 (Fig. 8B). Both double mutants, B103D4ΔIII (Fig. 8A) and C2H3D7ΔIII (Fig. 8B), were biologically active and protoplasts infected with each mutant accumulated larger DI-like RNAs similar to those observed previously for other region III deletion mutants (B10ΔIII and C2ΔIII; Figs. 6B and 7B). Therefore, these findings further demonstrate that very similar portions of regions II and III can be simultaneously deleted in the TBSV- and CNV-DI RNAs.

DISCUSSION

Our results on the characterization of CNV DI RNAs selected after host passage extend previous studies showing that native tombusvirus DI RNAs consist of four conserved regions of the helper genome (Burgyan *et al.*, 1989, 1991; Hillman *et al.*, 1987; Morris and Hillman, 1989; Knorr *et al.*, 1991; Rochon, 1991; Rochon and Johnston, 1991; Rubino *et al.*, 1990). These include region I, derived from the 5′-noncoding sequence, region II, containing sequences from the replicase gene, region III, which includes a small portion of the 3′ proximal nested genes and the adjacent 3′-noncoding sequences, and region IV, consisting of 3′-terminal noncoding sequences. These four motifs thus appear to have roles in replication, stability and/or movement of the DI molecules in plants.

A notable difference between the native CNV DI RNAs characterized in this study and the other described tombusvirus DI RNAs is the variable size of natural deletions that appear within region II. Another difference is the exclusive deletion of sequences between regions III and IV in all of the CNV DI RNAs we examined. These results complement those of an investigation in which the formation and evolution of TBSV DI RNAs was examined in protoplasts (White and Morris, 1994a). In the TBSV study, it was found that DI RNAs containing a deletion between region III and IV were more competitive than otherwise identical DI RNAs that did not contain the deletion. Furthermore, it was found that upon serial passage in protoplasts, DI RNAs containing a contiguous 3′ end (i.e., no deletion between region III and IV) evolved to smaller forms in which various segments between III and IV were deleted. The primary determinant of this transition appeared to be the selection for more highly replication competent molecules (White and Morris, 1994a). However, in the case of the present CNV experiments, selection in different host plants most likely requires competition for efficient replication, and for cell to cell and vascular movement.

The sequence analyses of the CNV DIs suggest that rearrangement and/or recombination events involving DI RNAs can have a significant role in their evolution. The identification of large CNV-DI RNAs which contain a long direct repeat of regions II, III, and part of IV provides evidence for such events *in planta*. Although such extensively duplicated regions have not been reported in other tombusviruses, Hillman *et al.* (1987) did describe a TBSV-DI RNA (DI-2-120) that contained an internal 78 nt direct repeat consisting of 5 bases from region I and 73 bases from region II. More recently, White and Morris (1994a) identified a similar DI RNA with a duplication of a segment in region II. In competition assays, this molecule was shown to be more competitive than its precursor, which did not contain the duplication (White and Morris, 1994b). Together, these observations support the hypothesis that DI RNA populations are dynamic and that duplication of certain sequences, such as those in region II, must confer some type of selective advantage; otherwise, similar DI RNAs would not have evolved under such diverse circumstances.

The comparative deletion analyses of the CNV and TBSV DI RNAs demonstrated that region IV and certain parts of region II are essential for efficient amplification of both TBSV and CNV DI RNAs in *N. benthamiana* protoplasts, but that region III is dispensable. Although the CNV-DI RNA mutant-C2Δ1a could replicate without a 5′

portion of region I, the level of accumulation was much lower than that of the region III deletion mutant (C2ΔIII) or the wild-type (C2) progenitor (Fig. 7B). From these results, we propose that nucleotides 27 to 105 have an important role in CNV-DI RNA accumulation *in vivo*, although they are not absolutely required for replication per se. Additional experiments demonstrated that a mutant with deletions in region Ia and III (C2ΔIa and III) was unable to accumulate in *N. benthamiana* protoplasts (Fig. 7B). The inability of this double mutant to accumulate may be the result of secondary and tertiary conformational changes that abrogate replication. The C2ΔIII mutant and similar region III deletion derivatives seem to be unstable DI RNA species that generate larger DI RNA molecules that appear to contain segment duplications. Thus, while region III is dispensable for accumulation, it must be important for some as yet undefined function because it is conserved in all naturally isolated DI RNAs characterized so far. A more comprehensive analysis of the possible roles of region III in attenuation of symptoms will be presented elsewhere.

The analysis of the nested deletion mutants in region II reveals the presence of similar regions in both TBSV- and CNV-DI RNAs that are essential for accumulation of the DI RNAs. One of the first characterized of the TBSV-DI RNAs (DI-2-111 in Hillman *et al.*, 1987) also contained deletions within region II, but these were different from those residing in the putative hairpin structure identified in our study (Fig. 9). This implies that some of the sequences dispensable for DI RNA replication may be selected against during evolution. For example, the derivatives B103D4ΔIII and C2H3D7ΔIII, which have substantial deletions in region II and III, may be close to the minimal biologically active DI RNAs. The infectivity assays in *N. benthamiana* protoplasts revealed that in order to have interfering capability, DI RNAs needed to be replication competent (Figs. 7B and 8B). Nevertheless, although region III deletion mutants could accumulate as well as wild-type DI RNAs, their abilities to interfere with the parental viral RNA were less pronounced than those of the wild-type DI RNAs. Interestingly, the larger DI-like RNAs that appeared upon inoculation of the deletion mutants B10ΔIII, B103D4ΔIII, C2ΔIII, and C2H3D7ΔIII were unable to effectively interfere with replication of the parental virus. In this regard, mutations within a Sindbis virus DI RNA that resulted in large internal deletions also facilitated evolution of heterogeneous DI RNA species larger than the parental mutant DIs (Levis *et al.*, 1986). However, it is possible that the larger Sindbis DI RNAs were generated *de novo* from the parental virus.

The serial passage experiments conducted with CNV closely parallel previous studies with the TBSV cherry isolate (Knorr *et al.*, 1991; Law and Morris, 1994). Both sets of experiments suggest that continuous passage of tombusviruses through a single host culminates in evolution of a homogeneous population of DI RNAs adapted for that particular host. Like the TBSV experiments, CNV passage resulted in appearance of a heterogenous DI RNA population during the first transfers through tobacco plants, but a single major class of DI RNA predominated after 8 to 10 passages at high m.o.i. Although experiments in protoplasts suggest that DI selection in the TBSV cherry system is primarily based on efficient replication (White and Morris, 1994a), the present results showing high CNV DI RNA multiplication in cucumber protoplasts suggest that factors in addition to replication influence DI RNA evolution during passage through alternate hosts. These factors may involve efficient cell to cell movement or long distance transport, or they could involve subtle associations with host factors that influence DI RNA persistence and competitive abilities in different hosts. Irrespective of the fundamental reasons for loss of the CNV-DI RNAs upon passage in the alternate cucumber host, our experiments indicate that the host has a major role in determining DI RNA destiny.

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