JOURNAL OF VIROLOGY, Aug. 1995, p. 4746–4751 Vol. 69, No. 8
0022-538X/95/$04.00+0

Characterization of Defective RNAs Derived from RNA 3 of the Fny Strain of Cucumber Mosaic Cucumovirus

MICHAEL V. GRAVES AND MARILYN J. ROOSSINCK*

Plant Biology Division, Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma 73402-2180

Received 21 February 1995/Accepted 19 April 1995

Two defective RNAs (designated D RNA 3α and D RNA 3β) were found to be associated with the Fny strain of cucumber mosaic cucumovirus but not with the Sny strain after serial passages in a tobacco host. The D RNAs were derived from RNA 3 by single, in-frame deletions within the 3a open reading frame. A full-length cDNA clone from which biologically active transcripts can be produced in vitro has been constructed for D RNA 3β. This transcript can be replicated in tobacco plants infected with subgroup I and II cucumber mosaic cucumovirus strains and with peanut stunt cucumovirus. Translation of D RNA 3β in vitro produced a 20-kDa peptide, which was consistent with the predicted coding capacity of the deleted 3a open reading frame. D RNA 3β was also associated with polyribosomes isolated from infected tobacco plants. The presence of the D RNAs had no apparent effect upon helper virus yield or symptom production.

Defective interfering (DI) RNAs have been well characterized for most animal RNA virus groups (9) and several plant RNA virus groups, including tombusviruses (4, 8), carmoviruses (13), furoviruses (3, 5), potexviruses (31), and bromoviruses (23). DI RNAs are derived from the helper virus genome, by simple internal deletions or by complex rearrangements of the genomic sequences (9). The DI RNA retains the *cis*-acting components necessary for efficient replication but depends upon the helper virus for replication and encapsidation functions (9). The presence of a DI RNA can have profound effects on the symptoms produced by the helper virus, ranging from symptom amelioration to enhancement to no effect at all (4, 5, 8, 9, 13, 23).

Cucumber mosaic cucumovirus (CMV) is a tripartite, message sense RNA virus in the family *Bromoviridae*. RNAs 1 and 2 each encode at least one protein (1a and 2a, respectively); together, these proteins form the viral components of the replicase complex (7, 17). RNA 3 encodes two proteins: the 3a protein, which is expressed directly from RNA 3, and the coat protein, which is expressed via a subgenomic mRNA (RNA 4) (21). The 3a protein has been proposed to be the CMV movement protein (21, 27, 29); however, other viral proteins appear necessary for virus movement as well (6, 27, 28). CMV has been divided into two subgroups on the basis of nucleic acid hybridization and serology of the capsid protein (21). The association of satellite RNAs with various CMV isolates and their ability to affect the symptoms produced by the helper virus have been well documented (for a review, see reference 25). However, no naturally occurring DI RNA associated with any wild-type CMV strain has been described. DI RNAs have been found to be associated with two strains of broad bean mottle bromovirus (BBMV), another member of the family *Bromoviridae* (23). The BBMV DI RNAs were derived from RNA 2 by small in-frame deletions in the 2a open reading frame (ORF). The presence of these DI RNAs in the viral infection in some hosts reduced the accumulation of BBMV RNA 2. In pea plants, the DI RNAs increased the symptoms produced by the helper virus, and the DI RNAs were not encapsidated in either pea or bean plants. Artificial DI RNAs have also been produced from brome mosaic bromovirus (16). Like the BBMV DI RNAs, brome mosaic bromovirus DI RNAs were also derived from RNA 2.

In this paper, we report the isolation of defective RNAs (D RNAs) produced upon serial passages of the wild-type Fny strain of CMV (Fny-CMV) in a tobacco host. The D RNAs were RNA 3 derivatives. The smaller of the two RNAs was replicated and maintained by both subgroup I and II CMV strains and by peanut stunt cucumovirus (PSV) and was associated with polyribosomes in vivo. However, the presence of the D RNAs had no apparent effect on helper virus accumulation or symptoms.

