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Cap-Independent Translational Enhancement by the 3′ Untranslated Region of *Red Clover Necrotic Mosaic Virus* RNA1

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Red clover necrotic mosaic virus **(RCNMV) is a member of the genus *Dianthovirus* and has a bipartite positive-sense genomic RNA with 3′ ends that are not polyadenylated. In this study, we show that both genomic RNA1 and RNA2 lack a 5′ cap structure and that uncapped in vitro transcripts of RCNMV RNA1 replicated to a level comparable to that for capped transcripts in cowpea protoplasts. Because the 5′ cap and 3′ poly(A) tail play important roles in the translation of many eukaryotic mRNAs, genomic RNAs of RCNMV should contain an element(s) responsible for 5′ cap- and poly(A) tail-independent translation of viral protein. By using a luciferase reporter assay system in vivo, we showed that the 3′ untranslated region (UTR) of RNA1 alone significantly enhanced translation of the luciferase reporter gene in the absence of the 5′ cap structure. Deletion studies revealed that the middle region (between nucleotides 3596 and 3732) in the 3′ UTR, designated the 3′ translation element of *Dianthovirus* RNA1 (3′TE-DR1), plays an important role in cap-independent translation. This region contained a stem-loop structure conserved among members of the genera *Dianthovirus* and *Luteovirus*. A five-base substitution in the loop abolished cap-independent translational activity, as reported for a luteovirus, indicating that this stem-loop is one of the functional structures in the 3′TE-DR1 involved in cap-independent translation. Finally, we suggest that cap-independent translational activity is required for RCNMV RNA1 replication in protoplasts.**

The genomic RNA of a positive-sense single-stranded RNA virus serves two essential functions at the start of the viral replication cycle in infected cells: as a template for negative strands and as mRNA for viral proteins. Virtually all eukaryotic cellular mRNAs are capped and polyadenylated. The 5′ cap structure (m^7GpppN) serves as the binding site for eukaryotic initiation factor 4F (eIF4F), composed of eIF4G, eIF4E, and eIF4A, which assists the binding of 40S ribosomes to mRNAs (15). The poly(A) tail serves as the binding site for the poly(A)-binding protein, which stabilizes eIF4F binding to the 5′ cap (43). Consequently, the 5′ cap and 3′ poly(A) tail are critical in recruiting translational machinery for the efficient translation of encoded proteins. However, a variety of eukaryotic viral mRNAs lack the 5′ cap and/or poly(A) tail and have therefore developed alternative strategies for translation regulation.

For *Tobacco mosaic virus*, which has a capped genome and which lacks the poly(A) tail, the pseudoknot domain in the 3′ untranslated region (UTR) appears to substitute functionally for the poly(A) tail and enhances translation (27). In addition, the poly(CAA) region in the 5′ UTR (termed Ω) is responsible for translation enhancement (9). The genomic RNA of picornaviruses has a poly(A) tail but lacks the 5′ cap. In this case, the internal ribosome entry site (IRES) in the 5′ UTR recruits ribosomes directly and enables cap-independent translation initiation to occur (21). In contrast to the IRESs of picornaviruses, the genomic RNA of *Barley yellow dwarf virus* (BYDV), *Satellite tobacco necrosis virus*, and *Tomato bushy stunt virus*, which lacks both the 5′ cap and poly(A) tail, harbors sequences in the 3′ UTR which confer cap-independent translation (32, 47, 48).

Red clover necrotic mosaic virus (RCNMV) is a member of the genus *Dianthovirus*. The genome of RCNMV consists of two positive-sense single-stranded RNAs. RNA1 encodes a functionally unknown 27-kDa protein (p27) and an 88-kDa protein (p88) with an RNA-dependent RNA polymerase (RdRp) motif (23) translated by the extension of p27 because of a ribosome frameshift (22, 52). Proteins p27 and p88 are components of the partially purified template-dependent RNA polymerase extracted from RCNMV-infected tissue (1). RNA1 also encodes a 37-kDa coat protein (CP) expressed from subgenomic RNA (53). RNA2 is a monocistronic RNA which encodes a 35-kDa movement protein (MP) required for virus movement in plants (29, 51). While RNA2 is not required for the replication of RNA1 in protoplasts (7, 38, 39), a 34-nucleotide (nt) sequence in RNA2 is required for the transcription of subgenomic RNA from RNA1 (44). It was previously reported that the RCNMV RNA1 and -2 extracted from virions were capped and lacked the poly(A) tail (29, 49). However, uncapped in vitro transcripts of RCNMV RNA1 and RNA2 show infectivity comparable to that of capped transcripts in *Nicotiana benthamiana* (50) and in cowpea protoplasts (this paper). Removal of the cap structure from virion RNAs or in vitro transcripts of the genomic RNAs reduces or abolishes infectivity in many viruses that have a 5′ cap structure. For example, the infectivity of *Tobacco mosaic virus* RNA was abolished by treatment of virus RNA with tobacco phosphodiesterase that releases m^7G from the 5′ terminal of mRNA (36). Therefore, the high infectivity observed in uncapped RCNMV RNAs implies that this virus utilizes a cap-independent mechanism to translate viral proteins.

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In this study, we reevaluated the status of the cap structure on the genomic RNAs of RCNMV and investigated the *cis*-acting element involved in translation regulation of RCNMV RNA1 using a luciferase (Luc) gene expression assay system in cowpea protoplasts. Our results show that genomic RNAs of RCNMV lack a 5′ cap structure and that the middle region in the 3′ UTR of RNA1 plays an important role in cap-independent translation of the RNA.

MATERIALS AND METHODS

Plasmid clones. Full-length cDNA clones of RCNMV Australian strain RNA1 (pRC1|G) and RNA2 (pRC2|G) were kindly provided by S. A. Lommel (50). Plasmids carrying the *Renilla* Luc gene (pSP64-RLUC), from which poly(A)$_{30}$-tailed mRNA transcripts could be generated by in vitro transcription with SP6 RNA polymerase (M. Ishikawa, Y. Chiba, and S. Naito, unpublished results), were kindly provided by M. Ishikawa. Plasmid p3′-8, which contains a 60-nt poly(A) tract, was a generous gift from K. A. White.

