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VIROLOGY 190, 579–586 (1992)

The Replication of Cymbidium Ringspot Tombusvirus Defective Interfering-Satellite RNA Hybrid Molecules

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Received April 9, 1992; accepted June 23, 1992

A DNA copy of DI RNA of cymbidium ringspot tombusvirus was cloned downstream of a phage T7 promoter. *In vitro*-transcribed RNA replicated in *Nicotiana clevelandii* when coinoculated with full-length viral genomic RNA transcripts and protected plants from apical necrosis. Artificial deletion mutants derived from the DI RNA clone showed that most of the central sequence block is necessary for replication. Hybrid DI RNA–satRNA clones were prepared and *in vitro*-synthesized RNA was inoculated to plants in the presence of helper viral RNA. There was replication only of *in vitro* transcripts derived from hybrid clones where satRNA sequences were inserted upstream or downstream from the central block, but not of those derived from clones where satRNA sequence replaced the central block. Progeny RNA of biologically active clones was either full-length or showed deletions depending on the insertion of satRNA sequences in DI RNA. DI RNA–satRNA constructs having part of the 5′ region exchanged were not replicated.

INTRODUCTION

Defective interfering (DI) RNAs are deletion mutants of animal and plant viruses that replicate only in the presence of the parent (helper) viral genome. Naturally occurring DI RNAs have been discovered in several taxonomic groups and genera of plant viruses, i.e., tombus- and carmovirus (reviewed by Roux *et al.*, 1991), rhabdovirus (Ismail and Milner, 1988), potexvirus (White *et al.*, 1991), geminivirus (Frischmuth and Stanley, 1991), and tospovirus (Resende *et al.*, 1991), but have also been artificially obtained, as with brome mosaic bromovirus (Marsh *et al.*, 1991).

For plant viruses, one of the most extensively investigated DI RNA systems is that of cymbidium ringspot tombusvirus (CyRSV), an icosahedral virus with a positive-sense ssRNA genome of 4733 nucleotides (nt) (Grieco *et al.*, 1989). CyRSV DI RNA was shown to consist of a mosaic molecule made up entirely of noncontiguous regions of genomic RNA (Burgyan *et al.*, 1989; Rubino *et al.*, 1990) generated *de novo* during viral replication through progressive genome deletions (Burgyan *et al.*, 1990, 1991).

In addition to DI RNA, CyRSV may also support the replication of a satellite (sat) RNA of 621 nt sharing limited sequence homology with genomic RNA (Rubino *et al.*, 1990). Similar to DI RNA, satRNA is completely dependent on helper virus for replication (Rubino *et al.*, 1992), but contrary to DI RNA, it is efficiently encapsidated (Gallitelli and Hull, 1985).

The biology of CyRSV DI RNAs is largely unknown. Sequences necessary for recognition by viral replicase and required for encapsidation have not been identified, nor it is understood how its replication interferes with that of genomic RNA, thus modifying the effects of viral infection on host plants (Burgyan *et al.*, 1989).

Some of these problems were addressed in the present study through the production of biologically active transcripts from a cDNA clone to a natural DI RNA. In addition, hybrid molecules containing sequences derived from CyRSV DI RNA and satRNA were constructed and inoculated to plants together with the helper virus, to explore the possibility that CyRSV genome in a reduced form (i.e., DI RNA) could carry foreign sequences inside host cells.

MATERIALS AND METHODS

Cloning biologically active DI RNA

An RNA preparation from CyRSV-infected *N. clevelandii* plants containing DI RNA of ca. 500 nt was used. Full-length cloning of DI RNA was obtained essentially as described (Burgyan *et al.*, 1991). Briefly, 10 μg total RNA was denatured in the presence of 10 m*M* methylmercuric hydroxide for 10 min at room temperature, and mixed with $\frac{1}{5}$ volume 700 m*M* 2-mercaptoethanol and 40 units RNase inhibitor (HPRI, Amersham). First cDNA strand synthesis was primed with 5′-phosphory-

