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SHORT COMMUNICATION

Mapping of the Red Clover Necrotic Mosaic Virus Subgenomic RNA

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The red clover necrotic mosaic dianthovirus capsid protein is expressed *in vivo* from a subgenomic RNA (sgRNA) identical to the 3′-terminal 1.5 kb of RNA-1. The 5′ leader sequence of the capsid protein sgRNA is 62 nucleotides, contains a 14-nucleotide putative promoter sequence homologous to the RNA-1 5′ terminus, and exhibits a high level of similarity with the tobacco mosaic virus 5′ leader translational enhancer element Ω. Analysis of the RNA-1 secondary structure, in the region adjacent to the position where the 5′ end of the sgRNA was mapped, predicts a stable stem–loop which includes the putative sgRNA promoter element. It is suggested that this structure is important for recognition of the sgRNA transcriptional initiation from the full-length negative-sense RNA-1.

The red clover necrotic mosaic dianthovirus (RCNMV) genome is composed of two single-stranded RNA components, RNA-1 (3.9 kb) and RNA-2 (1.45 kb), which are encapsidated together into 30- to 32-nm icosahedral virions by 180 copies of the 37-kDa capsid protein (*1–3*).

The 3′-proximal open reading frame (ORF) on the polycistronic RNA-1 encoding the 37-kDa viral capsid protein (CP) is expressed *in vivo* from a subgenomic RNA (sgRNA). A 1.5-kb sgRNA of nearly the same size as genomic RNA-2 was detected in protoplasts infected by RNA-1 alone (*4*). Northern blot analysis indicated that the sgRNA and the double-stranded (ds) form of the sgRNA hybridized to genomic RNA-1 (*5*). The accumulation of the CP sgRNA *in vivo* is very low and can only be detected sporadically by Northern blot analysis (S. Lommel, unpublished). However, the sgRNA can be detected consistently in infected plants in its double-stranded form (*5*). The lack of accumulation of the single-stranded form of the CP sgRNA has precluded mapping of the 5′ end of the sgRNA and has made it difficult to explain the large accumulation of CP *in vivo.*

In this paper we map the 5′ end of the RCNMV sgRNA and we hypothesize that a potential stem–loop-forming sequence element is involved in sgRNA synthesis. Furthermore, we identify a sequence element at the 5′ end of the sgRNA that is predicted to have translational enhancement activity, which can explain the high level of CP expression from a minimal level of corresponding sgRNA.

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The dsRNA form of the RCNMV CP sgRNA was isolated and used as a sequencing template to determine its site of initiation relative to genomic RNA-1. RCNMV (Australian isolate) was propagated in *Nicotiana benthamiana* and dsRNA was purified from infected leaves, 8 days postinoculation (dpi), by column chromatography on Whatman CF11 cellulose (*6*). The viral dsRNAs were separated by electrophoresis in 1% agarose gels. dsRNA-1 and dsRNA-2 together with the ds sgRNA were excised from the gels and extracted using the GENECLEAN II Kit (BIO 101, Inc.). Melted dsRNA was used as template in RNA sequencing reactions. The oligonucleotide primer 5′-CCGGTTCCTGGGCTGGG-3′, complementary to RNA-1 nucleotides 2480–2464 (*3*), was added to 1–2 μg dsRNA prior to the dsRNA being denatured by twice heating in a boiling water bath (1 min) followed by quenching in ice. The annealing, labeling, and sequencing reactions were performed according to the U.S. Biochemical RT RNA sequencing kit instructions (Cleveland, OH).

The 5′-terminal sequence of the CP sgRNA is presented in Fig. 1. Run-off sequence both from unfractionated RCNMV dsRNAs and from a preparation containing both ds CP sgRNA and dsRNA-2 clearly illustrates that the sgRNA initiates with an adenosine residue corresponding to RNA-1 nucleotide 2365. Based on location of the run-off, the CP sgRNA was calculated to be 1525 nucleotides, with a 62-nucleotide 5′ leader sequence prior to the CP ORF. Alignment of the 5′ termini of the sgRNA sequence and RNA-1 shows that 13 of the 5′-terminal 14 nucleotides are identical (Fig. 2). This observation supports the hypothesis of Giesman-Cookmeyer *et al.* (*7*) that the 5′-terminal nucleotides are replication

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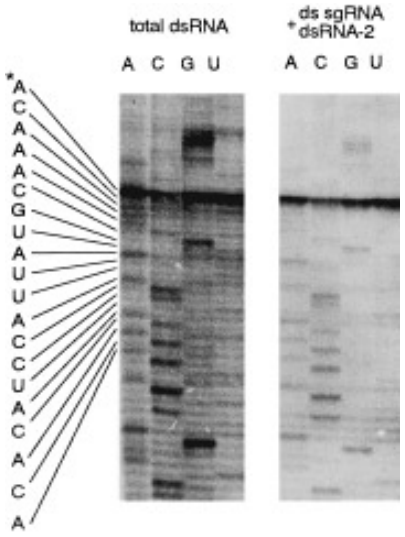