To facilitate the construction of deletion clones, an *Nco*I site was created at the p27 open reading frame (ORF) initiation codon in pRC1|G by using PCR-based in vitro mutagenesis (18). In the description of PCR primers, underlining indicates an introduced restriction enzyme site and boldface indicates a mutated nucleotide. A cDNA fragment of RCNMV RNA1 from nt 1 to 131 was amplified by PCR from pRC1|G using primers 5′RS1T7 (5′-GCGAGCTC*TAATACGACTCACTATAG*ACAAACGTTTTACCGGTTTG-3′; the T7 promoter sequence is in italics) and AC5′UTR/Nco− (5′-AAAACCCATGGACTGGTACGAAAAGTAG-3′). A cDNA fragment of RCNMV RNA1 from nt 113 to 3890 was amplified by PCR from pRC1|G using primers AC5′UTR/Nco+ (5′-CGTACCAGTCCATGGGTTTTATAAATC-3′) and 3′R/C1 (5′-TACCCGGGGTACCTAGCCGTTATAC-3′). Both DNA fragments were denatured and annealed, and the resulting mixture was used as a template for PCR. The newly synthesized DNA duplex was amplified by PCR using primers 5′RS1T7 and 3′R/C1. The amplified 3.9-kb DNA fragment was digested with *Sac*I and *Sma*I and cloned into the corresponding enzyme sites of pUC118 (Takara Bio Inc., Otsu, Japan) to create pURC1/Nco. Plasmids pR1-5′-NS, pR1-5′-XhS, pR1-5′-MS, and pR1-5′-SS were constructed as follows. pURC1/Nco was digested with *Nco*I/*Sph*I (to construct pR1-5′-NS), *Xho*I/*Sph*I (pR1-5′-XhS), *Mlu*I/*Sph*I (pR1-5′-MS), and *Sac*II/*Sph*I (pR1-5′-SS) and blunt ended with T4 DNA polymerase. Small DNA fragments from each reaction were purified by 1% low-melting-point agarose gel (FMC Bioproducts, Rockland, Maine) electrophoresis and cloned into pSP-luc+ (Promega, Madison, Wis.), which had been digested with *Xba*I and *Hpa*I, followed by treatment with T4 DNA polymerase. Small *Sac*I/*Nco*I DNA fragments of pURC1/Nco were cloned into the *Nde*I/*Nco*I sites of each plasmid. All DNA fragments with incompatible ends used here were ligated after blunting with T4 DNA polymerase. Plasmid pR1-XbS was constructed as follows. A cDNA fragment of RCNMV RNA1 from nt 3446 to 3890 was amplified by PCR from pURC1/Nco using primers Aus1-3′/Xba (5′-GCTCTAGATTGGTTCTTTTAAGTGTAGCC-3′) and M4 (5′-GTTTTCCCAGTCACGAC-3′), and a 0.5-kb *Xba*I/*Sph*I DNA fragment was cloned into the *Xba*I/*Hpa*I sites of pSP-luc+. Plasmids pR1-5′-XbS and pR1-5′ were constructed by cloning the *Sac*I/*Nco*I DNA fragment of pURC1/Nco into the *Nde*I/*Nco*I sites of pR1-XbS and pSP-luc+, respectively. To construct pR1-5′-XbSdSB, pR1-5′-XbS was digested with *Sac*II and *Bst*PI, blunt ended with T4 DNA polymerase, and religated. Plasmids pID1, pID2, p3′D1, p3′D2, p3′D3, and p3′D4 were constructed by ligation of *Sac*II/*Sma*I-digested pR1-5′-XbS with a similarly digested PCR fragment that had been generated from pR1-5′-XbS with primers ID1 (5′-TCCCCGCGGAACCGGCATCGGACCCTGGG-3′) plus 3′R/C1 (pID1), ID2 (5′-TCCCCGCGGCTAGCGTATTAATAGGTCGC-3′) plus 3′R/C1 (pID2), Aus1-3′/Xba plus 3′D1 (5′-CCCCCGGGAATATTCTCCTGTTTTATTAAC-3′) (p3′D1), Aus1-3′/Xba plus 3′D2 (5′-TCCCCCGGGTTTTCTACTCTCAGGGGCC-3′) (p3′D2), Aus1-3′/Xba plus 3′D3 (5′-TCCCCCGGGAAAAAGGAAATAACTACAAC-3′) (p3′D3), and Aus1-3′/Xba plus 3′D4 (5′-TCCCCCGGGTCTTCCGACAACGACGTGCGC-3′) (p3′D4), respectively. Plasmid p3′TE-DR1/Lm1 was constructed as follows. Two DNA fragments were amplified by PCR from pR1-5′-XbS using two primer sets, Luc+3′FW (5′-TGTGGACGAAGTACCGAAAGGTCTTACCGG-3′) plus Aus1/3′TE-Lm1− (5′-GGTACCTG**GGAAA**CAGGGTCCGATGCCGG-3′) and Aus1/3′TE-Lm1+ (5′-G**TTTCC**CAGGTACCTAGCGTATTAATAGG-3′) plus Luc+3′RV (5′-GCAGCCGAACGACCGAGCGCAGCGAGTCAG-3′). Both DNA fragments were denatured and annealed, and the resulting mixture was used as a template for PCR. The newly synthesized DNA duplex was amplified by PCR using primers Luc+3′FW and Luc+3′RV. The amplified 0.7-kb DNA fragment was digested with *Xba*I and *Sma*I and cloned into the corresponding enzyme sites of pR1-5′-XbS. Plasmid pRC1mL1 was constructed by cloning the *Sac*II/*Sma*I DNA fragment of p3′TE-DR1/Lm1 into the corresponding sites of pRC1|G. Plasmid pLUCpA30 was constructed by exchanging the *Renilla* Luc (R-Luc) gene of pSP64-RLUC with a firefly Luc gene. Plasmid pLUCpA60 was generated by ligating the *Hin*dIII/*Sac*I DNA fragment of pLUCpA30 into *Hin*dIII/*Acc*I-digested p3′-8, which contains a 60-nt poly(A) tract (11). All constructs were verified by sequencing with an ABI 310 automated sequencer.

