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Alanine Scanning Mutagenesis of a Plant Virus Movement Protein Identifies Three Functional Domains

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Alanine scanning mutagenesis was performed on the red clover necrotic mosaic virus (RCNMV) movement protein (MP), and 12 mutants were assayed in vitro for RNA binding characteristics and in vivo for their ability to potentiate RCNMV cell-to-cell movement. The mutant phenotypes that were identified in vitro and in vivo suggest both that cooperative RNA binding is not necessary for cell-to-cell movement in vivo and that only a fraction of the wild-type RNA binding may be required. The MP mutants defined at least three distinct functional regions in the MP: an RNA binding domain, a cooperative RNA binding domain, and a third domain that is necessary for cell-to-cell movement in vivo. This third domain may be required for targeting the MP to cell walls and plasmodesmata, interacting with host proteins, folding, or possibly binding RNA into a functional ribonucleoprotein complex capable of cell-to-cell movement.

INTRODUCTION

Plant viruses must be able to move from cell to cell in a systemic host to cause disease. Virus infection moves from the initially infected cell to neighboring cells and then throughout the host. The mechanisms of movement known for animal viruses, receptor-mediated endocytosis and cellular fusion, are not available to plant viruses because each plant cell is surrounded by a cell wall. Available evidence suggests that plant viruses move from cell to cell via the intercellular connections known as plasmodesmata (Gibbs, 1976; Atabekov and Dorokov, 1984). Furthermore, this movement generally requires a virus-encoded movement protein (MP) (reviewed by Hull, 1989; Atabekov and Taliansky, 1990; Robards and Lucas, 1990; Deom et al., 1992).

Comparison of MPs from several different groups of plant viruses revealed the presence of conserved amino acid motifs that may reflect a common functional ancestry (Melcher, 1990; Koonin et al., 1991). To date, there have been at least two functions associated with MPs. For tobacco mosaic virus (TMV), it has been shown that the 30-kD MP localizes to the plasmodesmata in cells of both TMV-infected plants and transgenic plants expressing TMV MP (Tomenius et al., 1987; Atkins et al., 1991) and can be found in the cell wall fraction of infected tissue (Deom et al., 1990). The TMV MP is capable of modifying the size exclusion limit of plasmodesmata, making it possible for larger molecules to pass from cell to cell (Wolf et al., 1989). A deletion mutant of the TMV MP lacking the C-terminal 73 amino acids does not localize to the cell wall fraction or to plasmodesmata. This mutant TMV MP does not modify plasmodesmata and is unable to facilitate cell-to-cell movement, but a smaller deletion of the C-terminal 55 amino acids, which does localize to plasmodesmata, allows cell-to-cell movement. However, this movement is somewhat impaired (Berna et al., 1991; Gafny et al., 1992).

Recently, it was also shown that the TMV MP was capable of binding to single-stranded nucleic acids in vitro (Citovsky et al., 1990). Because the TMV coat protein is not required for viral cell-to-cell movement (Culver and Dawson, 1989; Saito et al., 1990), this result suggests that it may be the TMV MP that both protects the viral RNA and facilitates its movement through the plasmodesmatal intercellular connections in the form of a ribonucleoprotein complex. Single-stranded DNA–MP and RNA–MP complexes have been visualized in vitro using electron microscopy. The dimensions of these complexes are such that they would probably not be excluded from movement through plasmodesmatal channels enlarged by the TMV MP (Citovsky et al., 1992). This supports the idea that TMV may move as a genomic RNA–MP complex.

Red clover necrotic mosaic virus (RCNMV) is similar to TMV in that it does not require its capsid protein for cell-to-cell movement (Xiong et al., 1993) and that the 35-kD MP is able to bind to single-stranded nucleic acids in vitro (Osman et al., 1992). Like the TMV MP, the RCNMV MP binds to both RNA and single-stranded DNA in a cooperative manner. A truncated MP, which is missing the C-terminal 88 amino acids, has no defect in RNA binding but is highly attenuated for viral movement and symptom development in cowpea (Osman et al., 1991b).

