JOURNAL OF VIROLOGY, Jan. 1997, p. 608–612 Vol. 71, No. 1
0022-538X/97/$04.00+0

Support of a Cucumber Mosaic Virus Satellite RNA Maps to a Single Amino Acid Proximal to the Helicase Domain of the Helper Virus

MARILYN J. ROOSSINCK,*[1] IGOR KAPLAN,[2] AND PETER PALUKAITIS[2]

Plant Biology Division, The S. R. Noble Foundation, Ardmore, Oklahoma 73402,[1] *and Department of Plant Pathology, Cornell University, Ithaca, New York 14853*[2]

Received 5 July 1996/Accepted 27 September 1996

Cucumber mosaic virus (CMV) is a tripartite RNA virus that can support the replication of satellite RNAs, small molecular parasites of the virus. Satellite RNAs can have a dramatic effect on the helper virus and the host plant in a manner specific to the helper, satellite, and host. Previously, we showed that the Sny-CMV strain is not able to support the replication of the WL1 satellite RNA in zucchini squash and that this phenotype maps to RNA 1. In the present study, we use recombinant cDNA clones of Fny- and Sny-CMV RNA 1 and a site-directed mutant of Fny-CMV RNA 1 to demonstrate that the inability to support WL1 satellite RNA maps to a single amino acid at residue 978 in the 1a protein, proximal to the helicase domain VI. Support of satellite RNA in whole plants and in protoplasts of zucchini squash is analyzed.

Cucumber mosaic cucumovirus (CMV) is a tripartite RNA virus of plus-sense polarity. Numerous strains of CMV that have different host ranges and induce different symptom types on plants have been isolated (26). RNAs 1 and 2 are required for viral replication, along with at least one host component (12, 24). RNA 1 contains a single open reading frame (ORF) encoding a protein of 110 kDa (the 1a protein). The 1a protein contains the putative methyltransferase domains (34) in the amino-terminal region and the conserved domains of all known helicases (15) in the carboxy-terminal portion. RNA 2 also encodes a large ORF of 98 kDa (the 2a protein), which contains the conserved GDD box found in many viral RNA polymerases (1). A second ORF that may play a role in long-distance movement of the virus is also present on RNA 2 (5). RNA 3 encodes both the movement protein and the viral coat protein, which is translated from the subgenomic RNA 4.

In addition to the viral RNAs, CMV strains may have a satellite RNA (sat RNA) associated with them. sat RNAs are completely dependent on their helper virus for replication and encapsidation. The CMV sat RNAs are generally around 335 nucleotides (nt) in length and do not contain any functional ORFs. They can dramatically alter the phenotype of the virus in a strain- and host-specific manner (for a review, see reference 32).

Two closely related strains of CMV, Fny- and Sny-CMV, were previously reported to have differential abilities to act as helper virus for replication of the WL1-sat RNA in zucchini squash (30). The inability to replicate the WL1-sat RNA to detectable levels in zucchini squash was unique to the Sny-CMV and was also host specific; i.e., Sny-CMV could support the replication of WL1-sat RNA in tobacco to normal levels. This difference in sat RNA replication was mapped to RNA 1 through the use of pseudorecombinant (reassorted) viruses. In this study, we show the further genetic mapping of this ability to act as a helper virus to a single amino acid residue immediately downstream of the last conserved helicase domain in the 1a protein.

* Corresponding author. Mailing address: Plant Biology Division, The S. R. Noble Foundation, P.O. Box 2180, Ardmore, OK 73402.

MATERIALS AND METHODS

Plants and virus strains. The plants used were zucchini squash (*Cucurbita pepo*) cv. Black Beauty and tobacco (*Nicotiana tabacum*) cv. Xanthi nc. All plants were grown under greenhouse conditions prior to inoculation. Squash plants and tobacco plants were inoculated at the cotyledon stage and at the three- to four-leaf stage, respectively, with viral RNA or in vitro-generated transcripts from cDNA clones at a concentration of 100 μg/ml, with or without the addition of either 5 or 50 μg of gel-purified WL1-sat RNA per ml (9), in 50 mM sodium phosphate (pH 9.2). Plants were maintained either under greenhouse conditions or in an environmentally controlled chamber with a day temperature of 26°C, a night temperature of 22°C, and a 16-h day length.