RNA preparation. RNA transcripts were synthesized in vitro from the linearized vector with T7 or SP6 RNA polymerase in the absence or presence of the cap structure analog (m^7GpppG; New England Biolabs, Beverly, Mass.) and purified with a Sephadex G-50 fine column (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The RNA concentration was determined spectrophotometrically, and its integrity was verified by 1% agarose gel electrophoresis. RCNMV RNA1 and RNA2 were transcribed from *Sma*I-linearized pRC1|G and pRC2|G, respectively, with T7 RNA polymerase. RC1mL1 was transcribed from *Sma*I-linearized pRC1mL1 with T7 RNA polymerase. The genomic RNA of a tobamovirus, tobacco mild green mosaic virus (TMGMV), was transcribed from *Sal*I-linearized pTGKJW (35) with T7 RNA polymerase. Control mRNAs, R-Luc mRNA, and firefly Luc mRNA were transcribed from *Eco*RI-linearized pSP64-RLUC and pLUCpA30, respectively, with SP6 RNA polymerase. Luc mRNA which contains a 60-nt poly(A) tract was transcribed from *Eco*RI-linearized pLUCpA60 with T7 RNA polymerase. Several Luc mRNAs which contained RCNMV-derived sequences were transcribed as follows. R1-5′-NS, R1-5′-XhS, R1-5′-MS, R1-5′-XbS, R1-5′-SS, R1-5′-XbSdSB, ID1, ID2, 3′D1, 3′D2, 3′D3, 3′D4, and 3′TE-DR1/Lm1 (named for their parent plasmids minus the "p" prefix) were transcribed from *Sma*I-linearized plasmids with T7 RNA polymerase. R1-5′ was transcribed from *Eco*RI-linearized pR1-5′ with T7 RNA polymerase, R1-XbS was transcribed from *Sma*I-linearized pR1-XbS with SP6 RNA polymerase, and R1-5′-XbB was transcribed from *Bst*PI-linearized pR1-5′-XbS with T7 RNA polymerase.

Protoplast transfection. Cowpea plants (*Vigna unguiculata* cv. California Blackeye) were grown as previously described (6). Cowpea protoplasts were prepared from 12- to 16-day-old plants essentially as previously described (17), except that 0.5 M mannitol was used as an osmotic stabilizer in all solutions to which the protoplasts were exposed. Transfection and incubation of protoplasts were carried out as previously described (25).

Dual-Luc assay. Protoplasts (3.0×10^5 cells) transfected with 2.0 pmol of Luc mRNA and 0.7 pmol of R-Luc mRNA were incubated at 17°C for 6 h. Cells were lysed in 500 μl of passive lysis buffer (Promega) and subjected to one or two freeze-thaw cycles to accomplish complete lysis of the cells. Aliquots (20 μl) of cell lysate were assayed with the Dual-Luciferase reporter assay system (Promega). The luminescence of firefly Luc was normalized with luminescence of R-Luc. Each experiment was repeated at least three times with different batches of protoplasts.

Northern blot analysis. Protoplasts (3.0×10^5 cells) inoculated with 1.5 μg of transcripts were incubated at 17°C. Total RNAs extracted from protoplasts were subjected to Northern blot analysis as previously described (4). The RNA probe specific to the RCNMV 3′ UTR was also previously described (34). The digoxigenin (DIG)-labeled RNA probe for plus strand-specific detection of TMGMV (35) was transcribed with T7 RNA polymerase according to the manufacturer's protocols (Roche Molecular Biochemicals, Indianapolis, Ind.). The DIG-labeled RNA probe specific to the Luc gene was transcribed from *Nco*I-linearized pSP-Luc+ (Promega) with T7 RNA polymerase. The RNA signals were detected with a luminescence image analyzer (LAS 1000 Plus; Fuji Photo Film).

Detection of 5′ capped RNA. Virions of RCNMV and TMGMV were purified as previously described (37). Virions of *Broad bean mottle virus* (BBMV) were isolated from *N. benthamiana* plants 10 days postinoculation by the method of Lane (26) with a minor change. Infected-leaf homogenate clarified with chloroform was subjected directly to two cycles of differential centrifugation (low-speed centrifugation of 5,000 × *g* for 20 min and high-speed centrifugation at 100,000 × *g* for 90 min). The final virus pellet was suspended in a storage buffer (50 mM sodium acetate, 10 mM acetic acid, 1 mM Na_2EDTA, 1 mM $MgCl_2$). Virion RNAs of RCNMV, TMGMV, and BBMV were extracted from purified virus (25). Virion RNAs were fractionated on 1.5% agarose gel, transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech), and then cross-linked on the blots by incubation at 80°C for 2 h. After blotting, membranes were immersed in a blocking solution (Roche Molecular Biochemicals) for 60 min and incubated with a 1/5,000-diluted monoclonal antibody against a 2,2,7-trimethyl guanosine-containing cap structure (Synaptic Systems, Göttingen, Germany) for 60 min. After being washed, membranes were reacted with an alkaline phosphatase-conjugated anti-immunoglobulin secondary antibody, followed by visu-

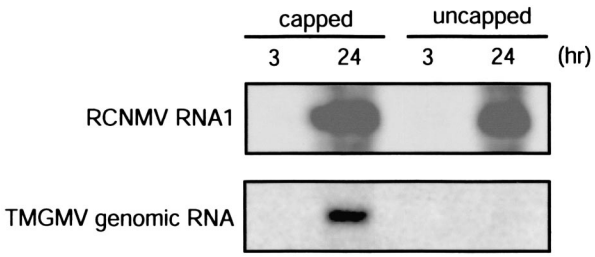

FIG. 1. Amplification of capped and uncapped transcripts of RCNMV RNA1 and TMGMV genomic RNA in cowpea protoplasts, which were incubated at 17°C. Total RNA extracted from protoplasts at 3 and 24 h postinoculation was separated by gel electrophoresis and blotted onto a membrane, which was probed with DIG-labeled RNA specific to RCNMV RNA1 or TMGMV genomic RNA.

alization with CDP-Star (Roche Molecular Biochemicals). Chemiluminescence was detected with a luminescence image analyzer (LAS 1000 Plus). Total RNAs blotted onto a membrane were detected with methylene blue staining (14). 5′ rapid amplification of cDNA ends (RACE) analysis was performed as previously described (34).

RESULTS

Virion RNAs of RCNMV lack a 5′ cap structure. It was previously reported that genomic RNAs of RCNMV, both RNA1 and RNA2, had an m^7GpppA cap structure at the 5′ ends (29, 49). However, the fact that uncapped in vitro transcripts of RCNMV RNA1 and RNA2 still showed infectivity comparable to that of capped transcripts in *N. benthamiana* (50) and in cowpea protoplasts (Fig. 1) indicated that the 5′ cap structure was not needed for viral RNA replication in RCNMV. In contrast, the 5′ cap structure is necessary for the replication of TMGMV, a member of the genus *Tobamovirus*, which has a capped genome (Fig. 1). These results led us to reexamine whether virion RNAs of RCNMV were capped.

