JOURNAL OF VIROLOGY, Jan. 2005, p. 978–986 Vol. 79, No. 2
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.2.978–986.2005
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The *Red Clover Necrotic Mosaic Virus* RNA2 *trans*-Activator Is Also a *cis*-Acting RNA2 Replication Element

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Received 17 June 2004/Accepted 8 August 2004

The expression of the coat protein gene requires RNA-mediated *trans*-activation of subgenomic RNA synthesis in *Red clover necrotic mosaic virus* (RCNMV), the genome of which consists of two positive-strand RNAs, RNA1 and RNA2. The *trans*-acting RNA element required for subgenomic RNA synthesis from RNA1 has been mapped previously to the protein-coding region of RNA2, whereas RNA2 is not required for the replication of RNA1. In this study, we investigated the roles of the protein-coding region in RNA2 replication by analyzing the replication competence of RNA2 mutants containing deletions or nucleotide substitutions. Our results indicate that the same stem-loop structure (SL2) that functions as a *trans*-activator for RNA-mediated coat protein expression is critically required for the replication of RNA2 itself. Interestingly, however, disruption of the RNA-RNA interaction by nucleotide substitutions in the region of RNA1 corresponding to the SL2 loop of RNA2 does not affect RNA2 replication, indicating that the RNA-RNA interaction is not required for RNA2 replication. Further mutational analysis showed that, in addition to the stem-loop structure itself, nucleotide sequences in the stem and in the loop of SL2 are important for the replication of RNA2. These findings suggest that the structure and nucleotide sequence of SL2 in RNA2 play multiple roles in the virus life cycle.

The genomic RNAs of positive-strand RNA viruses play multiple roles during the infection cycle. Upon entering host cells, they act as mRNAs that direct viral protein synthesis; they serve as templates for genomic RNA replication; they are packaged into progeny virions; and in some viruses they serve as templates for subgenomic RNA synthesis and act as regulators of gene expression. In these processes, viral RNAs function as *cis*-acting elements that recruit translation factors, RNA replicase component proteins, and structural proteins (1, 5, 6). Viruses achieve infection by properly regulating these processes, because these processes sometimes conflict with one another (2, 7, 26).

To investigate the RNA replication and gene expression mechanisms of RNA viruses, we used *Red clover necrotic mosaic virus* (RCNMV). RCNMV is classified in the family *Tombusviridae* and the genus *Dianthovirus*. The genome of RCNMV is divided into two RNA molecules, RNA1 and RNA2 (10, 12, 25), unlike the case for other viruses in the *Tombusviridae* family, which have a monopartite RNA genome. RNA1 has no cap structure at the 5′ end (22), has no poly(A) tail at the 3′ end (21, 35), and encodes putative RNA replicase components, a 27-kDa protein (p27), and an 88-kDa protein (p88). p88 has an RNA-dependent RNA polymerase motif (15) and is produced by programmed −1 ribosomal frameshifting (13, 38). RNA1 also encodes a 37-kDa coat protein (CP) that is expressed from a subgenomic RNA (40). The 3′ untranslated region (UTR) of RCNMV RNA1 functions as a primary determinant of temperature-sensitive viral RNA accumulation (21) and can function alone without its 5′ UTR as a cap-independent translational enhancer in cowpea protoplasts (22). RNA2 encodes a 35-kDa movement protein (MP) that is required for viral movement in plants (17, 37). RNA2 has no cap structure, and, unlike the 3′ UTR of RNA1, its 3′ UTR does not function as an enhancer of cap-independent translation, with or without its 5′ UTR (22; H. Mizumoto and T. Okuno, unpublished results). RNA2 is not required for the replication of RNA1 in protoplasts (21, 22, 27, 28), but a 34-nucleotide (nt) sequence in the protein-coding region of RNA2 is necessary for the synthesis of subgenomic RNA from RNA1 by an RNA-mediated *trans*-activation mechanism, which leads to the expression of CP (reference 33 and this study). Both the 5′ UTR and the 3′ UTR of RNA2 are important for its replication (34). However, the roles in RNA replication of the protein-coding region of RNA2, in which the *trans*-activator occurs, have not been investigated.

In this study, to gain further insight into the roles of the protein-coding region of RNA2, we determined the *cis*-acting sequences that are required for RNA2 replication in cowpea protoplasts, using a series of deletion mutants in the presence of RNA1. Our results show that a stem-loop structure (SL2) of 20 nt is critical for RNA2 replication. Interestingly, SL2 is the *trans*-activator required for RNA-mediated CP expression (reference 33 and this study). However, replication of RNA2 was not affected by mutations in the corresponding nucleotides in RNA1, which abolished the RNA-mediated expression of CP. This indicates that the interaction between RNA1 and RNA2 is not required for and does not affect the replication of RNA2. Further mutational analysis revealed the importance of nucleotide sequences in both the stem and loop of SL2 in the replication of RNA2. These findings suggest that the structure of SL2 and its nucleotide sequence play multiple roles in the infection process of the virus.

MATERIALS AND METHODS

Plasmid clones and their construction. pRC1/G and pRC2/G are full-length cDNA clones of RNA1 and RNA2, respectively, from an Australian strain of

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RCNMV, which were generously provided by S. A. Lommel (36). pRC2 G* contains an additional adenine residue at the second position upstream from the initiation codon of MP relative to pRC2/G, as reported by Xiong and Lommel (36). Viral RNA transcripts from pRC1/G and pRC2/G are designated wtRNA1 and wtRNA2, respectively. For other transcripts, plasmid names without the prefix p are used for viral RNAs. All viral RNA mutants with a deletion or nucleotide substitution, except for pRNA1fsCP, pfsMP-D1, and pfsMP-D4, were constructed by PCR-based in vitro mutagenesis with appropriate combinations of the oligonucleotides listed in Table 1. pRNA2fsMP, described below, was used as the template DNA for the construction of RNA2 mutants unless otherwise stated.

(i) pRNA1fsCP. pRC1 G was digested with BamHI, end filled with T4 DNA polymerase, and religated. The XhoI-SacII fragment of the ligated plasmid was used to replace the corresponding fragment of pRC1 G. RNA1fsCP expresses a truncated form of CP of 162 amino acids, which is not functional in the host plant (37).