Virus strains Fny- and Sny-CMV were previously described (31). Transcripts were generated from the previously described Fny-CMV RNA 2 and Fny-CMV RNA 3 clones pFny209 and pFny309, respectively (29), as well as from full-length and recombinant clones of cDNA of Fny- and Sny-CMV RNA 1 and from pFny1Ser978. All virus purifications were done as described previously (26). Viral RNA was purified as described by Palukaitis and Zaitlin (28). Virion RNAs were analyzed by agarose gel electrophoresis in 1.5% agarose and 1× Tris-borate-EDTA (TBE), followed by staining with ethidium bromide (35). Protoplasts were prepared from zucchini squash plants, inoculated, and analyzed by Northern blotting as described previously (8).

Cloning of Sny-CMV and RNA 1 recombinants. The initial cloning of Sny-CMV was described previously (31). Construction and sequencing of a full-length clone of Sny-CMV RNA 1 will be described elsewhere. Clone pSny83.3 contains a full-length cDNA of RNA 1 downstream of an SP6 promoter, such that transcription begins at the natural 5′ end of the CMV RNA. Clone pFny86.6 was derived from the Fny-CMV RNA 1 clone pFny109 (29) but was transferred to plasmid pBR322, in which the RNA 1 clones were more stable. The 5′ end was modified as for clone pSny83.3, so that the start of transcription coincides with the natural 5′ end of the viral RNA. Recombinant clones are summarized in Fig. 1 and are named to reflect the source of sequences (F for Fny-CMV and S for Sny-CMV) and the nucleotide position of the sites of recombination. Clone pFSF1621/2735 was generated by replacing an *Eco*RV-*Bgl*II fragment (1,114 nt, from nt 1621 to 2735) from pFny109 with the corresponding fragment from a partial cDNA clone of Sny-CMV RNA 1, pSny67. Clone pFS1551 was generated by cloning the 5′ portion of pFny86.6 up to the *Bcl*I site (nt 1551) and the 3′ portion of pSny67 from the *Bcl*I site to the 3′ end into vector pBR322 between the *Eco*RV and *Sph*I sites. Clone pSF1553 was generated from the reciprocal fragments of pSny83.3 and pFny86.6. The plasmids were amplified in *E. coli* GM272, a *dcm* strain, prior to digestion with *Bcl*I. All plasmids were linearized with *Pst*I and the ends were made blunt with T4 DNA polymerase prior to in vitro transcription with either T7 (pFSF1621/2735, pFny1ser978, pFny209, and pFny309) or SP6 (pFny86.6, pSny83.3, pFS1551, and pSF1553) RNA polymerase. Transcription reactions were essentially as described by Beckler (2). All transcripts were initially inoculated onto tobacco plants for amplification.

Sequencing of cDNA clones. The cDNA clone pSny67, containing the 3′ two-thirds of Sny-CMV RNA 1 cDNA, was partially sequenced by the dideoxynucleotide chain termination method with Sequenase (U.S. Biochemicals), and the following primers: the M13 reverse primer and primers complementary to nt 3089 to 3103, nt 2832 to 2846, and nt 1833 to 1847 of Fny-CMV RNA 1.

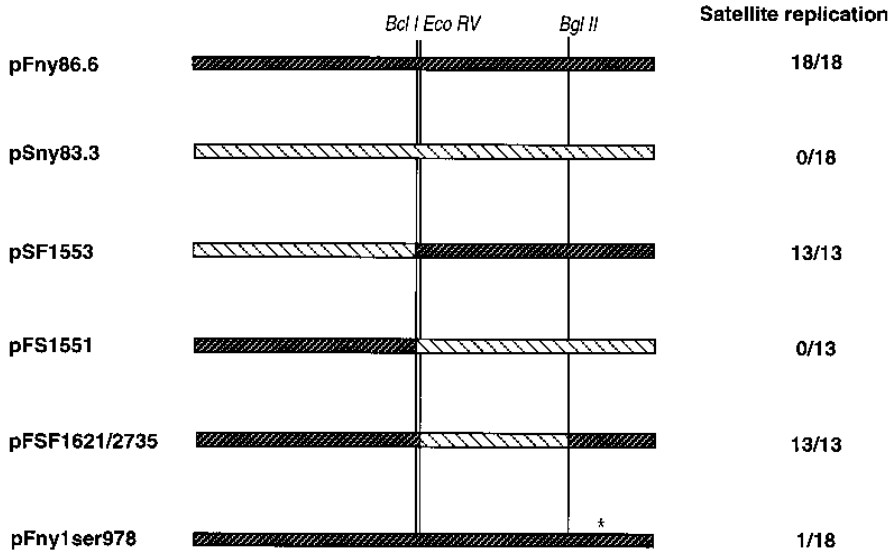

FIG. 1. RNA 1 recombinants. A schematic representation of the cDNA clones pFny86.6 and pSny83.3 is shown at the top, with the relevant restriction enzyme sites as marked. Fny sequences are denoted by ▬, and Sny-CMV sequences are denoted by ▨. Recombinants are shown below. The number of plants testing positive by dot blot analysis for satellite RNA and the total number tested are shown on the right. The results shown are the summary of results of two separate experiments.