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0042-6822/92 $5.00

lated oligonucleotide 5′ GGGCTGCATTGCTGCAA 3′ complementary to the last 17 nt of CyRSV genomic RNA sequence. Hybrid RNA–DNA molecules were melted at 100° for 1 min, and 5 μl cDNA were amplified by 35 cycles of the polymerase chain reaction (PCR) using as second primer the 5′-phosphorylated oligonucleotide5′ATCGATAATACGACTCACTATAGGAAATC-CTCCAGGACA 3′, containing the first 17 bases of genomic RNA, 17 bases of the bacteriophage T7 RNA polymerase promoter consensus sequence, and 5 bases contributing to formation of a *Cla*I restriction site. Amplitaq polymerase and a Perkin Elmer Cetus DNA Thermal Cycler were used. Four-microliters (4 units) of Klenow enzyme were added to the PCR mixture (100 μl), incubated for 30 min at 37°, and electrophoresed in 1.0% low-melting-temperature agarose (Bethesda Research Laboratories, BRL). The major PCR product corresponding in size to ca. 500 bp, was extracted, concentrated with ethanol, resuspended in water, ligated into *Sma*I-digested, dephosphorylated pUC18, and transformed into DH5α strain of *Escherichia coli.* The DI RNA clone thus obtained was designated DI-371.

Construction of mutant clones of DI RNA and hybrid clones DI RNA–satRNA

Two restriction sites, *Hpa*I and *Eco*RV, not present in DI RNA and vector sequences, were inserted in the DI RNA clone by site-directed mutagenesis (Kunkel *et al.,* 1987) at positions 141 and 299 in DI RNA clone using the oligonucleotides 5′ AGCAAAATGttAAcCAGTTTGT 3′ and 5′ ACCTTCGTGAtatcGAAAGCTAGTAGGA 3′, respectively (mutated bases in lower-case letters). The mutated clone was designated DI-3. Three deletion mutants of DI-3 were prepared by deleting regions between *Hpa*I and *Eco*RV (nt 141–299), *Hpa*I and *Bsm*I (nt 141–253), or *Bsm*I and *Eco*RV (nt 253–299) to give clones ΔHE, ΔHB, and ΔBE, respectively.

A full-length clone of CyRSV satRNA (pCS2B; Rubino *et al.,* 1992) was used as the source of satRNA sequences to be inserted in DI-3. In particular: (i) pCS2B was restricted with *Pfl*MI (nucleotide position 242 in satRNA sequence; Rubino *et al.,* 1990), made blunt ended with T4 DNA polymerase, then digested with *Bfr*I (position 542), and made blunt ended with Klenow enzyme (Sambrook *et al.,* 1989). The 300-nt-long fragment was extracted from 1% agarose and ligated to DI-3 digested with either *Hpa*I or *Eco*RV or both enzymes; (ii) pCS2B and DI-3 were digested with *Acc*I which cuts at positions 202 and 92, respectively, and in the polylinker upstream from the 5′ end of both clones. The ca. 210-nt-long fragment of pCS2B was purified and fused to DI-3 and the ca. 100-nt-long fragment from DI-3 was fused to pCS2B.

In vitro RNA transcription and inoculation

Recombinant plasmid (500 ng) was linearized with *Sma*I, extracted with phenol:chloroform, precipitated with ethanol, and transcribed with T7 RNA polymerase in a 25-μl reaction mixture using a Stratagene transcription kit. A full-length CyRSV genomic RNA DNA clone (G11; Dalmay *et al.,* unpublished) was used as helper inoculum: 2 μg was linearized with *Sma*I and transcribed in a 100-μl reaction mixture. Helper and DI RNA or hybrid clones transcription mixtures were combined and diluted with an equal volume of inoculation buffer (Heaton *et al.,* 1989) before inoculation. Aliquots (15 μl) of the mixture were spread with a glass spatula on each of four leaves of *N. clevelandii* plants.

Extraction and analysis of RNA

Virus purification and extraction of RNA from virus particles were done as described (Gallitelli *et al.,* 1985).

Total RNA was extracted from 200-mg tissue samples as described by White and Kaper (1989). About 100 ng RNA was denatured with formaldehyde and formamide, electrophoresed in formaldehyde-permeated agarose gels, and blotted to nylon membrane (Sambrook *et al.,* 1989). Northern blot analysis was performed using ^{32}P-labeled nick-translated probes of satellite or DI RNAs. Sequence analysis of DI RNA progeny clones was performed by amplifying cDNA by PCR and cloning the products as previously described. RNA was synthesized *in vitro* from all progeny RNA clones and back inoculated to plants. Only clones that gave infectious transcripts were sequenced with T7 DNA polymerase (Sequenase, USB) as in Hattori and Sakaki (1986).