MATERIALS AND METHODS

Virus strains and plant inoculations. The Fny- and Sny-CMV (subgroup I) strains have previously been described (24). LS-CMV (subgroup II) (18) was a gift from P. Palukaitis, Cornell University. 1997-PSV was a gift from S. Tolin, Virginia Polytechnic Institute. All CMV strains were passaged through tobacco (*Nicotiana tabacum* cv. Xanthi nc). In some experiments, *Nicotiana benthamiana* plants were used. Plant inoculations were performed with viral RNA, isolated from purified virus, at 100 μg/ml in 50 mM sodium phosphate buffer (pH 9.2). Leaves, two to three per plant, were mechanically inoculated with 10 μl of RNA when the plants were approximately 1 month old. In some experiments, transcript produced in vitro from D RNA cDNA clones was added at a molar ratio equal to that of the viral RNA inoculum.

Virus purification and RNA extraction. CMV virions were purified 12 to 14 days postinoculation from systemically infected leaf material, and viral RNA was isolated from the purified virus as previously described (14, 20). RNAs were separated under nondenaturing conditions on 1.5% agarose gels in 0.5× Tris-borate-EDTA buffer. Alternatively, samples were denatured by incubation at 65°C in formaldehyde–formamide–10× MOPS (morpholinepropanesulfonic acid) buffer (1.6:5:1, vol/vol/vol) followed by electrophoresis on 1.5% agarose gels in 1× MOPS buffer (26).

Northern (RNA) hybridization analysis. Agarose gels containing nondenatured RNA samples were treated with 50 mM NaOH to denature the RNAs, neutralized in three changes of 50 mM sodium borate (pH 8.0), and then transferred to nylon filters (Hybond N+; Amersham) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). RNAs denatured prior to electrophoresis were transferred directly to the filters. After transfer, the filters were baked at 80°C under vacuum for 2 h. RNA 3-specific negative-sense RNA probes labeled with [^{32}P]UTP were synthesized in vitro with T3 RNA polymerase from a plasmid clone of Sny-CMV RNA 3 which consisted of the capsid protein ORF and the entire 3′ untranslated region (24). The filters were pretreated with hybridization buffer (1% bovine serum albumin, 1 mM EDTA, 0.5 M sodium phosphate buffer [pH 7.2], 7% sodium dodecyl sulfate [SDS]) (30) at 65°C for 30 min and then probed with 3×10^6 cpm of labeled transcript in the same buffer at 65°C. The filters were subsequently washed in 0.1× SSC–2% SDS at 65°C.

Identification and cloning of D RNAs. First-strand cDNA was synthesized with

* Corresponding author. Mailing address: Plant Biology Division, Samuel Roberts Noble Foundation, Inc., P.O. Box 2180, Ardmore, OK 73402-2180. Phone: (405) 223-5810. Fax: (405) 221-7380.

purified viral RNAs and Superscript reverse transcriptase (Bethesda Research Laboratories, Inc.) and primer CN3nf (GGCTGCAGTGGTCTCCTT), which is complementary to the last 10 nucleotides (nt) of the 3′ ends of all four CMV RNAs. This primer contained an overhang to create a *Pst*I site for cloning purposes. Three specific primers were constructed and used to amplify double-stranded products from the first-strand cDNA via thermocycling reactions with *Pfu* thermostable DNA polymerase (Stratagene). Primer 332R (CTGACCATTT TAGCCGTWAGCTGGATGGAC) is complementary to all CMV RNAs near the 3′ end. Primer 1&2F (CAMGAGCGTAYGGTTCAAYCCCTGCCTCC) corresponds to a conserved region of CMV RNAs 1 and 2 near the 5′ end. Primer 351F (CGGGATCCATTTAGGTGACACTATAGTAATCTTACCAC TGT GTGTGT) contains the first 22 nt of the 5′ end of CMV RNA 3, an overhang to create a *Bam*HI site, and a modified SP6 RNA polymerase promoter. Primer D3F (GGATGCGCGCTGATAATGCT) corresponds to nt 256 to 275 of RNA 3, and primer D3R (GCCAATTACTACACACGCTA) is complementary to nt 644 to 663 of RNA 3. Thermocycling reactions (with 10-μl reaction mixtures) were carried out in capillary tubes in an Idaho Technologies model 1605 air thermocycler by utilizing the following programs: primers 1&2F and 332R, 35 cycles of 94°C for 0 s, 60°C for 0 s, and 78°C for 65 s; primers 351F and CN3nf, 35 cycles of 94°C for 0 s, 45°C for 0 s, and 78°C for 65 s; and primers D3F and D3R, 35 cycles of 94°C for 0 s, 54°C for 0 s, and 78°C for 15 s. All reactions began with incubation at 94°C for 30 s and ended with incubation at 78°C for 5 min. Amplification products were analyzed on 1% agarose–Tris-borate-EDTA gels (26).