FIG. 1. Run-off RNA sequence analysis of denatured unfractionated and partially separated RCNMV dsRNAs isolated from virus-infected plants. On the left is the run-off sequence from unfractionated RCNMV dsRNAs and on the right is the sequence from a mixture of CP sg- and RNA-2-specific dsRNA. Sequencing reactions were primed with an oligonucleotide complementary to RCNMV RNA-1 nucleotides 2464 to 2480.

signals for the synthesis of (+) strand genomic and subgenomic RNAs.

The 5′ untranslated leader sequence of tobacco mosaic virus RNA (Ω sequence) is known as a universal translation enhancer for both prokaryote and eukaryote gene transcripts *in vivo* and *in vitro* (*8–10*). Ω, as well as a deletion of the 25-nucleotide poly(CAA) sequence from the middle of the Ω element (designated ΩΔ3), significantly enhances the translation of β-glucuronidase and chloramphenicol acetyltransferase transcripts in tobacco mesophyll protoplasts (*9*). Alignment of the ΩΔ3 nucleotide sequence with the 5′ leader sequence of the RCNMV sgRNA shows a high level of nucleotide sequence similarity (Fig. 2). This observation may explain why, even though CP sgRNA accumulation is limited *in vivo,* significant quantities of CP and mature virions accumulate (3 μg virus per gram of infected tissue; *11*). The question remains as to why CP sgRNA synthesis and accumulation is low. Possibly, the sgRNA is synthesized efficiently, but its accumulation is limited as a result of rapid digestion and/or low stability. An alternative but less likely possibility is that the CP is also expressed from genomic RNA-1 by internal translational initiation.

By analogy with other single-stranded RNA viruses, it is suggested that the sgRNA is transcribed from the negative-strand copy of full-length genomic RNA-1 (*5*). The 14-nucleotide putative subgenomic promoter element (shaded nucleotides, Figs. 2 and 3) is predicted to reside within a stable stem–loop structure (*12*) (Fig. 3, WT). Since the promoter element resides within the genomic-length negative-sense strand of RNA, minus-sense sequences are presented in Fig. 3. As would be expected, a stable stem–loop structure with a similar stability energy is predicted in the same location in the positive-sense strand of RNA-1 (data not shown). The predicted structures in the positive and negative strands differ only in two CA residues which form GU base pairs in the positive strand.

If the stem–loop structure predicted in Fig. 3 is important for the recognition of polymerase and the synthesis of sgRNA, its destabilization should inhibit or completely abolish this process. To test this hypothesis, we generated five RNA-1 point mutations (M1–M5, see Fig. 3) designed to either destabilize the stem–loop and/or alter the putative promoter sequence without altering the

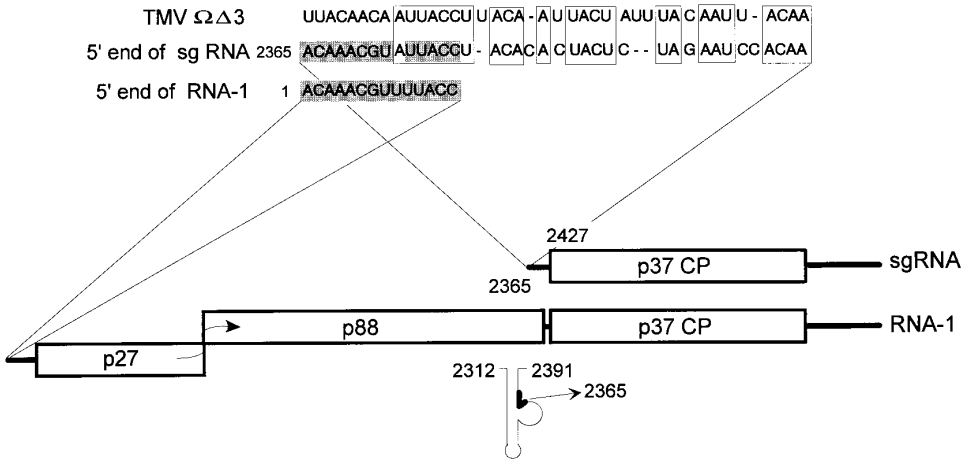

FIG. 2. Genome map of RCNMV RNA-1 and sequence alignments of the 5′ termini of the genomic and sgRNA with the TMV ΩΔ3 translational enhancer element. Identical nucleotides in the sgRNA 5′ terminus and TMV ΩΔ3 element are boxed. Dashes are introduced to effect maximal alignment. Gray-shaded nucleotides at the 5′ ends of RCNMV RNA-1 and the sgRNA identify the nearly identical putative promoter elements. Positions of the aligned sequences relative to the RNA-1 genome map are indicated. Location of the predicted stem–loop and nucleotides involved is presented in their position relative to the RNA-1 map.