RESULTS

Sequence analysis and biological activity of DI RNA clones

Sequence analysis of clone DI-371 showed it to be composed of 477 nt derived from, and identical to, tracts of viral genomic RNA, with the exception of two bases that were missing in DI RNA (Fig. 1A). In accordance to previous results (Burgyan *et al.,* 1991), the DI RNA sequence could be divided in three blocks (A, B, C) depending on the position of conserved sequences in genomic RNA (Fig. 1B). Block A was composed of the first 165 nucleotides of genomic RNA (i.e., 161 nt of the 5′ leader sequence and first 4 nt of the 33-kDa gene) with two deletions of 15 and 8 nt; block B had 157 nt, corresponding to nt 1348 to 1507 in genomic RNA; block C had 178 nt, corresponding to the carboxyl terminus of the 22-kDa gene (31 nt) and 147 nt of

A

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GGAAATCCTC CAGGACACCT ▽ATCCGGTGAT TTGCGCTACT GTTGCTTTGC     50
GTAGAGGATT TCTCTCCTGT ▽TATCAGGGGT TTGAAGGCAG GGTCTACCTT    100
TCGGGGGGAT AAATTGTAAC TTCCTGCAAA TAAGCAAAAT ▽GGGAAGCAGT    150
TTGTGAGAAG GTTGGGGTGG CCCATCGCTT AGGGTATGAC GGGTTCCTGT    200
CATACTACAG TGGTGCGAAA CTCCGTACTT ACACGCGAGC TGTGGAGAGT    250
CTGCATTCAC TCCTATTTCA GAGAGGGATA GTCATTGACT ACCTTCGTG▽A   300
GAAAGCTAGT AGGACAGACT CTTCAGTCTA GGTTGGTGGA AGCAGGAGAA    350
CACTGCTTG▽A CTAGCGGAGA ACATTTGTGG AAACCAACCA AGACACGGTT   400
GATCTCACCC TTCGGGGGGG CTATAGAGAT CGCTGGAAGC ACTACCGGAC    450
AACCGGAACA TTGCAGCAAT GCAGCCC                             477
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B

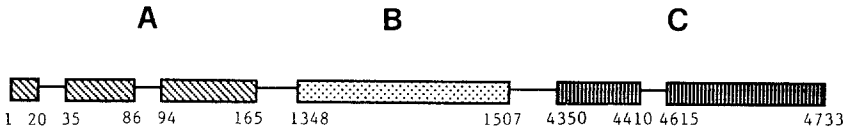

FIG. 1. (A) DNA sequence of CyRSV DI-371 RNA. Open triangles indicate the start of blocks of conserved sequence from the CyRSV genome Arrowheads indicate positions of nucleotides present in genomic RNA and absent in DI RNA. (B) Diagram of the structure of DI-371 RNA showing conserved regions (shaded blocks) and deletions (lines) in genomic RNA. Numbers indicate nucleotide positions in the CyRSV genomic RNA.

the noncoding region up to the 3′ end of genomic RNA (see Grieco *et al.*, 1989, for genome map of CyRSV).

Coinoculation to *N. clevelandii* plants of *in vitro* transcripts of G11 and DI-371 showed that *in vitro*-synthesized DI RNA replicated *in vivo* and protected infected plants from apical necrosis and death, which occurred in control plants inoculated only with G11 transcripts (not shown). *In vitro* transcripts from clone DI-3 (containing the two engineered restriction sites *Hpa*I and *Eco*RV) were also able to replicate in the presence of *in vitro* transcripts of G11 (Fig. 2, lane 5, and Fig. 4A, lane 10) and protect infected plants (not shown). DI-371 and DI-3 RNA appeared to be stable forms of DI RNA since smaller DI RNA molecules were not observed. Progeny molecules of DI-3 were sequenced and shown to be derived from inoculated molecules because of the presence of the two engineered restriction sites (not shown).

Artificial deletion mutants of DI-3 were constructed by deleting block B (157 nt; clone ΔHE), or sequences between *Hpa*I and *Bsm*I (112 nt; clone ΔHB) or *Bsm*I and *Eco*RV (45 nt; clone ΔBE). *In vitro* RNA transcripts from these clones were coinoculated with G11 RNA in *N. clevelandii* plants which were monitored for replication of mutant DI RNAs. Northern blot analysis showed that ΔHE and ΔHB RNAs did not replicate, whereas ΔBE RNA replicated and accumulated apparently as DI-3 RNA (Fig. 2), indicating that the sequence be-

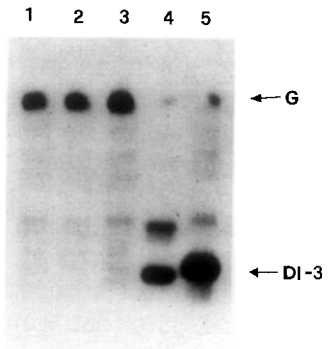

FIG. 2. Northern blot of RNA preparations from plants inoculated with CyRSV RNA (G11) and DI RNA (DI-3 or mutants ΔHE, ΔHB, or ΔBE) transcripts. G11 transcripts only (lane 1), G11+ΔHE (lane 2), G11+ΔHB (lane 3), G11+ΔBE (lane 4), and G11+DI-3 (lane 5). Hybridization with nick-translated DI-3 probe. G, genomic RNA. The band above the major species in lane 4 is a dimer of DI RNA.