Site-directed mutagenesis. A 1-kb fragment (*Hin*dIII [nt 2373] to *Pst*I, 3′ end) from pFny109 was subcloned into the vector pBS M13+ (Stratagene). Two mutagenesis primers, 5′CATTCCGCTACGAGTACAGTGGTGTATTG3′ (Mut II) and 5′ CAATACACCACTGTACTCGTAGCGGAATG 3′ (Mut I), were synthesized; underlined bases reflect the nucleotides that were changed. The mutagenesis reactions were carried out with 2 μl of a standard alkaline lysis miniprep (35) of the subclone (approximately 50 ng), treated with 5 μg of RNase A (Sigma) for 5 min at room temperature. The final reaction mixtures contained 20 mM Tris-HCl (pH 8.75), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg of bovine serum albumin per ml, 200 μM deoxynucleoside triphosphates, 50 ng of template DNA, 50 ng of each of either Mut I and the M13 reverse primer or Mut II and the M13−21 primer, and 2.5 U of *Pfu* DNA polymerase (Stratagene) in a volume of 100 μl. The sample was heated to 96°C for 2 min and then cycled 20 times at 96°C for 1 min, 55°C for 1.5 min, and 75°C for 1 min. A final incubation at 75°C for 10 min completed the cycles. A 30-μl portion of each reaction product was purified by low-melting-temperature agarose gel electrophoresis (1.5% agarose) in TAE buffer (35), the gel slices were melted at 65°C for 10 min, and 1 μl of each was mixed with 50 ng each of the M13 reverse primer and the M13−21 primer. The second amplification reaction was carried out as for the first. The final reaction product was digested with *Bgl*II and *Pst*I and used to replace the analogous fragment of pFny109 to create pFny1ser978. Colonies were subcultured in 15 ml of L broth supplemented with 100 μg of carbenicillin per ml, and plasmids were isolated by standard procedures. Plasmids were sequenced with an ABI automated sequencing system.

Dot blot analysis. Total nucleic acids were extracted from three leaf discs (approximately 50 mg) by a modification of the method of Routh et al. (33). Essentially, tissue samples were flash frozen in liquid nitrogen, pulverized, and extracted in 200 μl of a buffer containing 0.1 M NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA, and 1% sodium dodecyl sulfate (6) with an equal volume of 1:1 phenol-chloroform saturated with Tris-EDTA (TE). The phenol-chloroform extraction was repeated twice, and the aqueous phase was brought to 2 M LiCl and incubated overnight on ice (4). The final pellet was resuspended in 50 μl of 0.1 mM EDTA and stored at −20°C. Then 10 μl of extract was mixed with an equal volume of AMESS buffer (25) and spotted onto Hybond N+ membranes (Amersham). The blots were hybridized as previously described (10) with 3×10^6 cpm of minus-sense transcript generated from plasmid pWL47 (20).

RPAs. RNase protection assays (RPAs) were performed essentially as previously described (27), except that 30 μCi of [α-^{32}P]UTP was used in the transcription reaction and the probes were used within 2 days of synthesis. Plasmid pWL47 (20) was used to synthesize radiolabeled minus-sense and plus-sense transcripts, and 5×10^5 cpm of labeled transcript was used for each assay. Probes were annealed with approximately 1 μg of total nucleic acids extracted as described above.

Computer analysis of the 1a protein secondary structure. The secondary structures of the 1a proteins were predicted with the programs PEPTIDESTRUCTURE and PLOTSTRUCTURE of the Wisconsin Genetics Computer Group package (18).

RESULTS

Construction of recombinant RNA 1 clones. The recombinant virus clones are shown schematically in Fig. 1. The 3′ half of Sny cDNA was substituted for the 3′ half of Fny cDNA in the construct pFS1551. This recombination was at the *Bcl*I site (nt 1551). In the reciprocal recombinant pSF1553, the 5′ half was from Sny cDNA and the 3′ half was from Fny cDNA. The pFSF1621/2735 clone was obtained by substituting a 1.1-kb fragment from the *Eco*RV site (nt 1621) to the *Bgl*II site (nt 2735) of the Sny cDNA clone with the analogous fragment from Fny RNA 1 clone pFny109. Sequence analysis of the Sny cDNA demonstrated no sequence differences from the pFny109 clone in the region between the *Bcl*I and *Eco*RV sites (data not shown).