For D RNA cloning, the full-length, double-stranded cDNAs produced with the RNA 3-specific primers were extracted once with phenol-chloroform and precipitated with 0.3 M sodium acetate (pH 5.2) and ethanol. The cDNAs were then digested with *Bam*HI and *Pst*I and ligated into a *Bam*HI- and *Pst*I-digested pBluescript II SK− vector (Stratagene). For sequencing, a series of nested, insertional mutants were produced from two D RNA 3β cDNA clones with a Tn*1000* transposon system (Gold Biotechnologies). Individual clones were then sequenced by using dye-terminator chemistry and an Applied Biosystems, Inc., model 373A automated DNA sequencer according to the manufacturer's recommendations.

In vitro transcription and translation and polyribosome isolation. Full-length RNA transcripts were produced in vitro from cDNA clones that had been linearized with *Pst*I and had ends made blunt with T4 DNA polymerase (Promega); this was followed by transcription with SP6 RNA polymerase (Ambion) as previously described (2, 22). For infectious transcripts, the concentration of GTP was reduced to 0.6 mM, m^7GTP cap analog (New England Biolabs) was added to a final concentration of 3 mM, and the reaction mixtures were incubated at 37°C for 1 h. GTP was then added to a final concentration of 1.25 mM, and the reaction mixtures were incubated at 37°C for an additional 30 min. DNA was removed from the reaction mixtures with RQ1 RNase-free DNase (Promega), and the reaction mixtures were extracted with phenol-chloroform and precipitated with ethanol. The transcripts produced in this way would have exact 5′ ends and an additional C residue at the 3′ end. RNA concentrations were estimated by agarose gel electrophoresis followed by ethidium bromide staining.

Transcripts produced from full-length RNA 3 and D RNA 3β cDNA clones and total viral RNAs were translated in vitro in a nuclease-treated, rabbit reticulocyte lysate system by following the manufacturer's (Promega) recommendations, with [^{35}S]methionine as the labeled amino acid. The level of incorporation of radioactive amino acids was determined by trichloroacetic acid precipitation. Translation products were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer (26), placed in a boiling water bath for 5 min, and then subjected to electrophoresis on a discontinuous 10% polyacrylamide gel (12). Labeled translation products were visualized by incubation of the gels in a solution of 1% salicylic acid in 20% methanol, drying under vacuum, and exposure to X-ray film (26).

Both free polyribosomes and membrane-bound polyribosomes were isolated as described by Jackson and Larkins (11). Total RNA was isolated from the polyribosomal fractions by phenol-chloroform extraction followed by ethanol precipitation.

Nucleotide sequence accession number. Sequences reported here have been deposited in GenBank and assigned the accession number U20668.

RESULTS

Appearance of D RNAs with Fny-CMV. Wild-type Fny-CMV and Sny-CMV were passaged in tobacco, and the virus was purified from systemically infected leaf tissue. After the fourth passage, one additional encapsidated RNA which was not present in the Sny-CMV viral RNA was apparent in the Fny-CMV viral RNA (Fig. 1). This additional RNA migrated faster than RNA 3 on an agarose gel under nondenaturing conditions and appeared to be approximately equimolar with the genomic RNAs. Upon closer inspection of the gel, a second additional RNA, which migrated between RNA 3 and the first additional band, was visible in the Fny-CMV viral RNA. This RNA was much less abundant than the other viral RNAs. Similar electrophoretic patterns were observed when the samples were denatured (data not shown). These two additional RNAs have been designated D RNA 3α (larger, less abundant species) and D RNA 3β (smaller, predominant species). Both of these RNAs were maintained in an additional passage in the tobacco host (data not shown).