(ii) pRNA2fsMP. Four nucleotides (CATT) were inserted just after the initiation codon of the MP open reading frame (ORF) to create an EcoT22I site. This site was used to introduce a frameshift mutation into the MP ORF by using pRC2/G* as the template and two sets of primer pairs, P/T7/AR2 plus RC2dMP− and RC2dMP+ plus S/R2-3′, using recombinant PCR methods. The PCR product amplified with P/T7/AR2 and S/R2-3′ was digested with PstI and XbaI, and the 1.0-kb fragment was cloned into the corresponding enzyme sites of pUC118 (Takara, Otsu, Japan). The small NheI-SmaI fragment of the plasmid was used to replace the corresponding fragment in pRC2/G*.

(iii) pfsMP-D1 and pfsMP-D4. pRNA2fsMP was digested with EcoT22I and NheI or with PshAI and XbaI, blunt ended with T4 DNA polymerase, and religated with DNA ligase to produce pfsMP-D1 and pfsMP-D4, respectively.

(iv) pfsMP-D2. The two sets of primer pairs used were M4 plus d2nd− and d2nd+ plus S/R2-3′. Recombinant PCR products were amplified with M4 and S/R2-3′, digested with EcoT22I and PshAI, and used to replace the corresponding region of pRNA2fsMP.

(v) pfsMP-D3. The primer pairs used were M4 plus dright−, and dright+ plus S/R2-3′. Recombinant PCR products were amplified with M4 and S/R2-3′, digested with NheI and XbaI, and used to replace the corresponding region of RNA2fsMP.

(vi) pfsMP3′-D1, pfsMP3′-D2, pfsMP3′-D3, pDSL1, pDSL2, pDSL2/3, pDSL4, pDSL5, pDSL6, pDupSL2/3, pDmidSL2/3, pDdownSL2/3, pSL2-5′Sm, pSL2-3′Sm, and pSL2-5′3′Sm. The primer pairs used were P/T7/AR2 plus one each of the following: D3′H-1−, D3′H-2−, D3′H-3−, dSL1−, dSL2−, dSL2/3−, dSL4−, dSL5−, dSL6-, upSL2/3−, midSL2/3−, lowSL2/3-, SL2-5′S−, SL2-3′S−, and SL2-5′3′S−, respectively. Another primer, AC2-1210, was used together with each of the following: D3′H-1+, D3′H-2+, D3′-3+, dSL1+, dSL2+, dSL2/3+, dSL4+, dSL5+, dSL6+, upSL2/3+, midSL2/3+, lowSL2/3+, SL2-5′S+, and SL2-5′3′S+, respectively. Each recombinant PCR product was amplified with the primer pair P/T7/AR2 plus AC2-1210, digested with NheI and XbaI, and used to replace the corresponding region of pRNA2fsMP.

(vii) pfsMP3′-D4. The DNA fragment amplified with the primer pair P/T7/AR2 plus D3′H-4− was digested with NheI and XbaI and used to replace the corresponding region of pRNA2fsMP.

(viii) pTA1. DNA fragments were amplified from pRC1/G by using two primer pairs, AC1+1130 plus TA1− and TA1+ plus AC1-2520. The final recombinant PCR product was amplified with the primer pair AC1+1130 plus AC1-2520, digested with XhoI and BglII, and used to replace the corresponding region of pRC1 G.

(ix) pTA2. DNA fragments were replicated by using the primer pairs P/T7/AR2 plus TA2− and TA2+ plus AC2-1210. The final recombinant PCR product was amplified with the primer pair P/T7/AR2 plus AC2-1210, digested with NheI and BglII, and used to replace the corresponding region of pRNA2fsMP.

All constructs were verified by sequencing with an ABI 310 automated sequencer (Applied Biosystems, Foster City, Calif.).

RNA preparation. All RNA transcripts were synthesized in vitro from SmaI-linearized plasmids with T7 RNA polymerase and purified with a Sephadex G-50 (fine) column (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) (22). The RNA concentration was determined spectrophotometrically, and its integrity was verified by 1% agarose gel electrophoresis.

Protoplast experiments. Protoplasts (3.0×10^5) isolated from cowpea plants (*Vigna unguiculata* cv. California Blackeye) were inoculated with 1.5 μg of each transcript (22). Inoculated protoplasts were incubated at 17°C (21).

Northern blot analysis. Total RNAs extracted from protoplasts were subjected to Northern blot analysis, as previously described (4). The digoxigenin-labeled RNA probes specific to the 3′ UTRs of RCNMV RNA1 and RNA2 have been described previously (21). The RNA signals were detected with a luminescence image analyzer (Las 1000 Plus; Fuji Photo Film, Tokyo, Japan), and the signal intensities were quantified with the Image Gauge Program (Fuji Photo Film).

Western blot analysis. Protein samples were separated by electrophoresis on sodium dodecyl sulfate–12.5% polyacrylamide gels and then transferred electrophoretically with a semidry transfer cell (Bio-Rad) to polyvinylidene difluoride membranes (Millipore, Billerica, Mass.) (3). The membranes were blocked with skim milk and incubated with a primary polyclonal antibody specific for RCNMV coat protein, which was produced in rabbits. Proteins were detected as described previously (3).