We were interested in identifying the activities that are associated with the 35-kD RCNMV MP and required for cell-to-cell movement and viral infection. To this end, we generated 12 alanine scanning mutations in the RCNMV MP by site-directed mutagenesis and examined their effects on RNA binding in

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vitro and their infectivity in vivo. Alanine scanning mutagenesis (Cunningham and Wells, 1989) is based on the rationale that clusters of charged residues in the primary sequence of a protein are likely to be found on the surface of the folded structure. By mutagenizing these residues, regions of a protein involved in macromolecular interactions are targeted and the number of mutants that must be screened is minimized. Alanine residues are substituted for the charged residues to avoid disrupting, as much as possible, the folded structure of the protein.

We developed an in vitro RNA binding assay based on the work of Osman et al. (1992) and Citovsky et al. (1990) and used this assay to quantitatively and qualitatively characterize the binding of the wild-type and mutant RCNMV MPs to RNA. Also, using cDNA clones from which RCNMV infectious transcripts can be derived (Xiong and Lommel, 1991), we were able to test the effects of the alanine scanning mutations on cell-to-cell movement and systemic infection in vivo. Our findings indicated the presence of at least three functional domains in the RCNMV MP, including a region of the protein required for cooperative binding of the MP to RNA in vitro. We also showed that cooperative RNA binding in vitro by the RCNMV MP is not necessary for its role in cell-to-cell movement and systemic infection in vivo. Also, MP mutants that bind RNA at 20% of the wild-type level in vitro are still able to facilitate cell-to-cell movement of RCNMV in vivo.

RESULTS

In Vitro RNA Binding of the RCNMV MP

Binding of the RCNMV MP to single-stranded nucleic acids was first shown by Osman et al. (1992) using both mobility shift and UV cross-linking assays. We have utilized a similar mobility shift binding assay to study the RNA binding properties of the RCNMV MP in vitro. A uniformly radiolabeled transcript generated from the 5′ terminal 200 nucleotides of RCNMV RNA 2 was used as the probe in all of the binding studies. The RCNMV MP was produced in *Escherichia coli* using the pET vector system described by Studier and Moffat (1986) and Rosenberg et al. (1987).

As seen in Figures 1A and 1B, incubation of RCNMV MP purified from *E. coli* with the labeled RNA transcript prior to polyacrylamide gel electrophoresis retarded the mobility of the labeled RNA probe. The mobility shift was abolished when the reaction products were incubated with proteinase K prior to electrophoresis (Figure 1A, lanes 8 and 9). The addition of BSA neither interfered with the mobility shift caused by the MP (data not shown) nor bound to RNA by itself (Figure 1A, lane 10). The specificity of RNA binding was examined in a competition experiment. A 2.5- to 40-fold molar excess of unlabeled RNA was added to the binding reaction at the same time as the labeled probe (Figure 1B, lanes 2 to 10). Both the homologous competitor, the 5′ terminal 200 nucleotides of RNA