sat-RNA support by recombinant viruses. Transcripts generated in vitro from recombinant clones were initially inoculated onto tobacco plants, along with RNA 2 and RNA 3 transcripts from pFny209 and pFny309 (29). Progeny viral RNA from these initial infections was used to assess sat RNA replication in squash. Fny86.6-CMV refers to the progeny of the transcripts from pFny86.6, pFny209, and pFny309. Other strains are designated in a similar manner. Systemically infected tissues were analyzed by dot blot hybridization, using a minus-sense transcript from a WL1 cDNA clone variant, pWL47 (20). Fny86.6-CMV, FSF1621/2735-CMV, and SF1553-CMV were able to act as efficient helper viruses for WL1-sat RNA (9), whereas FS1551-CMV and Sny83.3-CMV were not able to replicate sat RNA to detectable levels in zucchini squash (Fig. 1). These results indicate that the determinant(s) for the inability to efficiently replicate satellite RNA in zucchini squash either maps to the region between the *Bgl*II site and the 3′ end of Sny-CMV or involves interactions between sequences upstream and downstream of the *Bgl*II site.

Sequence of the Sny cDNA 3′ end. The nucleotide sequence of the 3′ end of Sny cDNA clone pSny67, up to the *Bgl*II site, revealed only one nucleotide difference which resulted in an amino acid difference compared to pFny109 (Fig. 2). Two silent differences were also seen in the coding region, but no

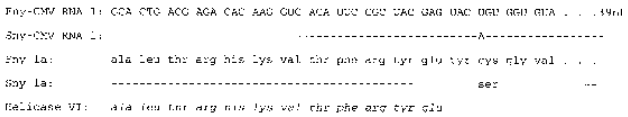

FIG. 2. Nucleotide and amino acid differences in the 3′ end of the Fny- and Sny-CMV 1a ORF. There is an additional 39 nt (13 amino acids) to the end of the ORF. The top line shows the Fny sequence, with differences in the Sny sequence indicated below. The highly conserved helicase motif VI (11, 14) is shown in italics below.

differences were found in the 3′ nontranslated region (data not shown). It seems most likely that the difference in sat RNA replication resides in the corresponding 1a protein sequence. Therefore, the effect of the single amino acid difference was further analyzed.

Site-directed mutant of pFny109. To determine if a change from a Cys to a Ser residue at position 978 of the 1a protein would prevent the support of sat RNA, nt 3027 of the cDNA insert in pFny109 was changed from a T to an A, resulting in a change of codon 978 from TGT (Cys) to AGT (Ser). The mutation was introduced by using a two-step thermocycling reaction, similar to that described by Higuchi et al. (13), except that *Pfu* polymerase was used instead of *Taq* polymerase. *Pfu* polymerase is a heat-stable polymerase with proofreading functions, and it has an error rate at least 12-fold lower than that of *Taq* DNA polymerase (21). Sequence analysis of 10 clones derived from the site-directed mutagenesis reaction revealed very few errors (data not shown). A clone that contained only the introduced mutation, and no other differences, was chosen for further study. Transcript from the resulting clone, pFny1ser978, was inoculated with transcript for RNAs 2 and 3 onto tobacco, and progeny viral RNA (Fny1ser978-CMV) was inoculated onto squash along with WL1-sat RNA. Dot blot analysis showed that the mutant was not able to support the replication of WL1-sat RNA, except in one plant, which had a low level of sat RNA (Fig. 1). When virion RNA from the single plant testing positive was analyzed by agarose gel electrophoresis, a very low level of sat RNA was found in the Fny1ser978-CMV sample (Fig. 3).

RPA. High-specific-activity RPAs were used to determine if a low level of sat RNA replication was occurring in plants that tested negative for sat RNA by dot blot analysis and to look for

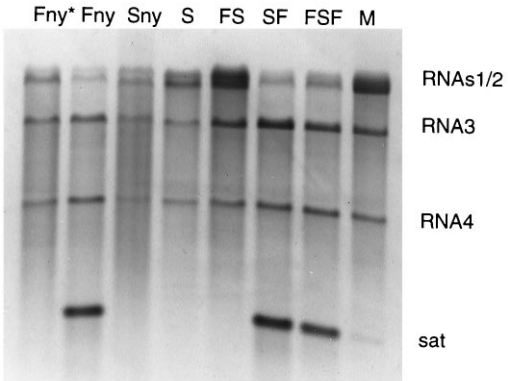

FIG. 3. Negative image of an ethidium bromide-stained agarose gel of virion RNAs purified from zucchini squash. Fny* was inoculated without WL1-sat RNA. All other inocula contained WL1-sat RNA. Lanes: Fny, Fny-CMV; Sny, Sny-CMV; S, Sny83.3-CMV; FS, FS1551-CMV; SF, SF1553-CMV; FSF, FSF1621/2735-CMV; M, Fny1ser978-CMV. The positions of the viral RNAs and sat RNA are marked.