To address this question directly, we used monoclonal antibodies that specifically reacted with the m^7G cap (3). RCNMV virion RNAs were fractionated on a denaturing gel, transferred to a membrane, and reacted with the cap structure-specific antibody (Fig. 2A) and stained with methylene blue to detect total RNAs (Fig. 2B). As controls, virion RNAs of TMGMV and BBMV were also fractionated on the same gel. Genomic RNA of TMGMV reacted positively to the cap structure-specific antibody (Fig. 2A). BBMV RNA1, -2, -3, and -4, which have the m^7GpppA cap structure at each 5′ end (5, 41), also reacted positively to the cap structure-specific antibody (Fig. 2A). However, the antibody did not react with virion RNA1 and -2 of RCNMV (Fig. 2A). This indicated that a detectable 5′ m^7GpppA cap structure was not present in RCNMV virion RNAs. The lack of 5′ cap structure in RCNMV RNAs was further confirmed by sequencing the products obtained by 5′ RACE with RCNMV virion RNAs in which cytidine residues complementary to the m^7G in the cap structure, if present, were not incorporated into the viral RNA 5′ ends of the RACE products. For *Brome mosaic virus*, *Striped jack nervous necrosis virus*, and TMGMV, which have capped 5′ ends, a cytidine residue complementary to the m^7G in the cap structure was incorporated into the 5′ RACE products (20; H. Hamada and T. Okuno, unpublished results). From these results, we conclude that RNA1 and RNA2 of RCNMV packaged into virus particles do not have detectable 5′ cap structures.

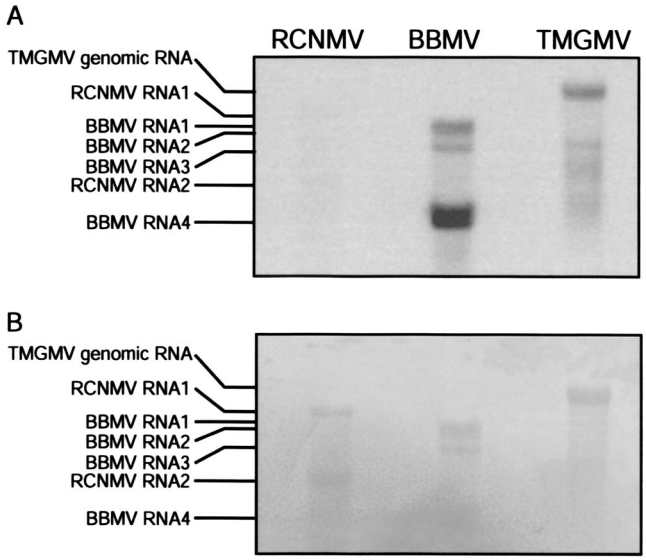

FIG. 2. Immunodetection of capped RNA with a cap-specific antibody. (A) Virion RNAs of TMGMV, BBMV, and RCNMV were fractionated on a denatured agarose gel, transferred onto a Hybond N+ membrane, and reacted with a cap-specific antibody. The concentration of each virion RNA was adjusted to load the same molar amount (1.5 pmol) of TMGMV genomic RNA, BBMV RNA3, and RCNMV RNA1. Identities of the RNAs are indicated on the left. (B) Total virion RNAs blotted onto the membrane used for immunoassay were detected with methylene blue staining.

The 3′ UTR of RCNMV RNA1 enhances cap-independent translation. It was expected that genomic RNAs of RCNMV would contain an element(s) responsible for cap-independent translation. To identify the sequence element(s) of RCNMV RNA1 involved in cap-independent translation, translational activity was quantitatively evaluated with the Dual-Luciferase reporter assay system (Promega). Plasmids with the firefly Luc gene placed between the complete 5′ UTR of RNA1 and a series of other regions of RNA1, including the 3′ UTR, were constructed (Fig. 3). Equimolar capped and uncapped in vitro transcripts from these plasmids were transfected into cowpea protoplasts together with capped and polyadenylated R-Luc mRNA. The luciferase activity was measured after 6 h of incubation at 17°C, an optimum temperature for RCNMV replication (34). The luminescence of R-Luc was used as an internal control.

Translational activity of an uncapped control Luc mRNA containing only vector-derived sequences and a poly(A) tail of 30 nt was less than 0.6% of that of a corresponding capped control Luc mRNA (Fig. 3, pA). Extending a poly(A) tail to 60 nt in the control Luc mRNA resulted in 1.3-fold increase in cap-dependent translational activity compared to that of capped Luc mRNA with a poly(A) of 30 nt but did not increase translational activity of uncapped Luc mRNA (data not shown). Capped R1-5′-NS, in which the Luc gene was inserted between the 5′ UTR and p27 ORF of RCNMV RNA1, produced a fourfold increase in activity compared to capped control Luc mRNA. In contrast, uncapped R1-5′-NS gave an over-200-fold increase in activity compared to uncapped control Luc