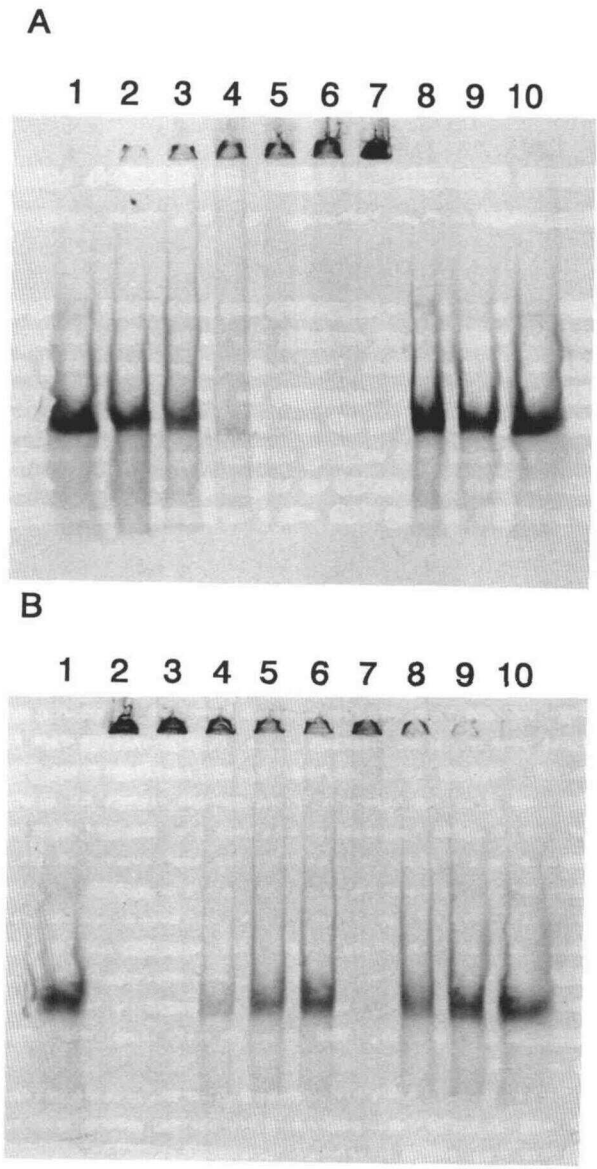

Figure 1. Mobility Shift Assay of Wild-Type RCNMV MP Binding to Labeled RNA.

(A) Mobility shift assay of wild-type RCNMV MP. Prior to electrophoresis on a 4% polyacrylamide gel, a ^{33}P-labeled transcript (10 ng) of the 5′ terminal 200 nucleotides of RCNMV RNA 2 was incubated with increasing quantities of *E. coli*–expressed MP at 4°C for 20 min in 40 μL of buffer B1. In lanes 1 to 7, 0, 0.1, 0.2, 0.4, 0.8, 1.0, and 2.0 μg of MP were incubated with probe, respectively. In lanes 8 and 9, 20 μg of proteinase K was added after the binding reaction, and the mixture was incubated for 20 min prior to electrophoresis. Two micrograms of MP was added in lane 8, and no protein was added in lane 9. In lane 10, 10 μg of BSA was incubated with the labeled RNA instead of MP. **(B)** Ten nanograms of ^{33}P-labeled transcript was used in a competition assay under the same conditions as given in **(A)**. Lane 1 contains only the radiolabeled RNA probe. In lanes 2 to 10, 2 μg of MP was incubated with the labeled RNA. In lanes 3 to 6, 25, 50, 100, and 200

2, and nonhomologous competitor, yeast ribosomal RNA, effectively competed with the labeled probe for MP binding, and the mobility shift was no longer observed (Figure 1B, lanes 3 to 6 and 7 to 10). This suggests that the in vitro binding activity of the MP is not sequence specific. The only specificity exhibited by the RCNMV MP is that it binds only to single-stranded nucleic acids and not to double-stranded nucleic acids (Osman et al., 1992).

The binding of the labeled RNA probe in the mobility shift assay appeared to occur in a highly cooperative fashion. The fact that the probe migrated either as the completely unbound or completely bound form in the polyacrylamide gel is consistent with the the idea that once one or a few molecules of MP are bound to a labeled RNA molecule, that molecule becomes a preferred substrate for the rapid binding of additional MP molecules (Citovsky et al., 1990). This would explain the absence of labeled RNA molecules with intermediate mobilities.

In Vitro RNA Binding Profiles of Mutant MPs

Alanine scanning mutations were generated in the RCNMV MP sequence using site-directed mutagenesis, as shown in Figure 2A, and mutant MPs were then purified from *E. coli*. Mutant RCNMV MPs 27-31, 144, 278, and 301 had RNA binding profiles similar to that of wild-type MP in the mobility shift assay (Figure 2B). Six other mutants (122, 128, 161, 204, 242, and 305) had RNA binding profiles that were unlike that of the wild type. In general, these mutant MPs retarded the mobility of the RNA probe much less than the wild-type MP. However, these mutant proteins did bind RNA; the predominant labeled species migrated more slowly than the unbound probe. Also, this labeled species represents the fully bound probe, even though its migration is between that of the unbound RNA probe and the RNA probe fully bound by the wild-type protein. It is not an intermediate; the addition of more protein does not produce any additional species, but rather more of the same species (data not shown). Mutants 280 and 291 exhibited two distinct RNA binding activities, one nonmigrating species like that of the wild-type MP and one quickly migrating species like the mutant MPs (Figure 2B). At lower MP concentrations (<100 ng/μL), only the wild-type binding (large mobility shift) was observed (data not shown).