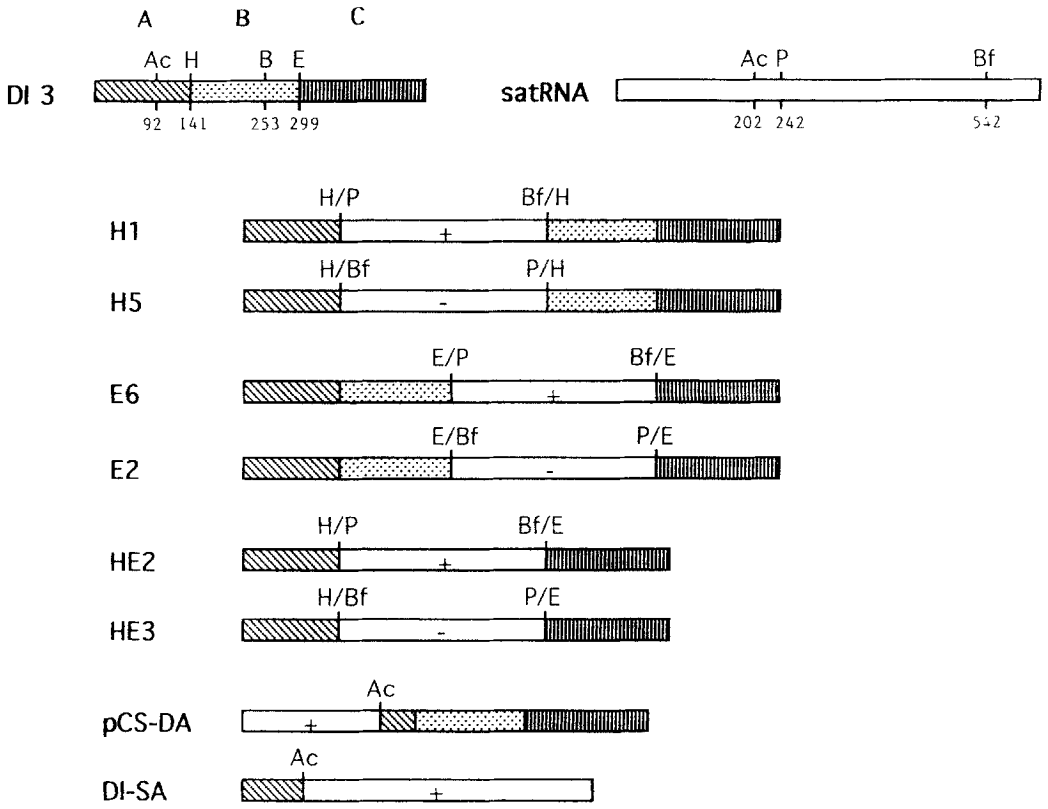

FIG. 3. Diagram of DI-3, satellite, and DI–satellite hybrid clones. Shaded blocks are DI RNA sequences (see also Fig. 1B); open blocks are satRNA sequences. Numbers are nucleotide positions in DI-3 and satRNA sequences of relevant restriction sites: Ac, *Acc*I; H, *Hpa*I; B, *Bsm*I; E, *Eco*RV; P, *Pfl*MI; Bf, *Bfr*I. + and − indicate orientation of satRNA sequences relative to the positive-sense sequence.

tween *Hpa*I and *Bsm*I in block B is indispensable for replication of DI RNA.