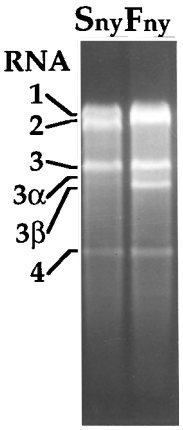

FIG. 1. Agarose gel electrophoresis of nondenatured RNAs isolated from purified virus after passages through tobacco. RNAs were stained with ethidium bromide. Sny, fourth passage of Sny-CMV; Fny, fourth passage of Fny-CMV. Additional RNAs present in the Fny viral RNA are indicated as RNA 3α and RNA 3β. The positions of the genomic RNAs are also shown.

Full-length minus-strand cDNA was synthesized from the fourth-passage Fny-CMV viral RNA containing the D RNAs as well as from the Sny-CMV RNA, which did not contain the D RNAs. Primers specific either for genomic RNAs 1 and 2 or for RNA 3 were used in a thermocycling reaction to synthesize and amplify double-stranded products from the cDNA. The products of each reaction were analyzed on agarose gels for the presence of the expected full-length molecule as well as for the two additional molecules. This method allows for the identification of only those RNA species which contain both ends of the viral RNA. Reactions with the RNA 1- and 2-specific primers (1&2F and 332R) and the RNA 3-specific primers (351F and CN3nf) produced the expected full-length products and no additional products when the Sny-CMV first-strand cDNA was used as the template (Fig. 2A). Reactions with the RNA 1- and 2-specific primers and the Fny-CMV cDNA template also did not produce any bands in addition to the full-length products. However, with the Fny-CMV cDNA template the RNA 3-specific primers produced two additional bands which migrated faster than the full-length RNA 3 product. Similar to the viral RNA concentrations, the 3β product was approximately equimolar with the full-length RNA 3 product, while the 3α product was much less abundant. These results indicated that the D RNAs were derived from Fny-CMV RNA 3.

Cloning and sequencing D RNA 3β. Eleven full-length cDNA clones for D RNA 3β were produced. To minimize misincorporation during the thermocycling reactions, *Pfu* DNA polymerase was used in the generation of the cDNAs (15). The complete nucleotide sequences of two of these clones were determined (Fig. 3). The sequences of both clones were identical and indicated that D RNA 3β was derived from a single 309-nt in-frame deletion from the 3a gene, producing an ORF that was missing amino acids 65 to 167 and that would encode a protein approximately 30% smaller than the wild-type 3a protein. Other than the deletion, there were a total of

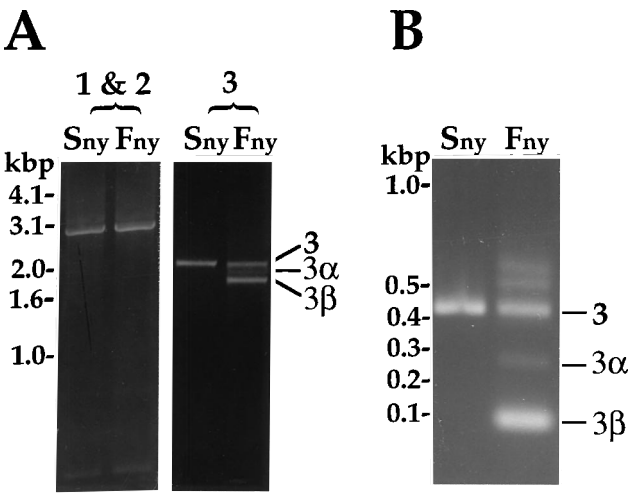

FIG. 2. Agarose gel electrophoresis of double-stranded DNA amplification products. DNAs were stained with ethidium bromide. (A) Identification of D RNAs as RNA 3 derivatives. Primers specific for RNAs 1 and 2 or RNA 3 were used to generate double-stranded DNA products via a thermocycling reaction as described in the text. First-strand cDNA was produced from either the fourth-passage Sny-CMV RNA (Sny) or the fourth-passage Fny-CMV RNA (Fny). Additional bands produced from the Fny cDNA by using the RNA 3-specific primers are indicated as 3α and 3β. (B) Primers D3F and D3R were used in a thermocycling reaction to amplify the deletion junction regions of RNAs 3, 3β, and 3α. The expected sizes of the full-length RNA 3 and D RNA 3β products are 407 and 98 bp, respectively (labeled 3 and 3β). Note the intermediate band at circa 250 bp (labeled 3α).