Under the conditions of the continuous, native gel system used for these mobility shift assays, the wild-type MP was unable to migrate into the gel. This assay could not determine whether the altered mobilities exhibited by the mutant MPs were actually caused by a mutant RNA binding profile. An alternative interpretation is that mutant MPs 122, 128, 161, 204, 242, and 305 (and to some extent 280 and 291) did not alter the RNA binding profile of the MP but were able to migrate

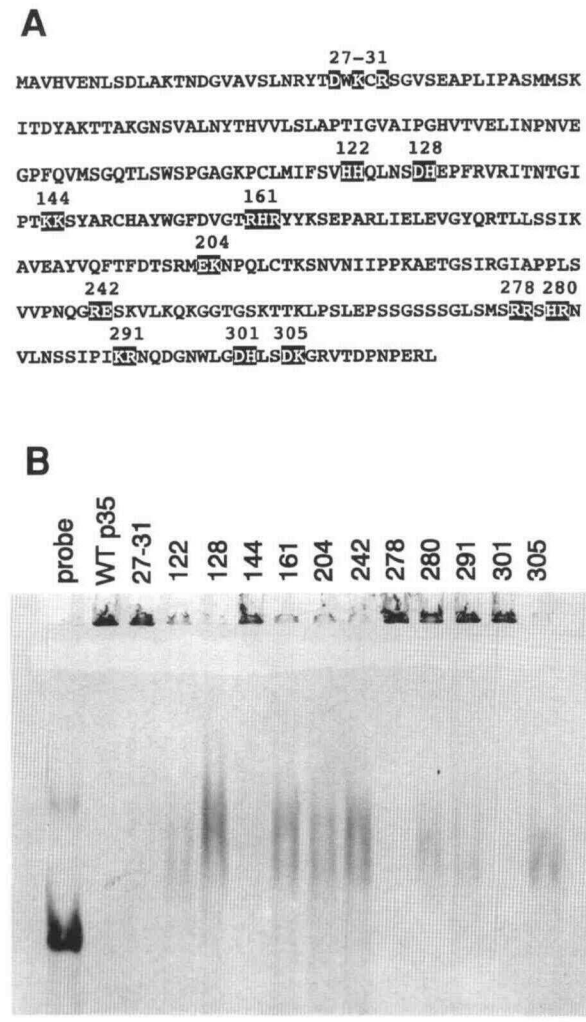

Figure 2. Alanine Scanning Mutations Introduced into the RCNMV MP and the RNA Mobility Shift Profile of Each Mutant.

(A) The primary amino acid sequence of the RCNMV MP in single-letter code. Reverse-shaded residues identify charged amino acids that were changed to alanine by site-directed mutagenesis. Above each mutagenized amino acid cluster is the mutant's label. For every mutant except 278, this label identifies the position of the first amino acid mutagenized in each cluster. For 278, it is the second amino acid.
(B) Autoradiograph of a mobility shift assay conducted with wild-type (WT) and mutant RCNMV MPs. Four micrograms each of the wild-type and mutant MPs were incubated for 20 min at 4°C with 10 ng of the ^{33}P-labeled RNA probe in a total volume of 40 μL of buffer B1. The reaction products were electrophoresed in the continuous gel system described in Methods. The gel was dried and exposed to film to produce the autoradiograph shown.

Figure 1. (continued).

ng, respectively, of unlabeled RNA transcript from the 5′ 200 nucleotides of RCNMV RNA 2 were added to the binding reaction as a competitor; in lanes 7 to 10, 50, 100, 200, and 400 ng, respectively, of ribosomal RNA from yeast were added as a competitor.

into the gel. The mutations introduced may have altered characteristics (such as net or effective charge) that affect the electrophoretic properties of the MP but not the RNA binding. To distinguish between these two possibilities, we conducted mobility shift assays using a discontinuous, native gel system. The resolution of different proteins and protein complexes was much greater in a discontinuous gel than in a continuous one (Chambrach et al., 1976), and the gel system that we employed had both a different pH and running buffer than the continuous gel system (see Methods).