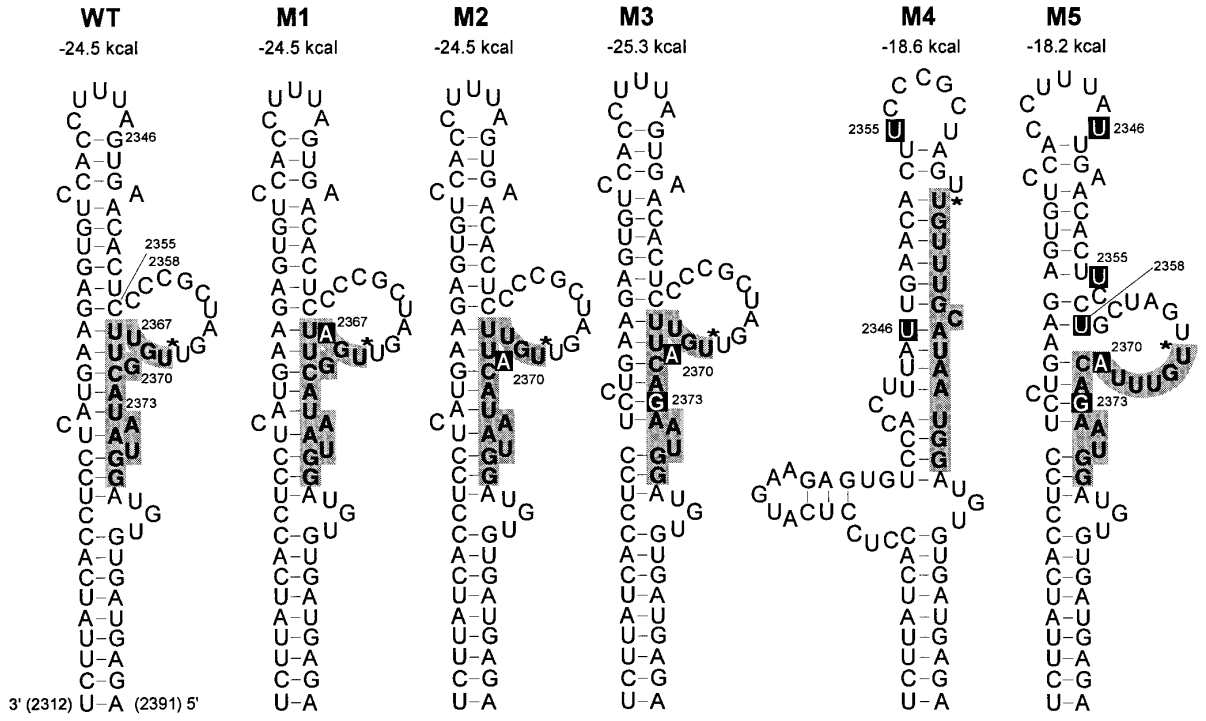

FIG. 3. Computer-predicted stem–loop structure in the region of RCNMV RNA-1 involved in the production of the capsid protein sgRNA. Stem–loop structures are presented with the complementary strand of RCNMV RNA-1. Nucleotide numbers are for the genomic-sense strand. Nucleotides highlighted with gray shading identify the putative subgenomic promoter element. Mutated nucleotides are reverse highlighted. Asterisk identifies the site of sgRNA initiation. Predicted structures of wild-type RNA-1 (WT) and five sets of point mutations (M1 through M5) are presented. Calculated thermal stabilities for each predicted structure are presented.

88-kDa replicase amino acid sequence. The muta-Gene *in vitro* mutagenesis kit (Bio-Rad, Richmond, CA) was used according to the manufacturer's instructions. Mutations were introduced into pRC1-IG (*13*), a plasmid containing a complete cDNA clone of RCNMV RNA-1. Sequence of the mutated constructs was confirmed by DNA sequencing. Plasmid DNAs were purified (*14*) and linearized with *Sma*I. *In vitro* transcription reactions were performed (*15*), and wild-type and mutant transcripts corresponding to RNA-1 and wild-type transcripts of RNA-2 were mixed, resuspended in GKP buffer (50 m*M* glycine, 30 m*M* K_2HPO_4, pH 9.2, 1% bentonite, 1% celite), and inoculated onto *N. benthamiana* plants (*13*). Wild-type RCNMV transcripts formed a systemic infection 5 dpi. Mutants M2, M3, and M4 also formed symptoms on the inoculated leaves as well as a systemic infection 5 dpi (Fig. 4). In contrast, mutants M1 and M5 produced typical symptoms on the inoculated leaves but no systemic symptoms by 14 dpi.