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
P/T7/AR2	AACTGCAGTTGTAATACGACTCACTATAGACAAACCTCGCTC
RC2dMP−	CCACATGAACAGCAATGCATCCAAACCTC
RC2dMP+	GAGGTTTGGATGCATTGCTGTTCATGTGG
S/R2-3′	CGGGGTGCCTAGCCGTTATA
M4	GTTTTCCCAGTCACGAC
d2nd−	CGAGTTCCCACCGAAAGAACTACGTGGG
d2nd+	GTAGTTCTTTCGGTGGGAACTCGACACCG
dright−	CCTTGGACTCGTCAAACCCCCAATACGC
dright+	GGGGTTTGACGAGTCCAAGGTGTTGAAAC
D3′H-1−	CCATCCTGCTTGCCAAACCCCCAATACGC
D3′H-2−	CCTCCTTTGTGTCAAAGGTAAACTGCACG
D3′H-3−	ATTTCTGTCTGTTTCAACACCTTGGACTC
dSL1−	GGATTTGGTGCGTGTCAAAGGTAAACTGC
dSL2−	GTACAACACTGGATTGAACCTGTCTCTGC
dSL2/3−	GCTGCCAGTTACAGTTGAGGATTCTTCTC
dSL4−	GTTCCAACGAACCTCCTTTCTGTTTCAAC
dSL5−	GCCGGAACGGCAGCTTCGTGGTTTTGCTG
dSL6−	CATTTCTGTTGGAACCGGAAGAAGGTTCC
upSL2/3−	GATTGAACCTGACAGTTGAGGATTCTTC
midSL2/3−	GGACTCTCGTCCTCTCTGCTTTGGGAGGG
lowSL2/3−	GCTGCCAGTTTTGGTTTGGTACAACACTG
SL2-5′S−	GGGGCGATCGGAGAGATTGAACCTGTCTC
SL2-3′S−	CACTGTCTCCAGGGGCGATACCTCTGATTG
SL2-5′3′S−	CACTGTCTCCAGGGGCGATCGGAGAGATTGAACCTGTCTCTGC
AC2-1210	AGAGACCCTACGAGGAGAAC
D3′H-1+	GGTTTGGCAAGCAGGATGGAGAAGAATCC
D3′H-2+	ACCTTTGACACAAAGGAGGAACTGGCAGC
D3′H-3+	GAAACAGACAGAAATGTTCTGAACTCATC
dSL1+	CTTTGACACGCACCAAATCCAATGTGAAC
dSL2+	CAGGTTCAATCCAGTGTTGTACCAAACC
dSL2/3+	CCTCAACTGTAACTGGCAGCAAAACCACG
dSL4+	GAAAGGAGGTTCGTTGGAACCTTCTTCCG
dSL5+	CGAAGCTGCCGTTCCGGCTTGAGTATGAG
dSL6+	CCGGTTCCAACAGAAATGTTCTGAACTC
upSL2/3+	CTCAACTGTCAGGTTCAATCAGAGGTATC
midSL2/3+	CAAAGCAGAGAGGACGAGAGTCCAAGGTG
lowSL2/3+	CCAAACCAAAACTGGCAGCAAAACCACG
SL2-5′S+	CAATCTCTCCGATCGCCCCGCCTCTCAG
SL2-3′S+	CGCCCCTGGAGACAGTGTTGTACCAAACC
SL2-5′3′S+	CAATCTCTCCGATCGCCCCTGGAGACAGTGTTGTACCAAACC
D3′H-4−	CGTCTAGAGAGATCTTCGGCTCATACTC
AC1+1130	GTTGAGGAAATTGTC
TA1−	GTTGAACGGCCTTCACAAGTGATTTCCAC
TA1+	GTGAAGGCCGTTCAACAAACGTATTACC
AC1-2520	ATGATTTGCGTCTTGGC
TA2−	GAGAGGCAGGCCGTTACCTCTGATTGAAC
TA2+	CAGAGGTAACGGCCTGCCTCTCAGTGTTG

RESULTS

CP and MP do not affect RNA2 accumulation. To facilitate the analysis of the *cis*-acting sequences of RNA2, with no requirement to consider the effects of possible *trans*-acting factors, we constructed a CP frameshift mutant of RNA1 (RNA1fsCP) and an MP frameshift mutant of RNA2 (RNA2fsMP), from

which intact CP and MP, respectively, are not expressed (Fig. 1A). These mutant RNAs accumulated to levels similar to those of wild-type RNA1 and RNA2 in cowpea protoplasts at 24 h postinoculation (hpi) (Fig. 1B), suggesting that CP and MP do not affect the replication or stability of either RNA1 or RNA2 in cowpea protoplasts. Therefore, a series of RNA2 mutants with a deletion in the MP ORF were constructed by using pRNA2fsMP and used in the search for *cis*-acting sequences.

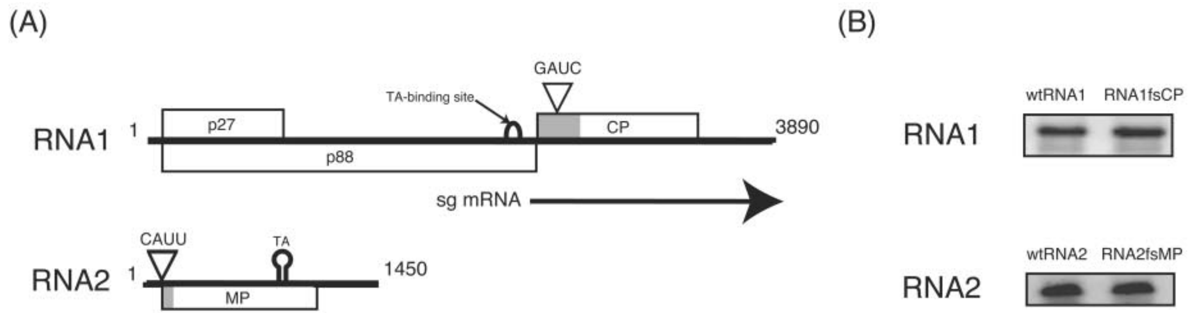
Nucleotide sequences in the MP-coding region are required for the accumulation of RNA2. Preliminarily, four RNA2fsMP mutants (fsMP-D1, fsMP-D2, fsMP-D3, and fsMP-D4) with deletions covering the MP-coding region (Fig. 1C) were tested for their ability to be replicated in cowpea protoplasts inoculated together with RNA1fsCP. fsMP-D1 and fsMP-D2, which have a deletion in the 5′ half of the MP-coding region, accumulated as efficiently as RNA2fsMP, whereas fsMP-D3 and fsMP-D4 did not accumulate or accumulated very poorly (Fig. 1D). This indicates the importance of the 3′ half of the MP-coding region in RNA2 replication. Next, four RNA2fsMP mutants (fsMP3′-D1, fsMP3′-D2, fsMP3′-D3, and fsMP3′-D4), which have a deletion in the 3′ half of the MP-coding sequence(Fig. 1C), were tested as described above. fsMP3′-D1 accumulated as efficiently as RNA2fsMP (Fig. 1E). In contrast, the accumulations of fsMP3′-D2, fsMP3′-D3, and fsMP3′-D4 were significantly lower than that of RNA2fsMP. In particular, fsMP3′-D2 and fsMP3′-D3 accumulated very poorly, to levels less than 10% of that of RNA2fsMP (Fig. 1E), suggesting that the region between nt 687 and 924 contains *cis*-acting elements that are critically required for RNA2 replication.