Several results emerged from the analysis of RNA binding by the wild-type and mutant MPs in the second gel system. Although the wild-type MP did not migrate into the gel in the discontinuous gel system, as seen when comparing Figures 2 and 3, the resolution of the unbound probe, bound complexes, and unincorporated nucleotides was much higher than in the original continuous system, as shown in Figures 3 and 4. Also, the background radioactivity characteristic of the continuous gel system (seen as a smear in each lane along the length of the gel in Figures 1 and 2) was virtually eliminated. This facilitated the quantification of RNA binding by radioanalytic imaging of the dried gels (see below).

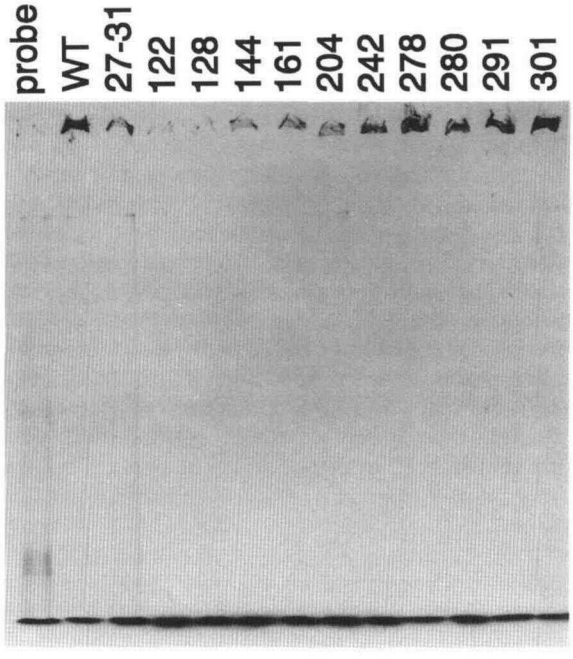

Figure 3. Autoradiograph of a Mobility Shift Assay Conducted with Wild-Type and Mutant RCNMV MPs Using a Discontinuous Gel System.

Two micrograms each of the wild-type (WT) and mutant MPs (excluding 305, which is presented in Figure 4) were incubated for 20 min at 4°C with 5 ng of the ^{33}P-labeled RNA probe in a total volume of 20 μL of buffer B1. The reaction products were electrophoresed on the discontinuous gel system described in Methods. The gel was dried and exposed to film to produce the autoradiograph shown. Unincorporated nucleotides are visible at the bottom of the gel in all lanes.

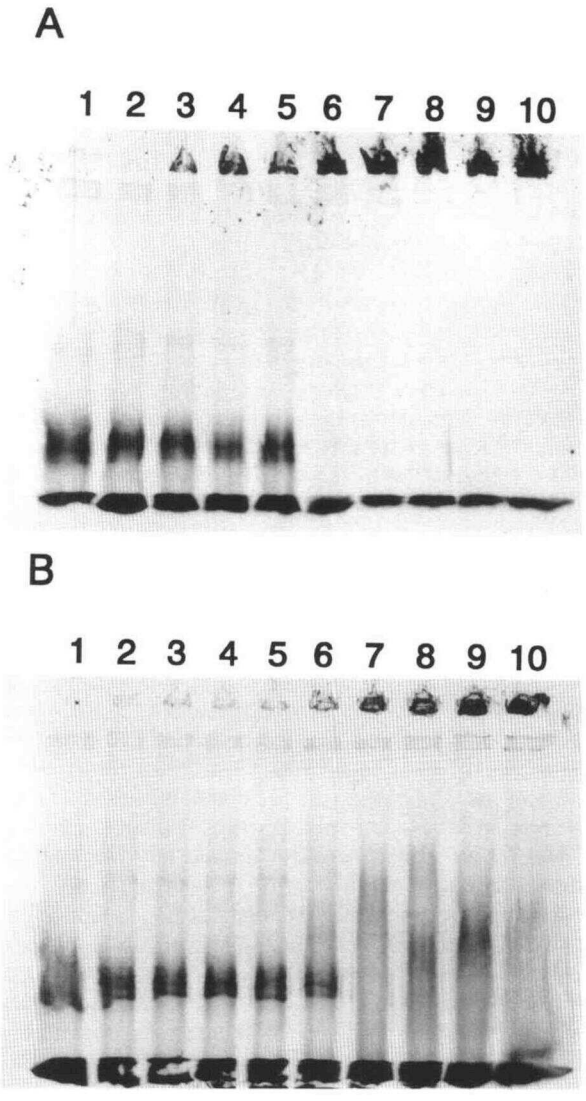

Figure 4. Comparison of RNA Binding profiles of Wild-Type and Mutant 305 RCNMV MPs in a Discontinuous Native Gel System.