six nucleotide differences (Table 1) between D RNA 3β and the previously published sequence of Fny-CMV RNA 3 (19), giving an overall nucleotide identity between the two of 99.7%. All of the differences resided within either the 5′ or 3′ untranslated regions. Since no clones were obtained for D RNA 3α, a thermocycling technique was designed to identify the D RNA 3α deletion by using primers D3F and D3R. These primers will amplify the deletion junction region of RNA 3 and D RNA 3β.

TABLE 1. Summary of the nucleotide differences between RNA 3 and D RNA 3β

RNA	Base at nt position[a]:					
	88	93	1921	1956	1957	2001–2006[b]
Fny RNA 3	T	G	G	C	G	T_6
D RNA 3β	C	T	A	G	C	T_5

[a] Numbers are published for Fny-CMV RNA 3 (19).
[b] Number of T residues within this range.

If the D RNA 3α deletion is in the same region, then an additional, intermediate-sized product should be produced. Several double-stranded DNAs were produced (Fig. 2B): one of circa 400 bp, one of circa 100 bp, and an intermediate-sized product of circa 250 bp (labeled 3, 3β, and 3α, respectively). The sizes of the 400- and 100-bp products agreed with the known sizes of this region from wild-type RNA 3 (401 nt) and D RNA 3β (98 nt) as determined from the cDNA sequences. The origin of the products larger than the wild-type RNA 3 product is unknown; however, their production in this reaction correlated with the presence of D RNAs in the original template RNA. The RNA 3, 3α, and 3β DNA products were gel purified and directly sequenced with the same primers used in the amplification. The sequence data indicated that D RNA 3α was derived by a deletion of 156 nt from the 3a ORF (Fig. 3). This deletion was contained entirely within the D RNA 3β deletion and was also in frame. However, in this case a new codon was created at the deletion junction site such that the ORF encoded amino acids 1 to 70 of the 3a ORF, a tyrosine residue, and amino acids 124 to 279 of the 3a ORF.

Translation of D RNA 3β. In vitro translation in a rabbit reticulocyte lysate system of transcript synthesized from a full-length RNA 3 cDNA clone resulted in the production of the 3a protein (30 kDa) as the major product (Fig. 4). A small amount of coat protein was also produced from this RNA. Translation of the transcript produced from the D RNA 3β cDNA clone resulted in the production of a 20-kDa protein as the major