The stem-loop structure of SL2 is essential but not sufficient for RNA2 replication. To identify the functional domains required for RNA2 replication in the region from nt 687 to 924, the RNA secondary structure was predicted by using the program Dynalign (19) together with the nucleotide sequence of RNA2 from another RCNMV strain (RCNMV-Can) (21). Six stem-loop structures (SL1, SL2, SL3, SL4, SL5, and SL6) were predicted in the region (Fig. 2A). SL2 and SL3 occur on a large, complex stem-loop structure predicted to occur after a 26-nt spacer sequence after SL1. The region from nt 714 to 840 was designated SL2/3. Five deletion mutants (D-SL1, D-SL2/3, D-SL4, D-SL5, and D-SL6) in which a single stem-loop structure or SL2/3 was removed from RNA2fsMP were constructed and tested. The accumulations of D-SL1, D-SL4, and D-SL5 did not differ significantly from that of RNA2fsMP (Fig. 2B). On the other hand, D-SL2/3 accumulated very poorly, to a level less than 5% of that of RNA2fsMP. D-SL6 also accumulated poorly, to a level about 30% of that of RNA2fsMP (Fig. 2B). This indicates that SL2/3 contains essential *cis*-acting elements required for RNA2 replication and that SL6 is also required for the efficient replication of RNA2.

To further delimit the domains in SL2/3 required for RNA2 replication, SL2/3 was divided into four parts (upSL2/3, midSL2/3, downSL2/3, and SL2) (Fig. 2A). Four RNA2fsMP mutants, each with one of these parts deleted (D-upSL2/3, D-midSL2/3, D-downSL2/3, and D-SL2) were tested as described above. D-upSL2/3 accumulated as efficiently as RNA2fsMP, whereas D-midSL2/3 and D-SL2 accumulated to a level less than 5% of that of RNA2fsMP, and D-downSL2/3 accumulated to a level about 40% of that of RNA2fsMP (Fig. 2C). This indicates that SL2 is an essential *cis*-acting element required for RNA2 replication and that downSL2/3 is also required for efficient replication of RNA2. Interestingly, the SL2 structure (nt 762 to 781) is conserved among the dianthoviruses and is involved in the RNA-mediated *trans*-activation of subgenomic RNA synthesis (33). In this study, we did not analyze the role of the downSL2/3 region any further.

Formation of the stem-loop structure of SL2 is important for efficient replication of RNA2. To further analyze the roles of SL2 in RNA2 replication, three RNA2 mutants (SL2-5′Sm, SL2-3′Sm, and SL2-5′3′Sm) (Fig. 3A, upper part) were constructed by introducing mutations in either or both sides of the stem of SL2 in RNA2fsMP. SL2-5′Sm and SL2-3′Sm, in which the stem structure is disrupted, accumulated poorly (Fig. 3B). Accumulation levels of SL2-5′Sm and SL2-3′Sm were about 1 and 17% of that of RNA2fsMP, respectively. Restoration of the SL2 stem structure by compensatory mutations significantly increased the accumulation of SL2-5′3′Sm to about 50% of that of RNA2fsMP (Fig. 3B), suggesting the importance of the structure of SL2 for RNA2 replication. It should be noted that the restoration of base pairing in the stem did not completely restore RNA2 accumulation to the wild-type level, suggesting the importance of the nucleotide sequence as well as the structure of SL2. Differences in the accumulation levels of SL2-5′Sm and SL2-3′Sm also support the involvement of the nucleotide sequence of the SL2 stem in RNA2 replication.