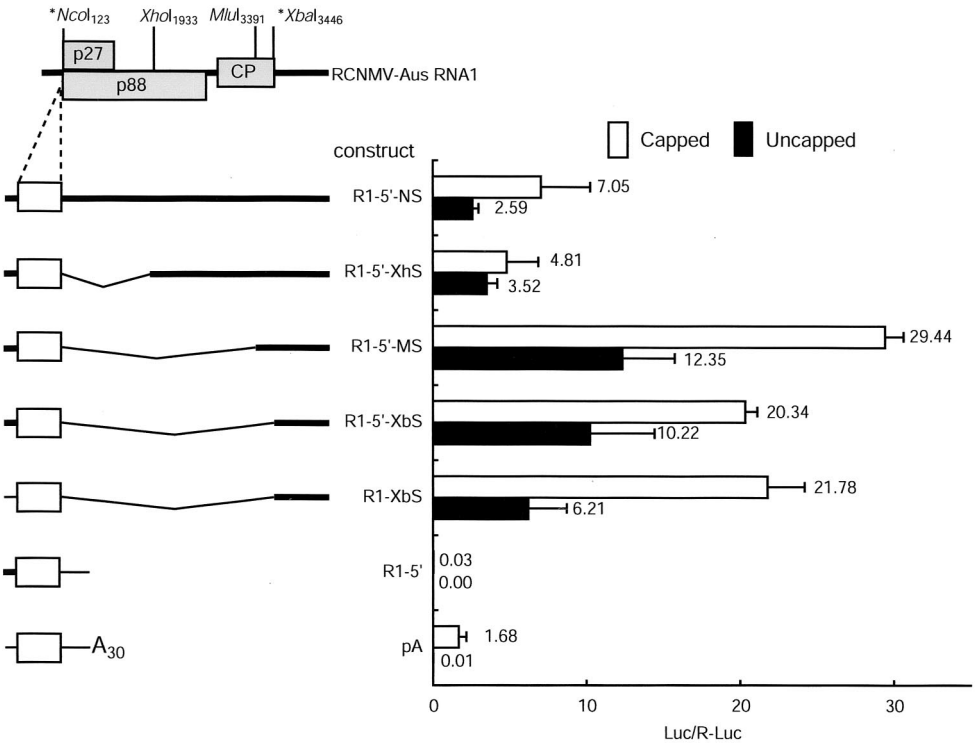

FIG. 3. Mapping of the RCNMV RNA1 sequences involved in cap-independent translation in vivo. Luc mRNAs containing various parts of RCNMV RNA1 diagrammed to the left were cotransfected with R-Luc mRNA (internal control) into cowpea protoplasts. The genome organization and restriction enzyme sites of RCNMV RNA1 are shown at the top. Asterisks, artificially introduced restriction enzyme sites; shaded boxes, ORFs for p27, p88, and CP; boldface black line, UTR. Maps below the genome depict transcripts coding for firefly Luc (open box) containing the viral sequence (boldface lines), vector sequence (thin lines), and poly(A) tails (A_{30}) in their UTRs. Bent lines, deleted regions. Firefly Luc activity from capped transcripts (white bars) and uncapped transcripts (black bars) for each construct are presented as firefly Luc/R-Luc ratios. Error bars, standard errors. Assays were performed at least three times.

mRNA (Fig. 3). These results indicate that RCNMV RNA1 has nucleotide sequences that enable uncapped mRNAs to be translated.

To define the sequence(s) of RNA1 required for cap-independent translation, Luc mRNAs having various regions of RNA1 sequences in their 3′ UTRs were tested for translational activity. All Luc mRNAs having the 3′ UTR of RNA1 were efficiently translated in the absence of a cap structure (Fig. 3). Interestingly, R1-5′-MS and R1-5′-XbS with large deletions from *Nco*I_{123} to *Mlu*I_{3391} or to *Xba*I_{3446} yielded enhanced translational activity in both cap-independent and cap-dependent manners compared to R1-5′-NS or R1-5′-XhS (Fig. 3). Spacing between the initiation codon of the Luc gene and the 3′ UTR of RCNMV RNA1 might affect cap-independent translational activity. Alternatively, nucleotide sequences of RNA1 between the *Xho*I_{1933} and *Mlu*I_{3391} sites might negatively affect translational efficiency. It is also possible that deletions of the viral sequences increase RNA stability.

The 5′ UTR of RNA1 alone did not confer translational activity to Luc mRNA with no poly(A) tail either in the presence or absence of the 5′ cap structure (Fig. 3, R1-5′). In contrast, the 3′ UTR of RNA1 alone significantly enhanced cap-independent translational activity (Fig. 3, R1-XbS), although the activity was approximately 60% that of R1-5′-XbS, which has both precise 3′ and 5′ UTRs of RNA1. These results indicate that the 3′ UTR of RCNMV RNA1 is a primary determinant for cap-independent translation.

Nucleotide sequences in the 3′ UTR of RCNMV RNA1 required for cap-independent translation. To delimit regions in the 3′ UTR of RNA1 required for cap-independent translation, we evaluated the translational activity of Luc mRNAs which contain the precise viral 5′ UTR and the Luc gene followed by viral 3′ UTRs with different regions deleted. Deletion from the *Xba*I site to the *Sac*II site (nt 3446 to 3568) in the viral 3′ UTR (R1-5′-SS) reduced translational activity of uncapped mRNA to 10% of that of uncapped R1-5′-XbS (Fig. 4A). Although deletion between nt 3571 and nt 3595 (ID1) also greatly reduced translational activity of uncapped mRNA to 2.9% of that of uncapped R1-5′-XbS, the activity was still 50-fold higher than that of uncapped control Luc mRNA (Fig. 4A). An additional deletion, extending to nt 3625 (ID2) or to nt 3672 (R1-5′-XbSdSB) abolished cap-independent translational activity, whereas capped constructs of these mRNAs showed 40 to 70% of the translational activity of capped R1-5′-XbS (Fig. 4A).

Deletion of the 3′-proximal stem-loop structure (nt 3862 to 3887), which is almost identical between RNA1 and RNA2 (29, 49), did not significantly reduce cap-independent translational activity (Fig. 4A, 3′D1). Further deletions extending to nt 3835 (3′D2) and 3761 (3′D3) reduced cap-independent transla-