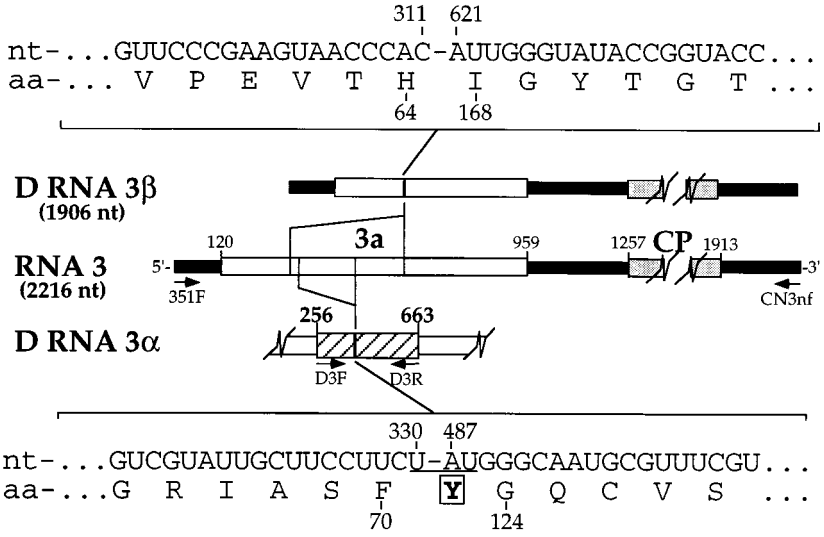

FIG. 3. Schematic representation of D RNA 3β and D RNA 3α, derived from nucleotide sequence analysis, and Fny-CMV RNA 3. The entire nucleotide sequence of D RNA 3β was determined. The region of D RNA 3α actually sequenced is indicated by the hatched rectangle. The nucleotide positions of the 3a and coat protein ORFs are shown above the RNA 3 diagram. Arrows indicate the positions of primers used in the thermocycling reactions. The sequences of 18 bases on either side of the deletion junctions and corresponding amino acid (aa) translations are shown for both D RNA 3β (top) and 3α (bottom). The hybrid codon at the deletion junction of RNA 3α, which encodes a Tyr residue (boxed Y), is underlined. Nucleotide and amino acid numbering is as published for Fny-CMV RNA 3 (19).

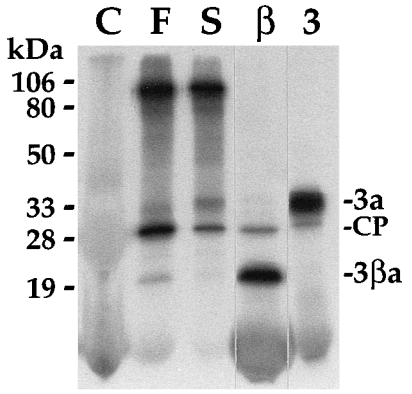

FIG. 4. Polyacrylamide gel electrophoresis, followed by autoradiography of in vitro translation products of D RNA 3β. Translation templates are as follows: lane C, water control; lane F, purified Fny-CMV genomic RNA containing D RNAs α and β; lane S, purified Sny-CMV genomic RNA; lane β, transcript produced in vitro from a D RNA 3β cDNA clone; lane 3, transcript produced in vitro from an Fny RNA 3 cDNA clone. Each lane contains approximately 50,000 cpm of trichloroacetic acid-precipitable [^{35}S]methionine. The positions of the 3a protein, coat protein (CP), and 3βa protein are shown to the right. Molecular mass standards are shown to the left.

product. The size of this product agreed with the coding capacity predicted for the truncated 3a gene. A small amount of coat protein was also produced from the D RNA 3β transcript. The 20-kDa product was also synthesized when total viral RNAs isolated from purified Fny-CMV that contained D RNA 3β were translated in the same system.

In order to determine the translation activity of D RNA 3β in vivo, both free and membrane-bound polyribosomes were isolated from healthy tobacco plants and plants inoculated with either Fny-CMV alone or Fny-CMV and the D RNA 3β transcript. Total RNA purified from the polyribosomal fractions, blotted, and probed with an RNA 3-specific probe shows that D RNA 3β is present in both the free polyribosome fraction and membrane-bound polyribosome fraction, although it appeared as a more diffuse band in the free polyribosome fraction (Fig. 5).

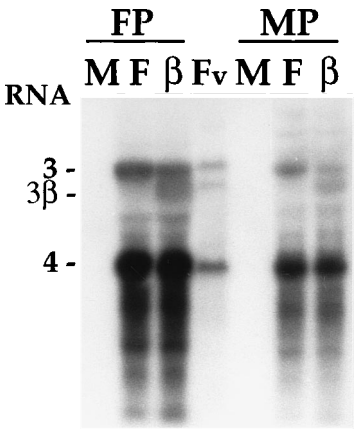

FIG. 5. Northern blot analysis of D RNA 3β for association with polyribosomes in vivo. RNAs isolated from tobacco polyribosomes were probed with an RNA 3-specific probe. FP, RNA isolated from free, cytoplasmic polyribosomes. MP, RNA isolated from membrane-bound polyribosomes. Samples are as follows: lanes M, mock-inoculated plants; lanes F, plants inoculated with Fny-CMV RNA; lanes β, plants inoculated with Fny-CMV RNA containing β RNA; lane Fv, 50 ng of purified Fny-CMV RNA containing β RNA. The positions of RNAs 3, 3β, and 4 are indicated to the left.