FIG. 1. Accumulation of RNA2 mutants with deletions in the MP-coding region. (A) Schematic representation of CP frameshift mutant RNA1 and MP frameshift-mutant RNA2 (used as a control) and (B) their accumulation competence in cowpea protoplasts. (A) The RCNMV genome is shown as a thick line, with protein-coding regions depicted as boxes above or below the lines of the encoded proteins. Shaded regions correspond to regions that are translated from the RNA1 CP frameshift mutant (RNA1fsCP) and the RNA2 MP frameshift mutant (RNA2fsMP). The four nucleotides used to generate the CP and MP frameshift mutants are indicated by the symbol ▽ at the insertion sites. Numbers on the right indicate the numbers of the nucleotides in the wild-type genomic RNAs. The region corresponding to CP subgenomic mRNA (sg mRNA) is shown as an arrow below RNA1. RNA elements involved in RNA-mediated CP sg mRNA synthesis are also indicated as TA-binding site in RNA1 and TA as a hairpin composed of 20 nt (SL2 in this paper) in RNA2. (B) Northern blot analysis of genomic RNA1 and RNA2 in cowpea protoplasts inoculated with wild-type RNA1 and RNA2 or with RNA1fsCP and RNA2fsMP at 24 hpi. Total RNAs extracted from cowpea protoplasts were separated by gel electrophoresis, blotted onto membranes, and probed with digoxigenin-labeled RNA probes specific for