Biological activity of DI RNA–satRNA hybrid molecules

Several DI RNA–satRNA hybrid clones were constructed (Fig. 3). SatRNA sequences inserted in the *Hpa*I site of DI-3 produced two types of hybrid clones which were designated H1 and H5. Clone H1 had the 300-nt satRNA sequence in the same orientation (positive sense) as in original satRNA molecule, whereas clone H5 had this sequence inverted (negative sense). This sequence was chosen because it lacks the ca. 50-nt stretch in common with DI and genomic RNAs, and includes other small stretches in common with genomic but not DI RNA (Rubino *et al.*, 1990). Should these sequences be important for encapsidation, progeny RNA from H1 and H5 clones would be found in virus particles. *In vitro* RNA transcripts from both clones were biologically active. Northern blot analysis of total RNA extracts showed that progeny RNA was of the same size as the inoculum RNA and hybridized both to DI RNA and satRNA probes (Fig. 4). Sequence analysis confirmed that progeny RNA was identical to the transcript RNA and that junction points between DI RNA and satRNA sequences were maintained (Fig. 5). Northern blot analysis of RNA preparations from purified virus particles showed that neither H1 or H5 progeny RNA was encapsidated (Fig. 6). However, H5 (but not H1) progeny was transmitted by sap inoculation (not shown).

E2 and E6 clones had the same sequence of satRNA as H1 and H5 inserted in *Eco*RV site in positive (E6) and negative (E2) orientation. Northern blot analysis of RNA progeny of E2 showed the presence of both full-length molecules, which hybridized with DI RNA and satRNA probes, and shorter molecules, which hybridized only to DI RNA probes (Fig. 4). Sequence analysis showed that full-size molecules were indeed identical to parent molecules, whereas shorter molecules lacked the satRNA sequence and, in addition, a tract of DI RNA sequence of 45 nucleotides upstream and 1 downstream or 20 nucleotides upstream and 8 downstream the *Eco*RV site (Fig. 5). Tissue extracts of plants inoculated with clone E6 *in vitro* transcripts showed the presence of only two types of RNA molecules, one of ca. 400 nt, which hybridized only to DI RNA probe, and one of ca. 700 nt which hybridized both to DI RNA and satRNA probes. No RNA of the size of the original inoc-

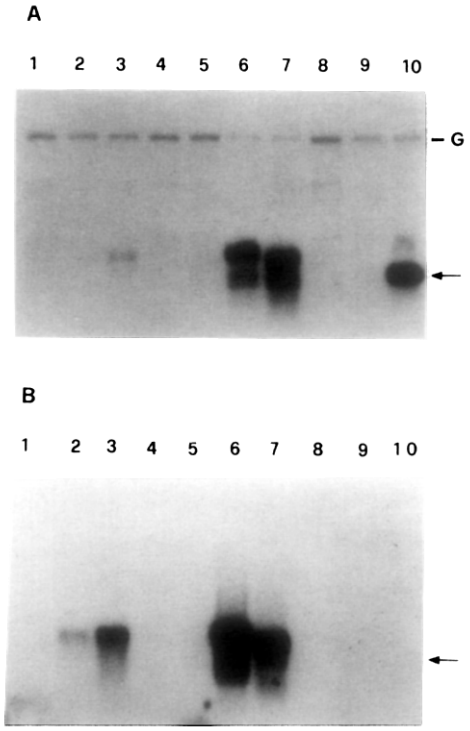

FIG. 4. Northern blots of RNA preparations from plants inoculated with CyRSV RNA and DI RNA–sat RNA hybrid transcripts. G11 transcripts only (lane 1), G11+H1 (lane 2), G11+H5 (lane 3), G11+HE2 (lane 4), G11+HE3 (lane 5), G11+E2 (lane 6), G11+E6 (lane 7), G11+pCS-DA (lane 8), G11+DI-SA (lane 9), and G11+DI-3 (lane 10). Hybridization with nick-translated clones of DI-3 RNA (A) and satRNA (B). Arrow points to position of DI-3 RNA. G, position of genomic RNA. Lane 2 in A has a band in the same position as in lane 2 in B, visible in the original autoradiograph.

ulum was present (Fig. 4). Sequence analysis showed that the shorter molecules lacked the satRNA sequence, and 46 and 15 nt of DI RNA, upstream and downstream from the *Eco*RV insertion site, respectively, whereas longer molecules lacked only 92 nt of the satRNA sequence (nt 352–443 in satRNA sequence) (Fig. 5). Progeny RNA from both E6 and E2 clones was not encapsidated (Fig. 6), but it was transmissible by sap inoculation and protected plants from necrosis (not shown).

To determine whether sequences in block B, besides keeping the DI RNA in the range of sizes that are replicated (ca. 400–700 nt; Burgyan *et al.*, 1991), were also essential for infectivity, clones HE2 and HE3 were produced in which DI RNA block B was replaced by satRNA sequence. No evidence of replication of RNA from these clones was obtained in spite of repeated attempts (Fig. 4).