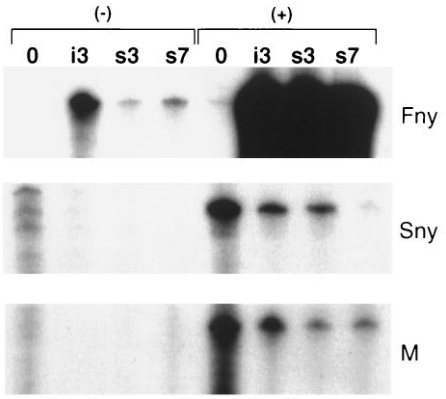

FIG. 4. RPAs of sat RNA from infected zucchini squash. (−), negative-sense RNA was detected with a positive-sense transcript probe; (+), positive-sense RNA was detected with a negative-sense transcript probe. Lanes: 0, zero time point; i3, inoculated leaf 3 days postinoculation; s3, systemic leaf 3 days postinoculation; s7, systemic leaf 7 days postinoculation. Fny, plants inoculated with Fny86.6-CMV; Sny, plants inoculated with Sny83.3-CMV; M, plants inoculated with Fny1ser978-CMV. The Fny lanes were exposed to X-ray film for 18 h, and the Sny and M lanes were exposed for 6 days, representing an approximately eightfold difference in exposure times.

the presence of negative-strand sat RNA. These assays are capable of readily detecting picogram quantities of RNA (data not shown). Zucchini squash plants were inoculated with viral RNA and 50 μg/ml of sat RNA, a 10-fold increase in the amount of sat RNA inoculum. Inoculated tissue was harvested 30 min after inoculation (zero time), both inoculated and upper leaf tissue were harvested 3 days postinoculation, and upper leaf tissue was harvested 7 days postinoculation. Total nucleic acid extracts were analyzed by RPA. A low level of positive-strand sat RNA was seen at zero time with Fny86.6-CMV, Sny83.3-CMV, and Fny1ser978-CMV, and high levels of positive-strand sat RNA were readily detectable in both inoculated and systemic tissue at later time points with Fny86.6-CMV (Fig. 4). Although sat RNA was also detected in the Sny83.3-CMV and Fny1ser978-CMV samples in both inoculated and systemic tissue at the later time points, the level was greatly reduced from that seen at zero time. In addition, no negative strand was detectable in these samples, although it was detected with Fny86.6-CMV (Fig. 4). Hence, it appears that Sny83.3-CMV and Fny1ser978-CMV are unable to support the replication of WL1-sat RNA in zucchini squash and that the small amount of sat RNA detected is probably residual inoculum. sat RNA is extremely stable in plant tissue, even in the absence of helper virus (17, 23).

Analysis of sat RNA replication in squash protoplasts. Previous work showed that Sny-CMV was unable to support WL1-sat RNA replication in squash protoplasts, whereas Fny-CMV supported high levels of WL1-sat RNA replication (7). In this study, we analyzed the recombinant virus strains and the mutant Fny1ser978 strain for their ability to support sat RNA replication in protoplasts (Fig. 5). Total nucleic acids were extracted 48 h after infection, separated by agarose gel electrophoresis, and blotted onto nylon membranes. First, the blots were probed for sat RNA, and then they were stripped and reprobed for CMV. SF1553-CMV and FSF1621/2735-CMV had high levels of sat RNA, but FS1551-CMV had a barely detectable level of sat RNA, which could be residual inoculum. The mutant Fny1ser978-CMV had a low level of sat RNA; however, the amount was only about 10% of that seen in SF1553-CMV and FSF1621/2735-CMV, indicating that the