RCNMV RNA1 or RNA2. (C) Schematic representations of RNA2 mutants. The shaded box indicates the MP-coding region. Boldface black lines indicate the viral genome, with the nucleotide numbers at the 3′ and 5′ ends. The bent lines indicate the deleted regions. Four nucleotides inserted after the initiation codon of MP are shown only for RNA2fsMP. (D and E) Northern blot analysis of RNA1fsCP and RNA2 deletion mutants (fsMP-Δ1, fsMP-D2, fsMP-D3, and fsMP-D4) (D) and RNA2 deletion mutants (fsMP3′-D1, fsMP3′-D2, fsMP3′-D3, and fsMP3′-D4) (E). Total RNAs were extracted at 24 hpi from cowpea protoplasts inoculated with RNA2 mutants together with RNA1fsCP. Relative values for the accumulation of the RNA2fsMP deletion mutants (RNA2 deletion mutant/RNA2fsMP) were calculated from four independent experiments, and the mean values (with standard deviations) are presented in the bar graph below the Northern blot.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(B)
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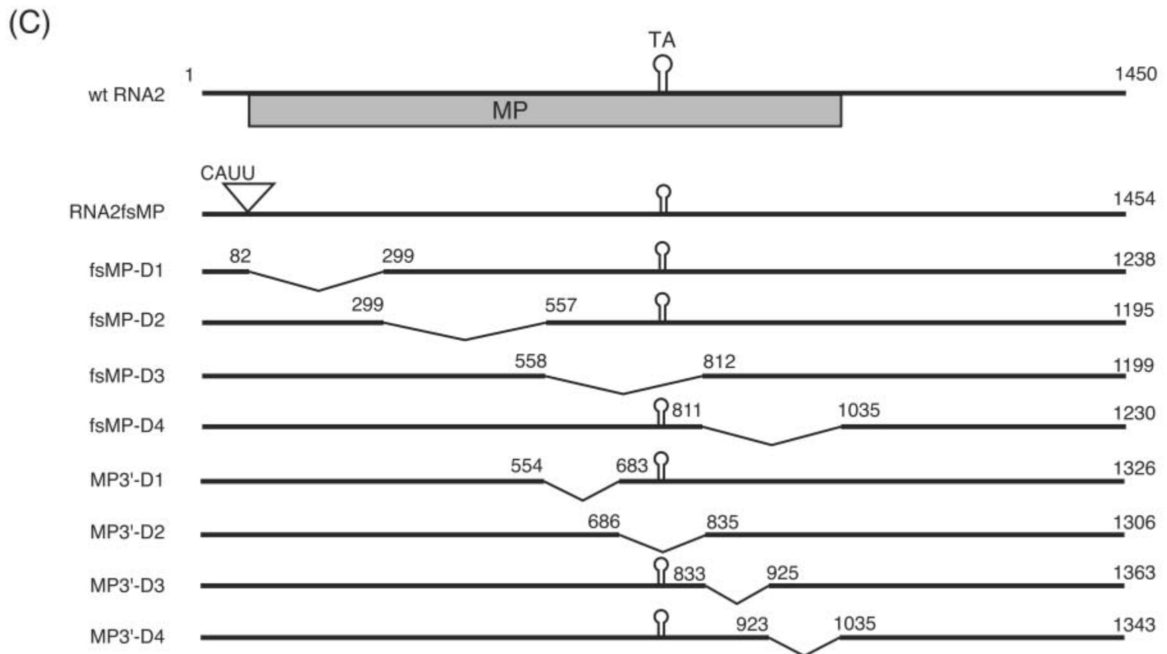
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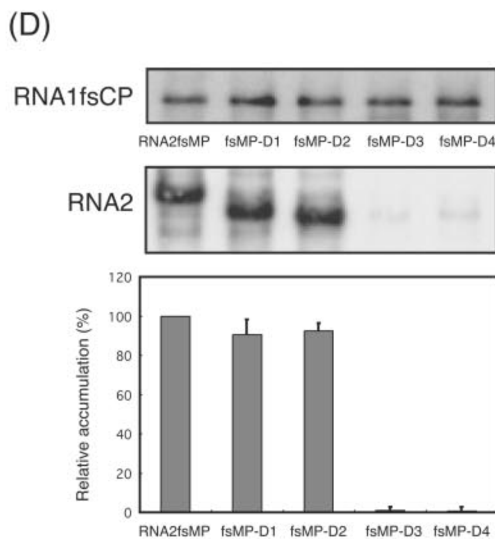
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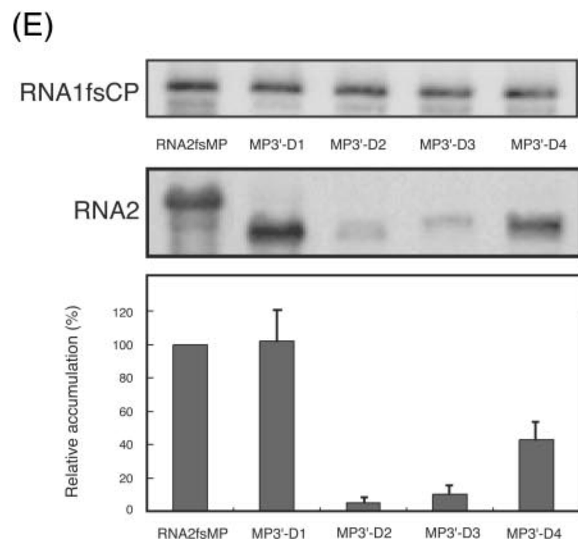
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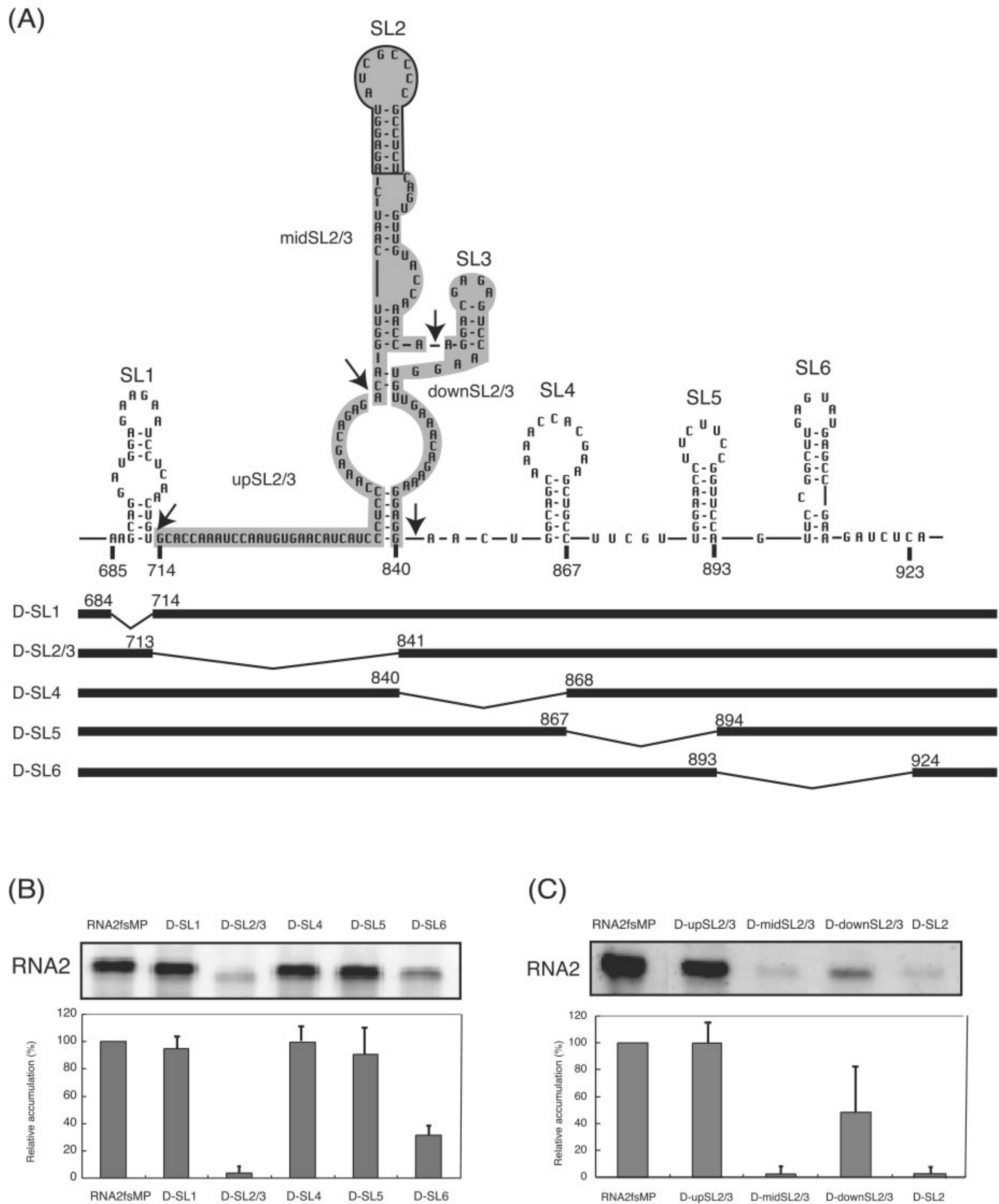