(A) Binding profile of wild-type MP in a discontinuous gel system. Five nanograms of ^{33}P-labeled RNA probe was added to increasing amounts of the wild-type MP isolated from *E. coli* in a 20-μL volume of buffer B1 at 4°C for 20 min prior to electrophoresis on a discontinuous gel system (described in Methods). 0, 0.03, 0.07, 0.1, 0.2, 0.4, 0.8, 1, 2, and 4 μL of protein were added in lanes 1 to 10, respectively. **(B)** Binding profile of mutant MP 305 in a discontinuous gel system. Five nanograms of ^{33}P-labeled RNA probe was added to increasing amounts of mutant MP 305 under the same conditions given in **(A)**. Unincorporated nucleotides are seen at the bottom of the gel for both **(A)** and **(B)**. The autoradiographs shown here were exposed seven to eight times longer than those in Figures 1 to 3.

Interestingly, all 12 mutant MPs also failed to migrate into the gel when bound to RNA in the discontinuous gel system (Figures 3 and 4). However, in some cases, it was possible to resolve RNA–MP complexes with a higher mobility in addition to the completely bound, nonmigrating complex (for example, see mutant 305, shown in Figure 4B, lanes 6 to 10). These additional complexes appeared to be intermediates in the binding process and may represent RNA molecules with only one or a few molecules of MP bound. These intermediates were seen only for mutant MPs 27-31, 122, 128 (data not shown), and 305 (Figure 4B). The loss of the "all-or-none" binding characteristic seen with the wild-type MP is consistent with a loss of cooperative binding, as originally described by Citovsky et al. (1990). It is also possible that one or more of these mutants still bind RNA cooperatively but not as efficiently as the wild-type MP. The other eight mutants formed only a single RNA–MP complex that did not migrate into the gel, as in the case of the wild-type MP (data not shown). In contrast, in the continuous gel system, several mutants exhibited RNA–MP complexes that migrated into the gel, migrating more quickly than the wild-type complex (compare Figures 2 and 3).

Because the wild-type and mutant MPs shift the mobility of the labeled RNA probe to the same extent in the discontinuous gel system, it was possible to directly compare the levels of RNA binding exhibited by the various mutant MPs. We observed that mutations 122 and 128 have the most significant effect on the levels of RNA binding (Figure 3) and that, in general, mutations in the 5′ half of the MP gene reduce the RNA binding capacity of the MPs more than those in the 3′ half of the MP gene. It is possible, therefore, that amino acids 122 to 129 in the MP represent an RNA binding site and that the surrounding regions are also important for RNA binding.

We quantified the RNA binding of the wild-type and mutant MPs in the mobility shift assay by measuring the radioactivity associated with the free and bound species in a dried polyacrylamide gel by radioanalytic imaging, as shown in Figure 5. The RNA binding profile of the wild-type MP was characteristic of cooperative binding and is consistent with results previously described for the TMV and RCNMV MPs (Citovsky et al., 1990; Osman et al., 1992).

From our analysis of the RNA binding profiles of the mutant MPs, three phenotypic classes could be distinguished. One class, composed of mutants 144, 161, 204, 242, 280, and 291, retained the cooperative binding properties of the wild-type MP but had lower levels of RNA binding (represented by mutant 242 in Figure 5). A second class, including mutants 27-31, 122, 128, and 305, not only affected the levels of RNA binding by the MP but also disrupted the cooperative binding of MP to RNA (represented by mutants 27-31 and 305 in Figure 5). The loss of cooperative RNA binding was demonstrated by the linear nature of the RNA binding curves (Figure 5) and was confirmed by the presence of bound species with intermediate mobilities in the polyacrylamide gel (Figure 4B, lanes 6 to 10). Mutants 278 and 301 defined a third class of mutants that was indistinguishable from the wild-type MP, affecting neither the level nor the cooperative RNA binding in vitro.