Inoculated and noninoculated leaves from the wild-type and each of the five mutant RCNMV infected plants were harvested. CP accumulation was determined by immunoblotting (*15*) and viral dsRNA accumulation was determined by extraction and by electrophoresis in 1% agarose gels (*6*). Wild-type RCNMV infection yielded three viral-specific dsRNA species corresponding to genomic RNA-1 and RNA-2 and the CP sgRNA in both inoculated (I) and systemic (S) leaves (Fig. 4). The dsRNA electrophoretic profile was the same as that observed by Osman and Buck (*5*). As expected, CP also accumulated in both the I and the S leaves. Mutants M2, M3, and M4 yielded wild-type dsRNA profiles and accumu-

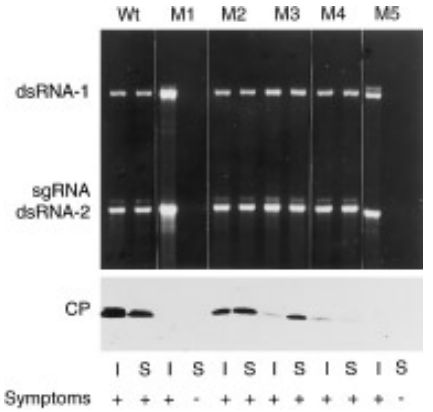

FIG. 4. Agarose gel electrophoresis of dsRNAs and immunoblot of capsid protein from wild-type and mutant RCNMV infected *N. benthamiana* plants. dsRNA and protein were isolated from 0.2 g of tissue from inoculated (I) and noninoculated, systemically (S) infected leaves 8 days postinoculation. Plants were inoculated with wild-type (Wt) RCNMV or each of the five point mutations (M1–M5). A (+) indicates the presence and a (−) indicates the absence of RCNMV infection symptoms at the time of harvest.

lated CP in both the I and the S leaves. While dsRNA accumulation was consistent among M2, M3, and M4, CP accumulation was quite variable. Mutant M2 accumulated near wild-type levels of CP in both the I and the S leaves, whereas with the M3 mutant, relatively little CP accumulated in the I leaves, even though systemic symptoms were apparent (Fig. 4). Mutant M4 exhibited greatly reduced CP accumulation in the I leaves and even less in the S leaves yet still gave a systemic infection. Collectively, these data indicate that mutants M2, M3, and M4 did not affect virus replication and sgRNA synthesis; however, M3 and M4 reduced CP translation.

Mutant M1 and M5 infections produced virus-specific dsRNAs only in the I leaves resulting in no CP accumulation in either the I or the S leaves. In addition, the CP sgRNA species was not detected in either M1- or M5-infected plants (Fig. 4). The M1 and M5 mutations prevented CP sgRNA accumulation and consequently CP accumulation, resulting in no systemic infection. This is consistent with previous observations that RCNMV CP expression (*15*) and apparently virion formation (*16*) are not required for cell-to-cell movement, but are essential for systemic infection. From these observations we conclude that the M1 and M5 mutations disrupted the subgenomic promoter element to the extent that CP sgRNA was not produced at sufficient levels to allow CP expression.

The single point mutation at 2367 constituting the M1 mutant is within the subgenomic promoter element and is not predicted to alter the secondary structure, and yet it abolished sgRNA synthesis eliminating systemic infection. In contrast M2, also a single point mutation at 2370, had no measurable effect on sgRNA or CP accumulation. The two point mutations comprising M3 are both within the subgenomic promoter and had the effect of reducing CP accumulation, but did not affect sgRNA accumulation. The two point mutations constituting M4 do not reside within the putative 14-nucleotide subgenomic promoter element and are predicted to drastically alter the shape and stability of the putative stem–loop structure (Fig. 3). CP sgRNA accumulation was at near to wild-type levels but CP was greatly diminished. The M5 mutation also reduced the stability of the stem–loop, but two of the five nucleotide substitutions were in the putative subgenomic promoter.

This analysis allows us to conclude that the complete subgenomic promoter is approximately 80 nucleotides (2312–2391) composed of the putative stem–loop structure including the core 14-nucleotide element homologous to the 5′ termini of the genomic RNAs (Fig. 2). We hypothesize that both the sequence of the subgenomic promoter element and the stem–loop structure are required for sgRNA synthesis and CP accumulation. It also appears that sequences controlling sgRNA synthesis and CP expression at least partially overlap. Additional mutations will need to be created and studied in order to dissect the subgenomic promoter and the region controlling CP translation.

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