FIG. 2. Accumulation of RNA2 mutants with each of the predicted stem-loop structures deleted in the MP-coding region which is required for RNA2 accumulation. (A) Secondary structure predicted by the computer algorithm Dynalign (18) for RNA2 between nt 685 and 924, together with the corresponding regions in the RNA2 of another RCNMV strain (Canadian strain). The names of the six stem-loop structures are given above the structures, and the regions deleted in D-upSL2/3, D-midSL2/3, and D-downSL2/3 from the large complex structure (SL2/3, shaded) correspond to those between the arrows, which indicate the deletion sites. The region deleted in D-SL2 is shown by a closed solid box. (B and C) Northern blot analysis and relative accumulation levels of RNA2fsMP, D-SL1, D-SL2/3, D-SL4, D-SL5, and D-SL6 (B) and of RNA2fsMP, D-upSL2/3, D-midSL2/3, D-downSL2/3, and D-SL2 (C) in cowpea protoplasts coinoculated with RNA1fsCP at 24 hpi. For other information, see the legend to Fig. 1.

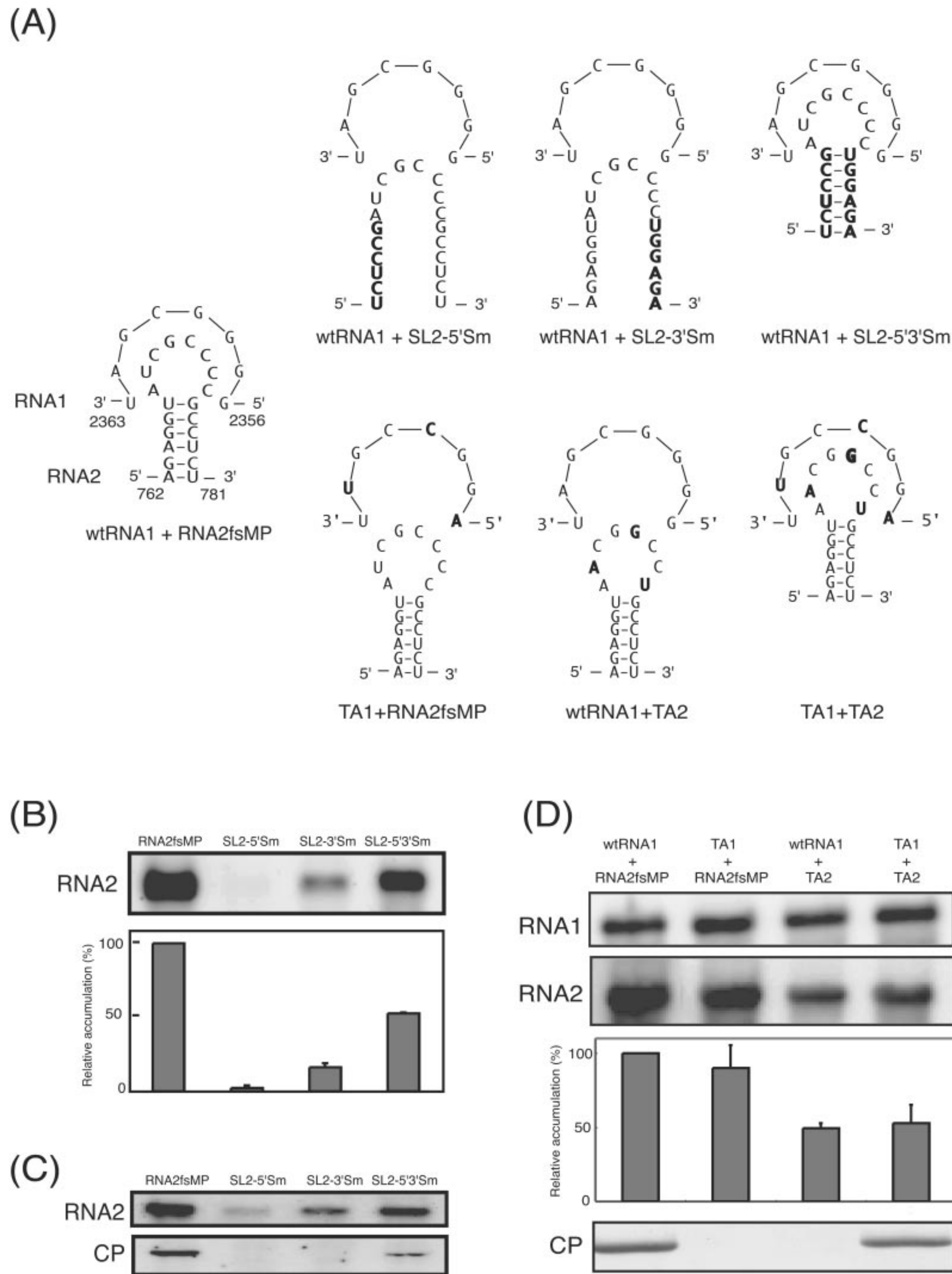

FIG. 3. Accumulation of RNA2 mutants with the SL2 stem structure or the SL2-mediated RNA interaction disrupted and restored. (A) Predicted secondary structures of SL2 with the TA-binding site in RNA1. Altered nucleotides are shown by boldface. (B) Northern blot analysis and relative values for the accumulation of RNA2fsMP, SL2-5′Sm, SL2-3′Sm, and SL2-5′3′Sm at 24 hpi in cowpea protoplasts inoculated with RNA1fsCP and RNA2 mutants. (C) Western blot analysis of CP accumulation with Northern blot results in cowpea protoplasts inoculated with wtRNA1 and RNA2 mutants. CP was detected by using an anti-RCNMV-CP antibody. (D) Northern blot analysis and relative values for the accumulation of RNA2fsMP and TA2 in cowpea protoplasts inoculated together with either wtRNA1 or TA1. Northern blot results for RNA1 and Western blot analysis of CP accumulation are also shown above and below, respectively. For other information, wee the legends to Fig. 1 and 2.

RNA1-RNA2 interaction does not affect RNA2 replication. SL2, identified as an essential element for RNA2 replication, is a stem-loop structure that is required for the interaction between RNA1 and RNA2 through direct base pairing. The interaction is thought to be required for the transcription of the CP subgenomic RNA leading to CP expression (11, 33). Therefore, we examined whether structural changes in SL2 affect the interaction between RNA1 and RNA2 by assessing CP accumulation by Western blotting with an anti-CP antibody as a probe. Cowpea protoplasts were inoculated with wtRNA1

instead of RNA1fsCP, together with the RNA2 mutants. The accumulation patterns of the RNA2 mutants were similar to those presented in Fig. 3B (Fig. 3C). Western blot analysis of protoplasts showed that the disruption of the SL2 structure compromised CP accumulation and that the restoration of the SL2 structure significantly restored CP accumulation (Fig. 3C). These results demonstrated that the formation of the SL2 stem structure in RNA2 is important for RNA-mediated CP subgenomic RNA synthesis in vivo, supporting the proposed premature termination model for subgenomic RNA synthesis in RCNMV (33).

Therefore, to test more precisely whether the RNA1-RNA2 interaction through SL2 affects RNA2 accumulation, we constructed an RNA1 mutant that does not interact with SL2 of RNA2. For this, the 8-nt element on RNA1 was altered at three positions that did not alter the amino acid sequence of the p88 polymerase (TA1) (Fig. 3A, lower part), because these eight nucleotides in the loop of SL2 base pair with an 8-nt element on RNA1 located 2 nt upstream from the start site of subgenomic RNA synthesis (33, 40). Therefore, TA1 was expected not to base pair with the corresponding sequence in SL2 of RNA2. Compensatory mutations were also introduced into the 8-nt loop sequence of SL2 at three positions (TA2). TA2 was expected not to interact with wtRNA1 but was expected to base pair with TA1 (Fig. 3A). The accumulation of RNA2fsMP did not differ significantly for inoculation with TA1 and wtRNA1 (Fig. 3D), suggesting that the RNA1-RNA2 interaction through SL2 does not affect RNA2 replication. On the other hand, although the accumulation of TA2 was about 50% of that of RNA2fsMP in coinoculation with wtRNA1, a similar reduction in TA2 accumulation was observed in coinoculation with TA1, which restores the complementarity between RNA1 and RNA2 that is necessary for their interaction (Fig. 3D). The expected interactions between RNA1 and RNA2 were confirmed by Western blot analysis with anti-CP antibody. CP was not detected in protoplasts inoculated with wtRNA1 and TA2 or with TA1 and RNA2fsMP, whereas after inoculation with TA1 and TA2, CP accumulated efficiently at 24 hpi (Fig. 3D). Together, these results indicate that the RNA1-RNA2 interaction through SL2, which is required for CP expression, is not required for and does not affect RNA2 replication. We conclude that the role of SL2 in RNA2 replication is independent of SL2-mediated RNA1-RNA2 interaction. Furthermore, it is clear that the nucleotide sequence of the loop of SL2, as well as that of the stem, are important for the efficient replication of RNA2, as described above (Fig. 3B and D).

DISCUSSION

In this study, we have shown that the stem-loop structure SL2 is required for the replication of RCNMV RNA2. Interestingly, SL2 is the stem-loop structure that acts as a *trans*-activator for subgenomic RNA synthesis, which is required for CP expression (11, 33; this paper). This suggests that SL2 of RCNMV RNA2 has at least three different functions: as a *cis*-acting sequence for the replication of RNA2, as a *trans*-acting sequence for CP expression through the production of subgenomic RNA, and as a coding sequence for MP.