Hybrid clone pCS-DA was 580 nt long and carried a 200-nt fragment from the 5′ terminal region of satRNA replacing 100 nt of the 5′ region of DI RNA. Clone DI-SA was similarly constructed, the 100 5′ terminal nucleotides of DI RNA substituting for 200 nt of satRNA (Fig. 3). *In vitro* transcripts from both these clones did not replicate (Fig. 4).

DISCUSSION

In this study, biologically active *in vitro* transcripts of a DI RNA molecule were prepared. These transcripts accumulated in infected cells when coinoculated with infectious full-length CyRSV genomic RNA. The presence of two engineered restriction sites in the DI RNA clone allowed the unequivocal identification of DI RNA in infected cells. The notion that this RNA derived from multiplication of the inoculum rather than *de novo* generation is supported by: (i) timing of appearance, i.e., inoculation with full-length genomic RNA transcripts never induces formation of DI RNA in the first passage (Burgyan *et al.*, 1991); (ii) stability of the 477-nt DI RNA used as inoculum which kept the same size regardless of the time period after inoculation. When they first appear, DI RNAs are molecules of 700 or more nt, which are shortened by progressive deletions to the stable size of ca. 400 nt as infection proceeds (Burgyan *et al.*, 1991).

DI-371 and DI-3 exhibited properties typical of DI RNAs, i.e., they multiplied at the expense of genomic RNA, interfering with symptom expression, and were not encapsidated. Plants infected with both DI RNAs did not cause necrosis and produced almost symptomless new leaves, whereas control plants inoculated only with full-length genomic RNA, showed top necrosis followed by death within 2 weeks. Moreover, similar to other CyRSV DI RNAs (Burgyan *et al.*, 1991), the progeny of DI-371 and DI-3 were not encapsidated at levels detectable by Northern blot analysis. This is significantly different from the DI RNAs of tomato bushy stunt tombusvirus (TBSV) and turnip crinkle carmovirus (TCV), both of which can be recovered from virions (Knorr *et al.*, 1991; Li and Simon, 1991).

A point of interest raised by the present investigation is that, apparently, not all dispensable sequences are eliminated in the process of formation of naturally stable forms of DI RNAs. In fact, a ca. 430-nt-long mutant of DI-3 RNA, in which the sequence between *Bsm*I and *Eco*RV in block B was deleted, was able to replicate and spread in infected plants. This sequence is evidently not essential for replication, as confirmed by its absence in some natural DI RNAs (Burgyan *et al.*, 1991), and appears to be downstream of the replicating signals contained in the sequence contained between restriction sites *Hpa*I and *Bsm*I (Fig. 3). Replacement of this deleted sequence with a satRNA sequence that brought the size of the molecule to a