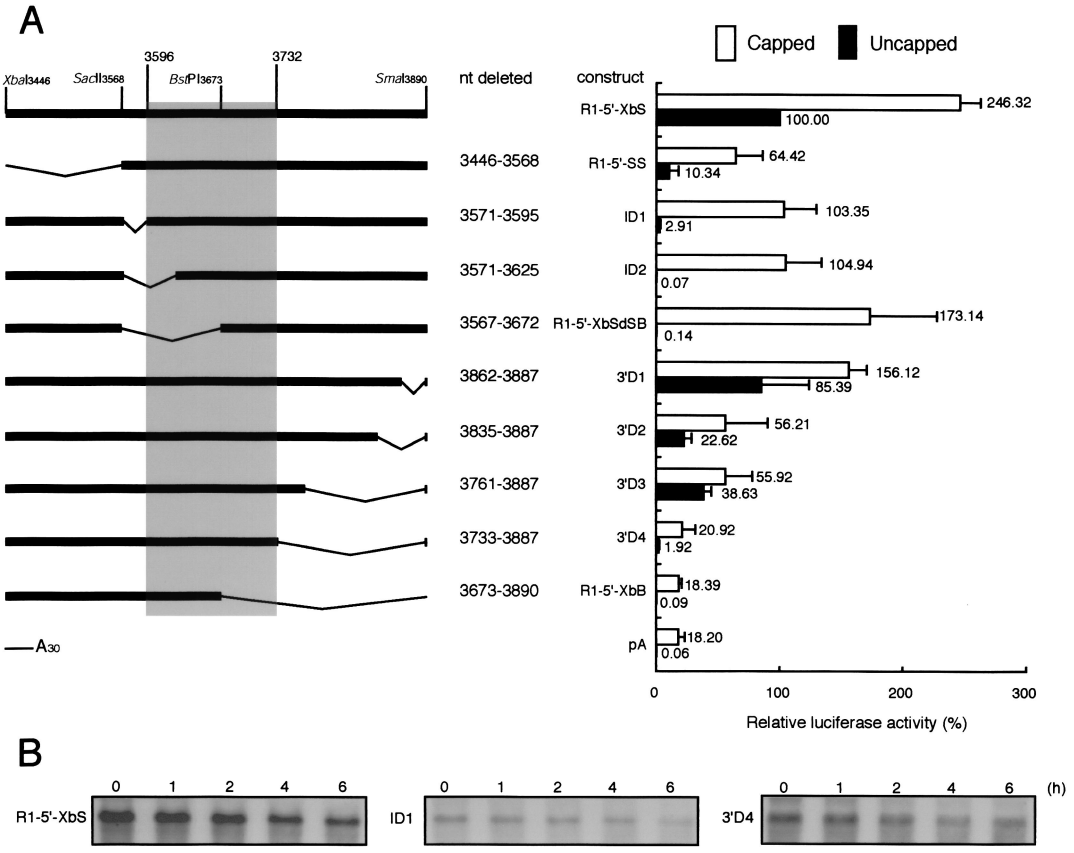

FIG. 4. (A) Deletion mapping of the 3′ UTR sequence of RCNMV RNA1 involved in cap-independent translation in vivo. Transcripts containing the precise viral 5′ UTR and the Luc gene followed by various parts of the 3′ UTR are diagrammed on the left. Restriction enzyme sites used for deletion are shown at the top. Boldface lines and thin lines, virus-derived and vector-derived sequences, respectively; bent lines, deleted regions. The 3′TE-DR1 (nt 3596 to 3732) is shaded. Firefly Luc activity from capped transcripts (white bars) and uncapped transcripts (black bars) for each construct are expressed as percentages of uncapped R1-5′-XbS activity; error bars, standard errors. Assays were performed at least three times. (B) Stability of RNAs in cowpea protoplasts transfected with uncapped R1-5′-XbS, ID1, or 3′D4. Total RNA extracted from protoplasts at the indicated times after transfection was separated by gel electrophoresis, blotted onto a membrane, and probed with DIG-labeled RNA specific to Luc mRNA.

tional activity, but the activities relative to those of corresponding capped mRNA remained high (Fig. 4A). Although deletion to nt 3733 (3′D4) greatly reduced translational activity of uncapped mRNA to 1.9% of that of uncapped R1-5′-XbS, the activity was still 30-fold higher than that of uncapped control Luc mRNA (Fig. 4A). Deletion to the *Bst*PI site from the 3′ end (nt 3673 to 3890) abolished cap-independent activity (Fig. 4A, R1-5′-XbB). It was possible that the deletions introduced into 3′ UTRs altered mRNA stability and affected cap-independent translational activity. To test this, uncapped RNA transcripts of R1-5′-XbS, ID1, and 3′D4 were transfected into cowpea protoplasts under the same conditions as those for the Luc assay. RNA degradation was monitored by Northern blot hybridization using an RNA probe that specifically hybridized with Luc mRNA. Results showed that accumulation patterns of the mRNAs with various deletions did not significantly differ from each other (Fig. 4B). Similar results were obtained with uncapped ID2 and R1-5′-XbB (data not shown). These results suggest that differences in translational activity of uncapped mRNA were not caused by differences in stability of mRNAs. Taken together, results obtained here suggest that sequences necessary for highly efficient cap-independent translation of RNA1 in vivo lie between nt 3446 and 3760 and that sequences between nt 3596 and 3732 are indispensable for cap-independent translation. Hereafter, we refer to this region (nt 3596 to 3732; highlighted in Fig. 4A) as a 3′ translation element of *Dianthovirus* RNA1 (3′TE-DR1).

A stem-loop structure conserved among several plant RNA virus genomes is responsible for cap-independent translation of RCNMV RNA1. To identify functional domains in 3′TE-DR1 required for cap-independent translation, we predicted its secondary structure by using the program Dynalign (30). The 3′TE-DR1 is predicted to have five stem-loop structures (SL1, SL2, SL3, SL4, and SL5) and base pairing (stem) between the ends of the region (Fig. 5A). SL1 is conserved in all dianthoviruses, RCNMV (34, 49), *Sweet clover necrotic mosaic virus* (10), and *Carnation ringspot virus* (42). Interestingly, as shown in Fig. 5B, the SL1 of RCNMV RNA1 is almost identical to one of three stem-loop structures predicted for the 3′ UTR of BYDV genomic RNA (11). The BYDV region, including the three stem-loop structures (TE105), facilitates cap-independent translation in vitro (11, 47). The loop sequence of SL-I, GGAAA, fits the consensus of a GNR(N)A pentaloop, a structure that forms a GNRA tetraloop fold (16). In BYDV,