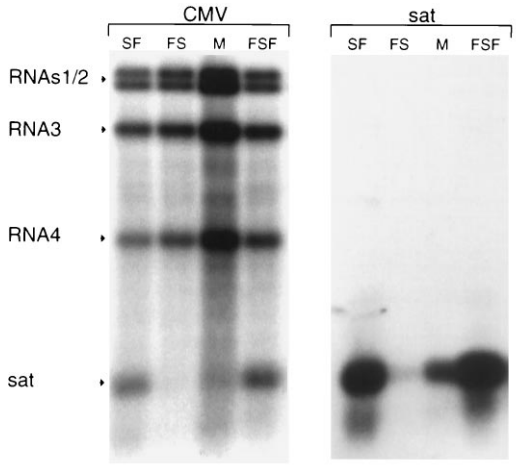

FIG. 5. Northern blot analysis of CMV and WL1-sat RNA replication in zucchini squash protoplasts. CMV lanes were probed with a ^{32}P-labeled RNA probe to the 3′ nontranslated region of all three viral RNAs; sat lanes were probed with a ^{32}P-labeled cDNA probe to sat RNA. Protoplasts were inoculated with WL1-sat RNA and SF1553-CMV (lanes SF), FS1551CMV (lanes FS) Fny1ser978-CMV (lanes M), or FSF1621/2735-CMV (lanes FSF). The positions of the viral RNAs and sat RNA are marked.

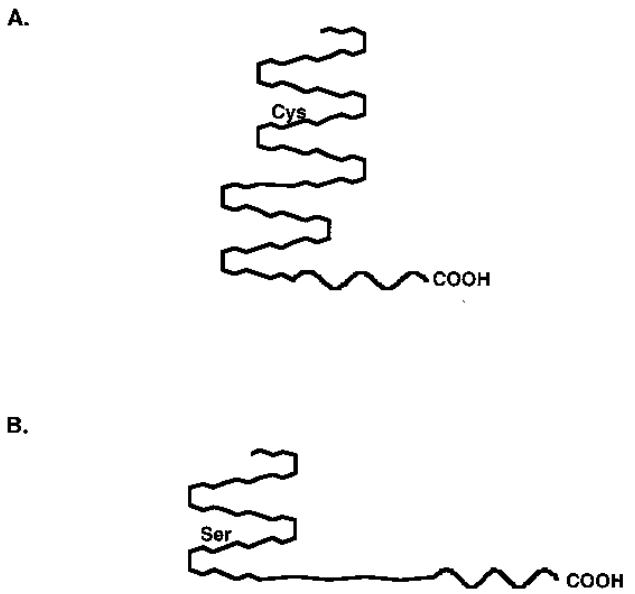

FIG. 6. Predicted secondary structure of the carboxy-terminal portion of 1a proteins from Fny-CMV (A) and Fny1ser978-CMV (B). The entire 1a proteins were used for the prediction, but the figure shows only amino acid residues 975 to 993, with the relevant difference, at amino acid 978, indicated.

Cys-to-Ser change had dramatically hampered the mutant from supporting the replication of WL1-sat RNA.

DISCUSSION

It is clear from numerous previous studies that there is a three-way interaction between sat RNAs, their helper viruses, and the host plants (32). The details of these interactions have not been elucidated and may involve different steps in the infection process. Many sat RNAs are not supported efficiently by most strains of CMV in cucurbit hosts (16, 19, 22). The nucleotide determinants for the unusually high level of sat RNA accumulation of the Ix-sat RNA in squash have been mapped to three domains in the sat RNA (3). In the present study, however, we saw an apparent lack of sat RNA replication by Sny86.6-CMV in zucchini squash, and this appears to be true in other cucurbit hosts as well (data not shown). The single plant testing positive for sat RNA with Fny1ser978-CMV could represent a low level of reversion of the helper virus to wild type. Alternatively, a small proportion of the RNA species in the WL1-sat RNA population may be competent for replication by Fny1ser978-CMV (as well as by Sny-CMV), because the cDNA clone pWL47 was derived from WL1-sat RNA. Transcript from this clone is supported by Sny-CMV in zucchini squash and other cucurbit hosts (data not shown). There are 20 nt differences in pWL47 compared to the consensus sequence for WL1-sat RNA (20); therefore, it seems likely that the WL47-sat RNA sequence represents a small minority of the WL1-sat RNA quasispecies swarm. The low level of sat RNA support by Fny1ser978 is also seen in protoplasts (Fig. 5).