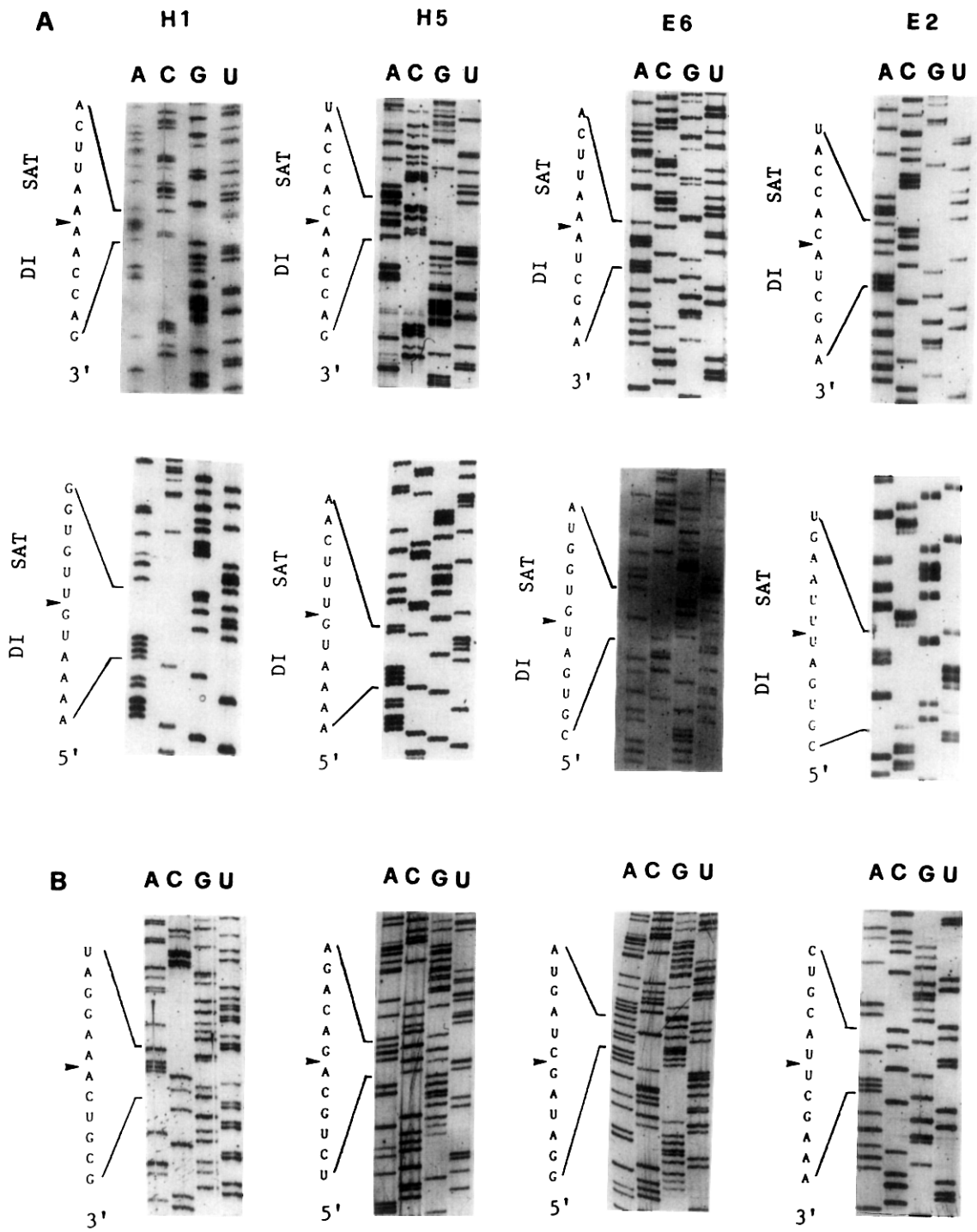

FIG. 5. (A) Sequences around the junction points (arrow heads) between DI and satRNA sequences in progeny RNA of clones H1, H5, E6, E2. (B) Sequences of E2 and E6 clones progeny around the junction points (arrowheads) generated by the deletions (from left to right) in satRNA sequence only (E6), of satRNA sequence plus 61 (E6), 28 (E2), and 46 nucleotides (E2) of DI RNA sequences. 5′ and 3′ indicate the orientation that should be followed in reading the sequence to match with the diagrams of Fig. 3.

length comparable to that of natural DI RNAs did not restore infectivity, indicating that with CyRSV, loss of infectivity is not simply a size effect as with TCV (Li and Simon, 1991).

Insertion of the same satRNA sequence in the *Hpa*I or *Eco*RV sites in full-length DI-3 produced clones from which infectious RNA was synthesized *in vitro.* However, there was a remarkable difference in the type of

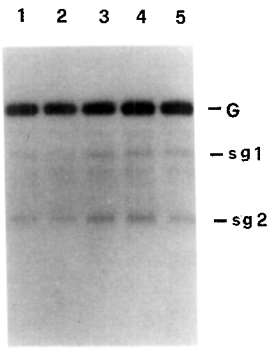

FIG. 6. Northern blot of RNA extracted from virus particles purified from plants inoculated with G11 transcripts only (lane 1), G11+H1 (lane 2), G11+H5 (lane 3), G11+E2 (lane 4), and G11+E6 (lane 5). Hybridization with nick-translated clone DI-3. G, sg1, and sg2 show the positions of genomic and subgenomic RNAs, respectively.

progeny RNA. Whereas progeny of both H1 and H5 transcripts were the same as the inoculum, i.e., with no deletions either in DI RNA or satRNA sequences, progeny of E2 transcripts consisted of full-length molecules, identical to the inoculum, and molecules that had lost the entire satRNA sequence and adjoining DI RNA sequence. Progeny RNA of E6 clone showed either a short deletion in the satRNA sequence only or the entire satRNA sequence together with part of the adjoining region of DI RNA.