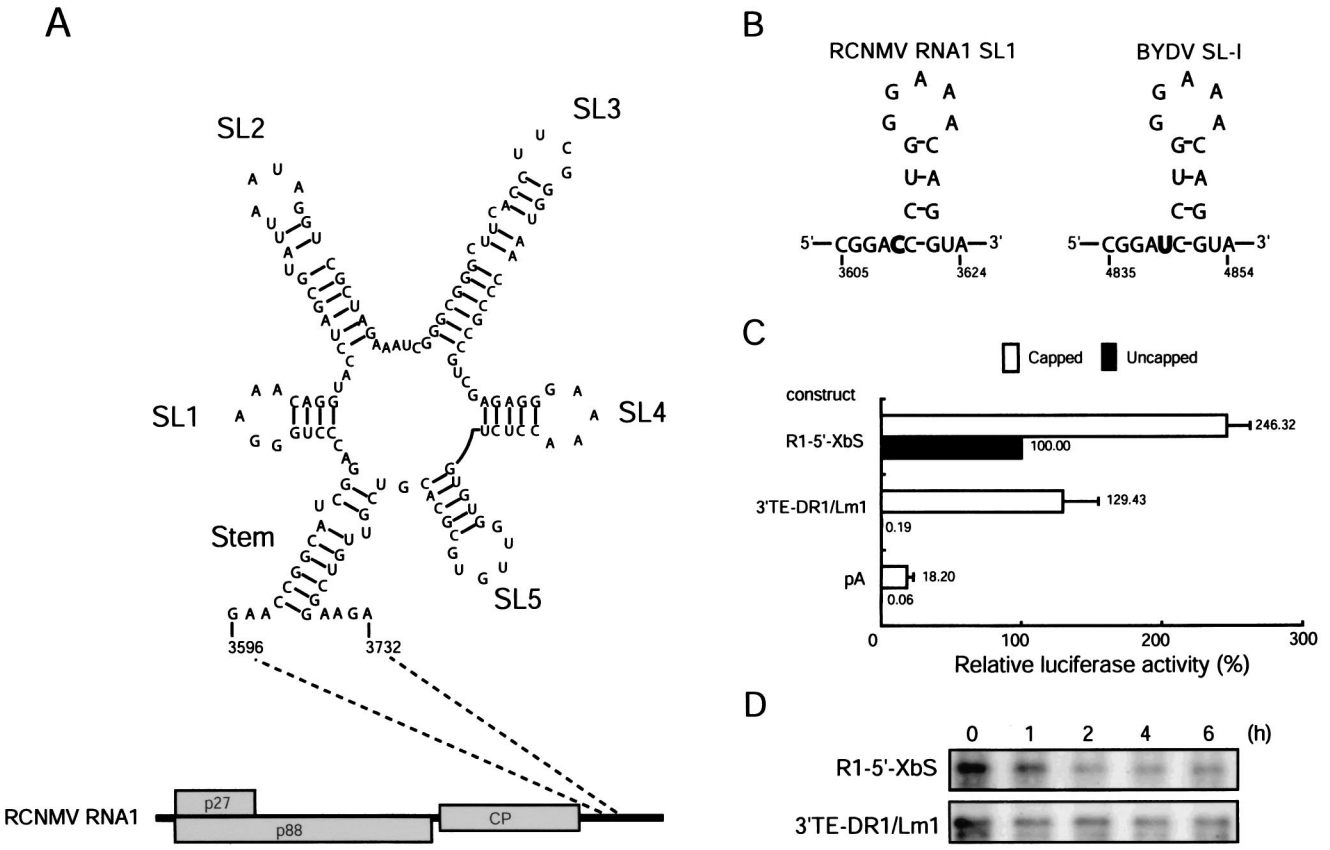

FIG. 5. (A) Secondary structure predicted by the computer algorithm Dynalign (30) for the 3′TE-DR1 of RCNMV (Australian strain) determined in concert with the 3′ UTR of the other RCNMV strain (RCNMV-Can) RNA1 (34). The calculation was performed with a maximum distance of 25, a gap penalty of 0.4 kcal mol^{-1}, and no single-base-pair inserts allowed. Numbering indicates nucleotide positions in RCNMV RNA1. (B) Secondary structure of SL1 of RCNMV RNA1 (left) and SL-I of BYDV (right). Boldface, nucleotide differences between RCNMV and BYDV. (C) Translational activity of the 3′TE-DR1 mutant (3′TE-DR1/Lm1) in vivo. For others, refer to Fig. 4A. (D) Stability of RNAs in cowpea protoplasts transfected with uncapped R1-5′-XbS or the SL1 mutant (3′TE-DR1/Lm1). For others, refer to Fig. 4B.

changing nucleotide sequences from GGAAA to UUUCC in the loop corresponding to RCNMV SL1 eliminates cap-independent translational activity in vitro (11). These previous results led us to test the effects of the same mutation in the SL1 of 3′TE-DR1 on translational activity. As shown in Fig. 5C, 3′TE-DR1/Lm1 possessing this mutation in R1-5′-XbS was not translated in the absence of the 5′ cap structure, although it was efficiently translated in the presence of the 5′ cap structure.

It is possible that 3′TE-DR1, including SL1, stimulates gene expression by increasing RNA stability. The RNA degradation pattern of uncapped RNA transcripts of R1-5′-XbS and 3′TE-DR1/Lm1 was monitored by Northern blot hybridization. The RNA transcripts with either the wild-type or the mutant SL1 did not differ significantly in degradation rate (Fig. 5D). These results and the results shown in Fig. 4 suggest that 3′TE-DR1 and sequences around 3′TE-DR1 are responsible for cap-independent translation.

The 3′TE-DR1 is required for replication of uncapped full-length RCNMV RNA1. To investigate whether the cap-independent translational nature observed in Luc mRNAs having 3′ UTR sequences of RCNMV RNA1 is related to the replication of RCNMV RNA1, we introduced the SL1 mutation into wild-type full-length RCNMV RNA1. Cowpea protoplasts were inoculated with the mutant RNA1 (RC1mL1), and wild-type RNA1 transcripts were synthesized with or without cap analogs. Total RNA was extracted from protoplasts at 3 and 24 h postinoculation and analyzed by the Northern blotting method using an RCNMV RNA1-specific probe. Uncapped transcripts of wild-type RNA1 were amplified to high levels, whereas RC1mL1 was not amplified in the inoculation of uncapped RC1mL1 transcripts (Fig. 6). Interestingly, inoculation with capped RC1mL1 transcripts did not cause viral RNA amplification, even though the SL1 mutation did not strongly affect translation efficiency in capped R1-5′-XbS (Fig. 5D). It is possible that the progeny RNAs from RC1mL1 were not translated in the absence of the 5′ cap and that the amount of viral replicases translated from the capped inoculum of RC1mL1 might be insufficient to produce progeny virus RNAs to the level detectable by Northern blot hybridization. Another possibility is that the mutation in SL1 knocked out a *cis*-acting sequence required for RNA replication as well as for cap-independent translation.

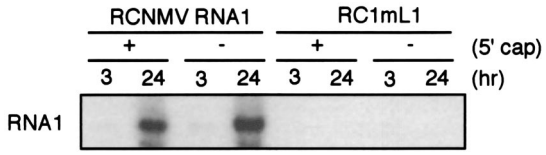

FIG. 6. Replication of RCNMV RNA1 and the SL1 mutant (RC1mL1) in cowpea protoplasts, which were incubated at 17°C. Total RNA extracted from protoplasts at 3 and 24 h postinoculation was separated by gel electrophoresis, blotted onto a membrane, and probed with DIG-labeled RNA specific to RCNMV RNA1.

DISCUSSION

Our results show that genomic RNAs of RCNMV, RNA1 and RNA2, did not have the 5′ cap structure (Fig. 2). This is supported by high infectivity of uncapped in vitro transcripts of RCNMV RNA1 shown in this study (Fig. 1), by similar results in a previous study (50), and also by the presence of 3′TE-DR1 found in the 3′ UTR of RCNMV RNA1. 3′TE-DR1 confers cap-independent translation initiation on Luc mRNAs in cowpea protoplasts (Fig. 5C). We also showed that a mutation in 3′TE-DR1 which abolished the cap-independent translational activity by 3′TE-DR1 abolished replication of the uncapped RCNMV RNA1 in protoplasts (Fig. 6). These results suggest that the RCNMV RNA1 utilizes the cap-independent translational mechanism.