SL2 is predicted to occur in the protein-coding region of RNA2. The importance of *cis*-acting replication elements in protein-coding regions has been reported for many RNA viruses (8, 9, 16, 20, 29, 31, 39). For example, the picornavirus *cis*-acting replication element (*cre*) located within the protein-coding region is required for positive-strand RNA synthesis through the uridylation of the protein primer, VPg (23).

Structure and nucleotide sequences of SL2. Disruption of the stem structure in SL2 by nucleotide substitutions greatly reduced RNA2 accumulation, and restoration of the structure by complementary mutations restored RNA2 accumulation by 50% (Fig. 3B), suggesting the importance of the predicted SL2 structure in the replication of RNA2. The incomplete recovery by complementary mutations might be due to the change of base paring from U · G to G · U at the top of the SL2 stem, because it is thought that a U · G base pair is not structurally and functionally equivalent to a G · U base pair in RNA stem-loop structures (18). Indeed, this change causes a severe reduction in the SL2-mediated subgenomic RNA synthesis as assessed in the *Tomato bushy stunt virus* vector system and affects thermal denaturation properties of RNA1 and RNA2 oligomer complex formation in vitro (11). These results also support the importance of the SL2 structure in RNA2 replication as well as in *trans*-activator function.

Nucleotide substitutions in the loop sequence of SL2 reduced RNA2 accumulation to 50% of that of RNA2fsMP, independently of its *trans*-activator function in CP expression (Fig. 3D). This indicates that the loop sequence of SL2 is also involved in RNA2 replication. The loop sequence of SL2 might be directly involved in the interaction with the RNA replication machinery, including viral replicase, or in interaction with other parts of RNA2, including downSL2/3 (nt 815 to 840), SL6 (nt 894 to 923) (Fig. 2C), or the 3′ UTR (34). A small stem-loop structure predicted in the 3′-terminal region of RCNMV RNA2 is required for minus-strand RNA synthesis (34). These RNA elements might be important in the formation of tertiary structures that guide the replication complex, as has been proposed for an internal *cis*-acting replication element found in the genome of bacteriophage Qβ (14, 32). Alternatively, the SL2 structure in RNA2 itself might be directly involved in plus-strand RNA synthesis. A stem-loop structure predicted on plus-strand RNA has been proposed to act in the production of positive-strand RNAs in *Brome mosaic virus* (30).

Despite the importance of the SL2 stem structure in RNA2 replication as discussed above, it is also possible that nucleotide sequences of SL2 are important in maintaining the structure on the corresponding minus-strand RNA for RNA replication. Substitutions in the nucleotide sequence of the stem of SL2 may affect RNA structures on the minus-strand RNA and disrupt RNA elements required for plus-strand RNA synthesis. A stable stem-loop structure can be predicted by computer analysis to occur in the minus strand corresponding to the region of SL2; six nucleotides on the 5′ side of the SL2 stem correspond to the loop sequence in the SL of the minus strand (data not shown). The importance of stem-loop structures predicted on minus-strand RNAs has been reported for *Tomato bushy stunt virus* (31) and *Turnip crinkle virus* (24), in which predicted hairpins are thought to act as replication enhancer RNA elements involved in plus-strand RNA synthesis. Analysis of minus-strand RNA synthesis will provide important information with which to address this question.

RNA elements other than SL2 are involved in RNA2 accumulation. Despite retaining SL2, fsMP-D4 and fsMP3′-D3 lost the ability to be replicated, like those RNA2 mutants in which SL2 was deleted (Fig. 1D and E). This suggests that the nucleotide sequence from nt 812 to 924 also contains *cis*-acting elements required for the replication of RNA2, like SL2. Delimitation analysis suggested that these elements occur in the regions deleted in D-downSL2/3 (nt 805 to 840) and D-SL6 (nt 894 to 923), because these two mutants accumulated very poorly compared with RNA2fsMP (Fig. 2B and C). This may explain the loss of replication capacity by fsMP-D4 and fsMP3′-D4, because both of the regions deleted in D-downSL2/3 and D-SL6 are absent from fsMP-D4 and fsMP3′-D4. Alternatively, the deletion of the region from nt 812 to 1034 or from nt 834 to 924 might reduce the stability of the mutant RNAs. However, this is unlikely because the Northern blot signals for fsMP-D4 and fsMP3′-D3 did not differ from that of RNA2fsMP in the absence of RNA1 in cowpea protoplasts at 24 hpi (data not shown).

Functional independence of SL2 in RNA2 replication and the *trans*-activation of CP expression. SL2 plays a key role in RNA-mediated *trans*-activation of subgenomic RNA transcription from RNA1 through direct base pairing between RNA1 and RNA2 (11, 33; this study). Therefore, the disruption of SL2-mediated base pairing between RNA1 and RNA2 might affect RNA2 replication by freeing RNA2 from interaction with RNA1. However, disruption of SL2-mediated base pairing between RNA1 and RNA2 did not affect RNA2 accumulation (Fig. 3D), indicating the functional independence of SL2 in RNA2 replication and RNA-mediated CP expression. Because CP expression required efficient RNA2 accumulation (Fig. 3D) (M. Tatsuta and T. Okuno, unpublished results), lack of competition between the roles of SL2 described above might be important in the temporal regulation of CP expression in the RCNMV life cycle. The different mechanisms by which RNA1 and RNA2 are translated also suggest a temporal regulation of replicase component proteins and MP; the 3′ UTR of RNA1 functions as an efficient cap-independent translation element, but that of RNA2 does not (22; Mizumoto and Okuno, unpublished results).

ACKNOWLEDGMENTS

We are grateful to S. A. Lommel, North Carolina State University, for the RNA1 and RNA2 cDNA clones of the Australian RCNMV strain.

This work was supported in part by a grant-in-aid for scientific research (A) (13306005) from the Japan Society for the Promotion of Science and in part by a grant-in-aid for scientific research on priority areas (A) (12052201), "Molecular Mechanisms of Plant-Microbe Interaction toward Production of Disease Resistant Plants," from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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