These results confirm the importance of the *Hpa*I–*Bsm*I sequence of block B (and perhaps of the 3′ terminal part of block A, too) for the viability of DI RNA. In fact, any deletion in the satRNA sequence of progeny RNA of clones H1 and H5 is likely to involve part of the *Hpa*I–*Bsm*I region, because deletion of satRNA sequence is not precisely defined and may go beyond the artificial junction between the two foreign sequences. Deleted molecules would not be viable, thus no selective pressure against the replication of full-length molecules would be exerted. Conversely, progeny RNA of clones E2 and E6 are not affected by deletions, involving satRNA sequence and part of the junction regions. In this case deleted molecules would be viable and be replicated preferentially with respect to full-length molecules.

Published data indicate that no putative initiation signals (i.e., repeated consensus sequences) could be identified for certain in noncontiguous portions of CyRSV or TBSV genomes (Burgyan *et al.,* 1991; Knorr *et al.,* 1991), which would support the idea that a "copy choice" mechanism could underly the discontinuity of RNA polymerase activity leading to DI RNA generation. As shown in the present paper, recombination points may occur in quite different sequence contexts, including the satRNA sequence (progeny of E6), where no natural deletion mutants are known to occur. Full-length hybrid DI RNA–satRNA molecules possess long regions with highly stable computer-generated secondary structure (not shown), which may constrain the action of RNA polymerase *in vivo* and force it to leave the template, but there is no evidence of possible consensus sequences where the enzyme may resume synthesis. On the other hand, if resumption of synthesis occurs at random rather than at specific initiation signals, it may be expected that polymerase carrying the nascent RNA strand would attach to any viral or cell RNA molecule, so that recombinant production would be very frequent. It is possible that mechanisms alternative to the model presented by Cascone *et al.* (1990) for the generation of TCV DI RNA may operate leading to the formation of deletion mutants of CyRSV genomic RNA.

Surprisingly, hybrid clones pCS-DA and DI-SA did not produce infectious transcripts, although each contained the authentic termini of both DI RNA and satRNA in exchanged portions (Fig. 3). If RNA polymerase requires correct template termini to recognize and replicate a molecule, then it was reasonable to expect that transcripts from both the above clones should have replicated in the presence of the helper genome, which is assumed to encode an enzyme suitable for replication of viral genome, DI RNA, and satellite RNA. Could the mechanism for the replication of DI RNA and satRNA require both termini from the same molecule? It is interesting to note that a natural CyRSV hybrid genomic satRNA molecule has never been observed. This may be explained either assuming that the replication machineries for genomic/DI RNA and satRNA are different, although based on the same virus-encoded viral RNA polymerase, and/or that there is no release at any time of the replicase from the original template during the *de novo* generation of DI RNA.

In this and previous studies (Burgyan *et al.,* 1991), no evidence of efficient encapsidation of CyRSV DI RNA was obtained, contrary to what is known for CyRSV satRNA (Gallitelli and Hull, 1985). Hybrid DI RNA–satRNA clones were not encapsidated at a level detectable by Northern blots using a DI (Fig. 6) or satRNA (not shown) probe, even when full-length progeny was present (clones H1 and H5). If a specific signal is required for encapsidation, it may not reside in the sequence between *Pfl*MI and *Bfr*I sites spanning the central part of satRNA. As previously reported, the ca. 50-nt sequence that satRNA shares with genomic RNA do not seem to be involved in packaging, as the same sequence is conserved in DI RNA (Rubino *et al.,* 1990). This may be taken as an indication that determinants for encapsidation may be of higher order than primary structure as suggested by Wei *et al.* (1990) for TCV

coat protein/RNA interactions. Size also does not appear to be a prerequisite for encapsidation, since DI RNAs very close in size to satRNA are formed which are not encapsidated (Burgyan *et al.,* 1991), and subgenomic RNAs of 2.1 and 0.9 kb are efficiently encapsidated (Russo *et al.,* 1988). Encapsidation is considered an important factor in the selection of DI RNA species, packaged RNA having an advantage for survival (Makino *et al.,* 1990; Knorr *et al.,* 1991; Li and Simon, 1991). This may not be the case of CyRSV DI RNA, where only an exceedingly small fraction of it is encapsidated, which can be detected only because it is transmissible with inoculated virus particles and multiplies. Most CyRSV DI RNA exists as free RNA in infected cells, so that its resistance to cell nucleases may be primarily due to the presence of secondary structures. Hence, selection would act toward the formation of compact molecules.

ACKNOWLEDGMENTS

We thank Prof. G. P. Martelli for critically reading the manuscript and Hajnalka Csákány and Antonella Antonacci for the skilful technical help given during the course of this work. This research was supported by National Research Council of Italy, Special Project RAISA, Subproject N. 2. Paper N. 507.

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