The specificity of WL1-sat RNA support is also found at the level of the host, since the WL1-sat RNA is supported by Sny-CMV in solanaceous hosts (7). The precise mapping of this inability to support sat RNA by Sny-CMV to an amino acid adjacent to the conserved helicase domains suggests that the helper virus, along with unknown host factors, may be unable to efficiently unwind the secondary structure of the sat RNA to replicate it. If Sny83.3- or Fny1ser978-CMV were capable of supporting negative-strand sat RNA synthesis, it should have been detected in the RPAs, especially in the inoculated cotyledons; therefore, the block in replication could be at the negative-strand level. The ability to synthesize positive strand from negative strand could not be determined, and the analysis will require a cDNA clone of WL1-sat RNA, which is capable of synthesizing a functional negative strand. The low level of positive-strand sat RNA detectable in systemic tissue from plants inoculated with Sny83.3- or Fny1ser978-CMV by RPA may indicate that the inoculum sat RNA is being packaged and transported by the helper virus even in the absence of replication. Since levels in the inoculated leaf did not increase but, rather, decreased, there is no evidence of any replication. However, given the presence of sat RNA in virion RNA (Fig. 3), we cannot completely rule out that the Fny1ser978-CMV is capable of some low level of replication of WL1-sat RNA. There is a cluster of five conservative amino acid differences between the Fny- and Sny-CMV 1a proteins, in the region upstream of the *Bgl*II site, that could have a secondary effect on sat RNA support.

A computer analysis of the predicted secondary structure of the Fny-CMV 1a protein was compared to that of the predicted secondary structure of the 1a protein when the Cys at position 978 was changed to Ser. The single difference of a Cys in Fny 1a protein, vis-à-vis a Ser residue, resulted in a significant difference in the predicted secondary structure of the carboxy-terminal region of the protein. An extended alpha helix predicted in the carboxy-terminal region of the 1a protein of Fny-CMV is shortened in the predicted Sny-CMV 1a protein and replaced by a random-coil region (Fig. 6). Hence, the change in the predicted structure of the 1a protein when the Cys at amino acid 978 is changed to a Ser residue supports the hypothesis that unwinding of secondary structure is the block in replication of WL1-sat RNA by Sny-CMV, since this region is directly adjacent to the helicase domain VI of the 1a protein and is most probably a part of the functional motif. However, the role of the host in this unwinding activity is not clear. Future studies on the secondary structure of the WL1-sat RNA and the closely related WL47-sat RNA, which is supported by

Sny-CMV, should help to elucidate the precise relationship between sat RNA structure and replication.

ACKNOWLEDGMENTS

We thank Stan Flasinski and Maria Harrison for careful review of the manuscript and Robert Gonzales and Valerie Graves for sequence analysis of mutant clones.

This work was supported by the S. R. Noble Foundation and grant DMB-9106293 from the National Science Foundation to P.P.