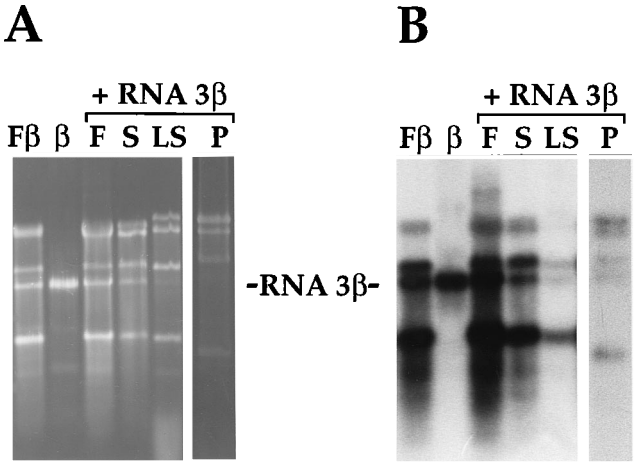

FIG. 6. Agarose gel electrophoresis of D RNA 3β passaged with different helper viruses in tobacco. (A) Ethidium bromide-stained gel of viral RNAs isolated from purified virus. (B) Northern blot of gel in panel A probed as for Fig. 4. The position of RNA 3β is indicated. The right four lanes contain RNAs isolated from virus purified from plants inoculated with the indicated helper virus and D RNA 3β transcript. Lanes Fβ, Fny-CMV containing α and β RNAs; lanes β, transcript produced in vitro from a D RNA 3β cDNA clone; lanes F, Fny-CMV; lanes S, Sny-CMV; lanes LS, LS-CMV; lanes P, 1997-PSV.

Passage of D RNA 3β transcripts with different helper viruses. Transcripts produced from the D RNA 3β cDNA clone were passaged in tobacco plants in combination with different helper viruses. The transcript was replicated and encapsidated through a systemic infection by both Fny-CMV and Sny-CMV (both subgroup I strains) and by Fny-CMV in *N. benthamiana* (Fig. 6 and data not shown). The transcript was also inoculated with LS-CMV, a subgroup II strain, and although D RNA 3β does not appear to be present in the ethidium bromide-stained gel, it was detected by Northern analysis (Fig. 6B). This indicated that LS-CMV was able to replicate and encapsidate D RNA 3β in tobacco plants, albeit at reduced levels compared with the levels produced by the subgroup I strains. The probe also hybridized weakly to LS-CMV RNA 3 and strongly to subgenomic RNA 4. Since the probe used in this experiment was derived from a subgroup I CMV RNA 3, which cross-hybridizes only weakly with subgroup II RNAs (18), the strong hybridization to RNA 4 in the LS-CMV lane of Fig. 6B indicates that the subgenomic promoter of D RNA 3β is recognized by LS-CMV, producing subgenomic RNA 4 derived from D RNA. Transcripts were also passaged with 1997-PSV which was also able to replicate and encapsidate the D RNA in tobacco, again at reduced levels compared with the levels produced by the subgroup I CMV strains. No additional RNAs were detected after experiments involving passage of these virus strains without the D RNA 3β transcript (data not shown).

There was no apparent difference in the yield of virus from the plants inoculated with D RNA 3β compared with that from the control plants (data not shown). The relative proportions of the encapsidated genomic RNAs were unchanged as well (Fig. 1 and 5), suggesting that the D RNAs had no effect on helper virus replication and accumulation. In addition, no obvious symptom differences between the control plants and those inoculated with the D RNAs could be detected.