Biological Activity of the Alanine Scanning Mutant MPs

RNA 2 transcripts were produced in vitro from cDNA clones containing each of the 12 alanine scanning mutations and were coinoculated with the wild-type RNA 1 transcript onto *Nicotiana benthamiana* plants. Symptom development was monitored on both the inoculated and noninoculated leaves, and viral infection was assayed by RNA gel blot analysis. Symptoms observed on the inoculated leaves were indicative of the ability of an MP to facilitate cell-to-cell movement. On the other hand, symptoms on the noninoculated leaves demonstrated the ability of the virus to move long distance or systemically (Xiong et al., 1993). Plants inoculated with viral transcripts of the MP mutants 27-31, 204, 242, 280, 301, and 305 developed symptoms on the inoculated and noninoculated leaves of *N. benthamiana* between 4 and 6 days postinoculation. Gel blot analysis of total RNA extracted from inoculated leaves showed that RNA 2 was only detected in tissue isolated from plants exhibiting symptoms. MP mutants that produced no symptoms did not accumulate a detectable amount of viral RNA in the inoculated leaves, as shown in Figure 6. Each mutant was inoculated on at least two plants at a time and the inoculations were repeated at least three times.

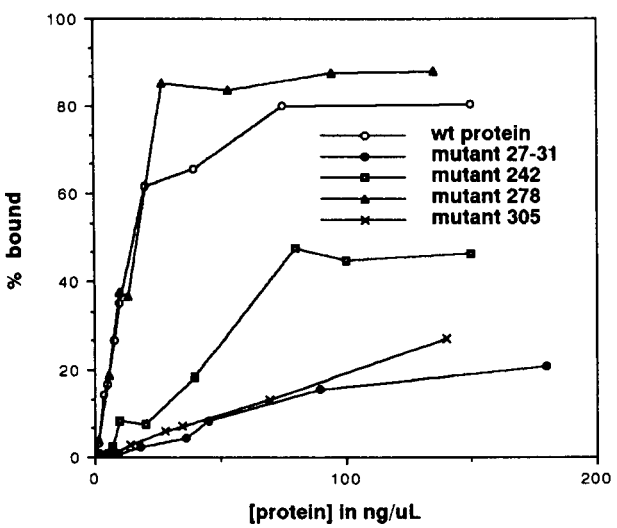

Figure 5. Quantification of Mobility Shift Assays of the Wild-Type RCNMV MP and Three Phenotypic Classes of RCNMV MP Alanine Scanning Mutants.

Five nanograms of the ^{33}P-labeled transcript described above was added to increasing amounts of the *E. coli*–produced MPs (as described in Methods) in a total volume of 20 μL. The RNA binding profiles of the four mutants, 27-31, 242, 278, and 305, are compared to the wild-type (WT) MP. RNA binding data were obtained from quantification of reaction mixtures electrophoresed on a discontinuous gel system, as described in Methods. The dried gel was quantified on an Ambis scanner, and the resulting data were plotted, as described in Methods, to produce the graph shown.

Viral RNA was isolated from plants inoculated with each of the six infectious MP mutants, and the RNA was sequenced. Five of the mutants retained the mutations that were originally introduced (data not shown). However, mutant 280 consistently reverted to the wild type in three separate experiments. All six infectious mutants elicited symptoms on *N. benthamiana* that were consistent with infection by RCNMV, such as necrosis, stunting, ringspots, and mosaic patterns, but the severity of these symptoms varied widely. Mutants 204 and 242 caused much more severe necrosis and stunting than the wild-type MP, whereas mutants 27-31 and 301 did not cause necrosis, stunting, or mosaic patterns but rather exhibited only small circular lesions (ringspots) on the inoculated and systemically infected leaves. Plants inoculated with mutant 305 exhibited predominantly mosaic symptoms and stunting with little necrosis; those inoculated with mutant 280 were, not surprisingly, indistinguishable from the wild type. Although there appears to be a correlation between the severity of symptoms elicited by the mutant MPs and the quantity of viral RNA observed in our RNA gel blot analysis, it is important to note that the gel blot analysis was conducted using total RNA from the inoculated leaves, whereas the majority of symptom differences observed were on the systemically infected leaves. Also, the timing and severity of symptoms do not necessarily correlate with virus titer or viral spread. Nevertheless, these data do suggest that the RCNMV MP has a significant role in symptom development and that mutations in the MP may affect the rate and/or severity of viral infection.