The cap structures are present at the 5′ ends of nearly all eukaryotic cellular mRNAs and many viral mRNAs (8). The 5′ cap structure possesses several functions of biological significance, especially in the translational initiation step. According to the ribosome scanning model of eukaryotic translation initiation, the initiation factor eIF4F specifically recognizes the 5′ cap structure and, with the help of other initiation factors such as eIF3, recruits the 43S ribosomal subunit initiation complex that scans 5′ to 3′ along the mRNA (24, 31). A growing body of evidence shows that the genomic RNAs of several viruses lack the 5′ cap structure and therefore employ alternative strategies for translational initiation. An example is the internal entry of ribosomes, which is mediated by internal sequences in the 5′ UTR of mRNAs, called IRESs. The picornavirus IRES recruits 40S ribosomal subunits and various canonical and noncanonical translational initiation factors to promote translation in the absence of the 5′ cap structure (13, 46). Another cap-independent translational initiation mechanism is proposed for several plant viruses (32, 40, 47, 48). For example, BYDV, a member of the genus *Luteovirus* closely related to RCNMV in its RdRp gene, possesses 105-nt cap-independent translational elements (TE105) in the 3′ UTR of genomic RNA, which facilitates cap-independent translation in vitro (11, 47). TE105 folds into a cruciform structure with three stem-loops (SL-I, SL-II, and SL-III) (11). SL-I is conserved in the 3′ UTRs of genomic RNA of luteoviruses and *Tobacco necrosis virus*, as well as RNA1 of dianthoviruses (47). The cap-independent translational element in RCNMV RNA1, 3′TE-DR1, contains this conserved stem-loop structure (Fig. 5B). Changing the conserved pentaloop of GGAAA to UUUCC in this structure of RCNMV RNA1 eliminated the cap-independent translational activity (Fig. 5C), as reported for BYDV (11). These results suggest that the conserved stem-loop structure can function in the cap-independent translational initiation of distinct viruses belonging to different genera. This conserved loop of GGAAA fits the consensus of a GNR(N)A pentaloop, a structure that forms a GNRA tetraloop fold (16). The GNRA tetraloop structure in *boxB* of nascent mRNA of bacteriophage λ is bound specifically by the bacteriophage λ N protein and host NusA protein in the transcription antitermination process (28). Thus, it was considered that the conserved stem-loop structure might be involved in recruiting one or several proteins involved in cap-independent translation in a fashion similar to that of *boxB* of the nascent mRNA of bacteriophage λ (11).

Despite similarities in cap-independent translation elements in the 3′ UTRs of RCNMV RNA1 and BYDV genomic RNA, there are apparent differences in the translational mechanism between RCNMV and BYDV. The 5′ UTR of RCNMV RNA1 was not required for cap-independent translation of reporter mRNA conferred by the viral 3′ UTR. In contrast, both the 5′ UTR and 3′ UTR of the genomic RNA of BYDV were required for cap-independent translation of reporter mRNA (47). A recent report has demonstrated that direct base pairing between the 5′ UTR and 3′ UTR of BYDV RNA is necessary for cap-independent translation initiation (12). It has been demonstrated that communication between the 5′ cap structure and poly(A) tail, resulting in circularized mRNA, enhances the translation of cellular mRNAs (43). In addition, uncapped polyadenylated (2, 33) and uncapped nonpolyadenylated viral mRNAs (19) are likely to form a closed-loop structure. Therefore, it is possible that RCNMV RNA1 also forms circular structures by a mechanism different from that used by BYDV. Other than for SL1, 3′TE-DR1 shows no apparent similarity to the TE105 of BYDV RNA. Instead of the reported direct base pairing between 5′ UTR and 3′ UTR in BYDV, the sequence element(s) and/or secondary structure(s) in 3′TE-DR1 could be involved in the formation of a structure that includes a closed loop to facilitate cap-independent translation. Chemical structure analysis and structure-directed mutagenesis will shed light on the function of 3′TE-DR1.

Besides 3′TE-DR1, vicinities of 3′TE-DR1 (between nt 3446 and 3595 and between nt 3733 and 3760) also enhance cap-independent translational activity, possibly by stabilizing RNA structure and/or supporting the function of 3′TE-DR1 without affecting apparent RNA stability, since deletion from nt 3446 to 3595 and from nt 3733 to 3760 significantly reduced cap-independent translational activity (Fig. 4A, R1-5′-SS, ID1, and 3′D4). In contrast, deletion from nt 3862 to 3887 did not significantly reduce cap-independent translational activity (Fig. 4A, 3′D1). This region includes stem-loop structures for RNA1 and RNA2 of RCNMV which are almost identical (29, 49). These sequence and structural similarities imply that the stem-loop structure is likely to be a *cis*-acting element required for viral RNA replication. It was previously reported that mutation in the stem-loop structure of RNA2 abolished the replication of RNA2 (45). In addition, single nucleotide changes from U to C in the left arm of the stem-loop structure in the RCNMV Canadian strain (RCNMV-Can) RNA1 were sufficient to change the temperature-sensitive replication of RCNMV-Can and to direct virus RNA replication at nonpermissive temperatures (34). These results suggest that, while the

3′-proximal stem-loop structure of RNA1 is not important for the translation of RdRp, it could be important for the replication of RNA1 by functioning as the binding site for RdRp.

RCNMV RNA1 encodes viral replication proteins p27, p88, and CP; p27 and p88 are translated directly from RNA1, whereas CP is translated from a subgenomic RNA transcribed from RNA1. Because these two RNA species contain the same 3′ UTR, it is considered that CP would also be translated from subgenomic RNA in a cap-independent manner. Another virus-encoded protein, MP, is translated from RNA2. In this study, we showed that RNA2, as well as RNA1, did not have detectable 5′ cap structures (Fig. 2). These results imply that MP encoded by RNA2 would also be translated in a cap-independent manner. However, a conserved stem-loop structure involved in the cap-independent translational initiation of RCNMV RNA1, as well as BYDV, does not exist in RCNMV RNA2. In addition, Luc mRNA in which the firefly Luc gene was inserted between the 5′ UTR and MP ORF of RCNMV RNA2 was not translated in the absence of the 5′ cap structure (H. Mizumoto and T. Okuno, unpublished results). These results suggest that RNA2 employs translational strategies different from those employed by RNA1. This difference of translation initiation strategy between RNA1 and RNA2 of RCNMV may be involved in controlling the temporal expression and/or total expression level of viral proteins.

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