DISCUSSION

This is the first report of a D RNA associated with a member of the *Cucumovirus* genus. Although the D RNAs were not

apparent in the original Fny-CMV stock or after the first, second, or third passage through the tobacco host, they were present after the fourth passage. These RNAs are maintained by the virus, since they were still present after an additional passage. However, these D RNAs do not appear to outcompete the genomic RNAs, since the amount of D RNA remains either much less than or approximately equal to that of the genomic RNAs. The D RNAs were derived from RNA 3 of the Fny strain, and their formation appears to be unique to this strain, since no D RNAs have been produced by the Sny strain during similar passage experiments, nor have they been reported for any other CMV strain. D RNA 3α could be an intermediate in the production of D RNA 3β since it is maintained at a very low level, while D RNA 3β reaches levels equal to those of the genomic RNAs, and the α deletion is smaller and resides totally within the region deleted from D RNA 3β.

Although many D RNAs can affect helper virus replication and symptom production (4, 5, 8, 9, 13, 23), the CMV D RNAs described here are apparently benign and are packaged in the virion at the same level as or at a much-reduced level compared with that of the helper virus RNAs, without reducing the amount of helper virus packaged or affecting the symptoms. This raises interesting questions as to the mechanisms by which a D RNA, which does not outcompete the helper virus, is maintained in a plant during a viral infection. Also of interest is the fact that the deletions from both of the D RNAs maintained an ORF. Similar results were found for the D RNAs of clover yellow mosaic potexvirus (31) and the DI RNAs of BBMV (23). Furthermore, mutations which disrupted the ORF of the clover yellow mosaic potexvirus D RNA produced an RNA which was not maintained by the helper virus in an infected plant (32).

Translation in vitro of D RNA 3β resulted in the production of the expected 20-kDa truncated 3a protein, indicating that the D RNA can serve as a viable mRNA. This finding was supported by the presence of D RNA 3β on free polyribosomes, suggesting that this RNA was translationally competent in vivo as well. D RNA 3β and RNAs 3 and 4 were also present in the membrane-bound polyribosome fraction; however, since we have found viral replicase activity associated with this same fraction (6a), these RNAs could be involved in replication rather than translation. D RNA 3β appeared as a diffuse band in the free polyribosome preparations compared with a distinct band in the membrane-bound polyribosome fraction and other total RNA preparations. This appearance of D RNA 3β could be an indication of the presence of a population of D RNAs within the infected tissue.

Although formation of the D RNAs has been observed only for the Fny-CMV strain, other CMVs appear capable of supporting a D RNA once it is formed. Of special note was the other subgroup I strain, Sny-CMV. This strain was passaged in the same tobacco host simultaneously with the Fny-CMV strain and did not produce any D RNAs. Further passage of Sny-CMV has also failed to produce D RNAs (data not shown). Yet this strain was capable of supporting D RNA 3β. The Sny-CMV strain will therefore be useful in studying the genetics of D RNA formation.

The mechanism of formation of the CMV D RNAs is still unknown. The polymerase "copy choice" originally proposed by Huang is the generally accepted mechanism for D RNA formation (9, 10). This mechanism, which includes template switching induced by local complementarities and the secondary structure of the replicating RNA, was also proposed for the formation of the DI RNAs from BBMV (23), the only other member of the *Bromoviridae* for which D RNAs have been found. Such a mechanism would suggest that Fny-CMV RNA 3 is primarily responsible for the formation of the D RNAs by the presence of sequences and/or structures not found on other CMV RNA 3 segments which are capable of inducing the switching mechanism. There is extensive secondary structure in and around the deleted region of RNA 3 predicted by computer simulation with the program STAR, version 3.0 (1). However, there is no experimental evidence that these structures actually exist in vivo or that they play a role(s) in D RNA formation. It is also possible that the formation of the D RNAs is due to some characteristic unique to the Fny-CMV replicase and its interaction with host factors. Genetic mapping experiments with reassorted viruses should help to distinguish between these possibilities.

ACKNOWLEDGMENTS

We thank P. S. White for help with primer design for the thermocycling reactions; R. A. Gonzales, V. A. Graves, and A. Harris of the Molecular Analysis and Synthesis Section for synthesis of oligonucleotide primers and for sequencing the D RNA 3β cDNA clones; P. Palukaitis for providing CMV strains; S. Tolin for providing 1997-PSV; and N. Paiva and S. Flasinski for critical reviews of the manuscript.

This work was entirely funded by the Samuel Roberts Noble Foundation, Inc., Ardmore, Okla.

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