REFERENCES

1. **Argos, P.** 1988. A sequence motif in many polymerases. Nucleic Acids Res. **16:**9906–9917.
2. **Beckler, G. S.** 1992. Production of milligram amounts of highly translatable RNA using the RiboMAX™ system. Promega Notes **39:**12–16.
3. **Bernal, J. J., and F. Garcia-Arenal.** 1994. Analysis of the determinants of the satellite RNA of cucumber mosaic cucumovirus for high accumulation in squash. Virology **205:**262–268.
4. **Diaz-Ruiz, J. R., and J. M. Kaper.** 1978. Isolation of viral double-stranded RNAs using a LiCl fractionation procedure. Prep. Biochem. **8:**1–17.
5. **Ding, S.-W., W. X. Li, and R. H. Symons.** 1995. A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. EMBO J. **14:**5762–5772.
6. **Dunsmuir, P., D. Bond, K. Lee, D. Gidoni, and J. Townsend.** 1988. Stability of introduced genes and stability in expression, p. 1–17. *In* S. B. Gelvin, R. A. Schilperoort, and D. P. S. Verma (ed.), Plant molecular biology manual, vol. 2. Kluwer Academic Publishers, Dordrecht, The Netherlands.
7. **Gal-On, A., I. Kaplan, and P. Palukaitis.** 1995. Differential effects of satellite RNA on the accumulation of cucumber mosaic virus RNAs and their encoded proteins in tobacco vs zucchini squash with two strains of CMV helper virus. Virology **208:**58–66.
8. **Gal-On, A., I. Kaplan, M. J. Roossinck, and P. Palukaitis.** 1994. The kinetics of infection of zucchini squash by cucumber mosaic virus indicate a function for RNA 1 in virus movement. Virology **205:**280–289.
9. **García-Arenal, F., M. Zaitlin, and P. Palukaitis.** 1987. Nucleotide sequence analysis of six satellite RNAs of cucumber mosaic virus: primary sequence and secondary structure alterations do not correlate with differences in pathogenicity. Virology **158:**339–347.
10. **Graves, M. V., and M. J. Roossinck.** 1995. Characterization of defective RNAs derived from RNA 3 of the Fny strain of cucumber mosaic cucumovirus. J. Virol. **69:**4746–4751.
11. **Habili, N., and R. H. Symons.** 1989. Evolutionary relationship between luteoviruses and other RNA plant viruses based on sequence motifs in their putative RNA polymerases and nucleic acid helicases. Nucleic Acids Res. **17:**9543–9555.
12. **Hayes, R. J., and K. W. Buck.** 1990. Complete replication of a eukaryotic virus RNA in vitro by a purified RNA-dependent RNA polymerase. Cell **63:**363–368.
13. **Higuchi, R., B. Krummel, and R. K. Saiki.** 1988. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16:**7351–7367.
14. **Hodgman, T. C.** 1986. An amino acid sequence motif linking viral DNA polymerases and plant virus proteins involved in RNA replication. Nucleic Acids Res. **14:**6769.
15. **Hodgman, T. C.** 1988. A new superfamily of replicative proteins. Nature **333:**22–23.
16. **Jacquemond, M., and J.-P. Leroux.** 1982. L'ARN satellite du virus de la mosaïque du concombre. II. Étude de la relation virus-ARN satellite chez diers hôtes. Agronomie **2:**55–62.
17. **Jacquemond, M., and H. Lot.** 1982. L'ARN satellite du virus de la mosaïque du concombre. III. La propriété de survie *in vivo*. Agronomie **2:**533–538.
18. **Jameson, B. A., and H. Wolf.** 1988. The antigenic index: a novel algorithm for predicting antigenic determinants. CABIOS **4:**181–186.
19. **Kaper, J. M., and M. E. Tousignant.** 1977. Cucumber mosaic virus-associated RNA 5 I. Role of host plant and helper strain in determining amount of associated RNA 5 with virions. Virology **80:**186–195.
20. **Kurath, G., and P. Palukaitis.** 1989. RNA sequence heterogeneity in natural populations of three satellite RNAs of cucumber mosaic virus. Virology **173:**231–240.
21. **Lundberg, K. S., D. D. Shoemaker, M. W. W. Adams, J. M. Short, J. A. Sorge, and E. J. Mathur.** 1991. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. Gene **108:**1–6.
22. **Mossop, D. W., and R. I. B. Francki.** 1979. Comparative studies on two satellite RNAs of cucumber mosaic virus. Virology **95:**395–404.
23. **Mossop, D. W., and R. I. B. Francki.** 1978. Survival of a satellite RNA *in vivo* and its dependence on cucumber mosaic virus for replication. Virology **86:**562–566.
24. **Nitta, N., Y. Takanami, S. Kuwata, and S. Kubo.** 1988. Inoculation with RNAs 1 and 2 of cucumber mosaic virus induces viral RNA replicase activity in tobacco mesophyll protoplasts. J. Gen. Virol. **69:**2695–2700.
25. **Palukaitis, P., S. Cotts, and M. Zaitlin.** 1985. Detection and identification of viroids and viral nucleic acids by 'Dot-Blot' hybridization. Acta Hortic. **164:** 109–118.
26. **Palukaitis, P., M. J. Roossinck, R. G. Dietzgen, and R. I. B. Francki.** 1992. Cucumber mosaic virus. Adv. Virus Res. **41:**281–348.
27. **Palukaitis, P., M. J. Roossinck, and F. Garcia-Arenal.** 1994. Application of the ribonuclease protection assay in plant virology. Methods Mol. Genet. **4:**237–250.
28. **Palukaitis, P., and M. Zaitlin.** 1984. Satellite RNAs of cucumber mosaic virus: characterization of two new satellites. Virology **132:**426–435.
29. **Rizzo, T. M., and P. Palukaitis.** 1990. Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2 and 3: generation of infectious RNA transcripts. Mol. Gen. Genet. **222:**249–256.
30. **Roossinck, M. J., and P. Palukaitis.** 1991. Differential replication in zucchini squash of a cucumber mosaic virus satellite RNA maps to RNA 1 of the helper virus. Virology **181:**371–373.
31. **Roossinck, M. J., and P. Palukaitis.** 1990. Rapid induction and severity of symptoms in zucchini squash (*Cucurbita pepo*) map to RNA 1 of cucumber mosaic virus. Mol. Plant-Microbe Interact. **3:**188–192.
32. **Roossinck, M. J., D. Sleat, and P. Palukaitis.** 1992. Satellite RNAs of plant viruses: structures and biological effects. Microbiol. Rev. **56:**265–279.
33. **Routh, G., J. A. Dodds, L. Fitzmaurice, and T. E. Mirkov.** 1995. Characterization of deletion and frameshift mutants of satellite tobacco mosaic virus. Virology **212:**121–127.
34. **Rozanov, M. N., E. V. Koonin, and A. E. Gorbalenya.** 1992. Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. J. Gen. Virol. **73:**2129–2134.
35. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.