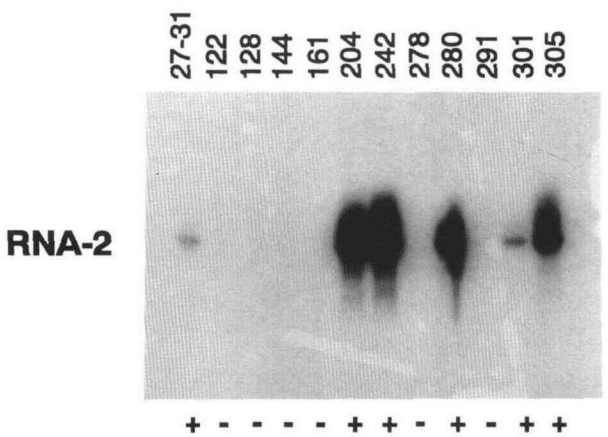

Figure 6. Summary of Data from Plants Inoculated with the RCNMV MP Alanine Scanning Mutants and RNA Gel Blot Analysis of Total RNA from Inoculated Leaves.

Autoradiograph of a gel blot with total RNA isolated from the inoculated leaves of plants infected with each alanine scanning mutant. Approximately 20 μg of total RNA isolated from plants infected with the indicated mutant was added to each well of a 1.0% agarose gel. RNA was transferred onto a nylon membrane and hybridized with a labeled RNA 2 cDNA clone. The blot was exposed to film for 5 hr at room temperature. A (+) below the gel lane indicates the observation of symptoms and a (−) a lack of symptoms on both the inoculated and systemic leaves of *N. benthamiana* 6 to 10 days after inoculation with each of the 12 alanine scanning mutants.

Cell-to-Cell Movement Activity Does Not Correlate with Cooperative RNA Binding in Vitro

The mobility shift assay allowed us to measure the ability of each MP mutant to cooperatively bind RNA and to determine the relative levels of RNA binding. In addition, we were able to test each mutant for its ability to facilitate cell-to-cell movement of the virus infection in the plant. Figure 7 summarizes the three activities assayed for each RCNMV MP alanine scanning mutant. From the comparison of these activities, we concluded that the cell-to-cell movement function of the MP was not dependent upon cooperative binding or on a high level of RNA binding. For example, mutants 27-31 and 305, which facilitated cell-to-cell movement and caused systemic infection in *N. benthamiana*, bound to RNA poorly and did not bind to RNA in a cooperative manner. In this study, no mutant MP was identified that had absolutely no RNA binding activity. However, mutants 27-31 and 305 displayed 20% of the level of RNA binding activity exhibited by the wild-type MP (Figures 3 to 5, and 7). In contrast to mutants 27-31 and 305, mutant 278 bound cooperatively to RNA as efficiently as wild-type MP (Figures 3 to 5, and 7) but was not capable of potentiating the cell-to-cell movement of RCNMV. Consequently, we concluded that mutation 278 disrupted a region of the MP that was essential for biological activity but not for cooperative RNA binding.

DISCUSSION

Deletion mutagenesis has been used previously to identify the various functional domains in plant virus MPs. Using this approach, Citovsky et al. (1990, 1992) were able to identify two putative RNA binding domains in the TMV MP. Similarly, Osman et al. (1991b) and Xiong et al. (1993) demonstrated that the C terminus of the RCNMV MP is not necessary for cell-to-cell movement in a limited number of systemic hosts. In addition, Berna et al. (1991) and Erny et al. (1992) were able to identify sequences that are apparently necessary for cell wall localization of the TMV and alfalfa mosaic virus MPs, respectively. We have used alanine scanning mutagenesis to investigate functional domains of the RCNMV MP. We believe this method is both more informative and more subtle than deletion analysis because alanine substitutions are not likely to significantly perturb tertiary structure (Cunningham and Wells, 1989). Therefore, this type of mutagenesis may facilitate the isolation of mutants with interesting phenotypes.

Using this strategy, we have demonstrated the presence of at least three functional domains in the RCNMV MP by characterizing MP mutants both in vitro and in vivo. We confirmed

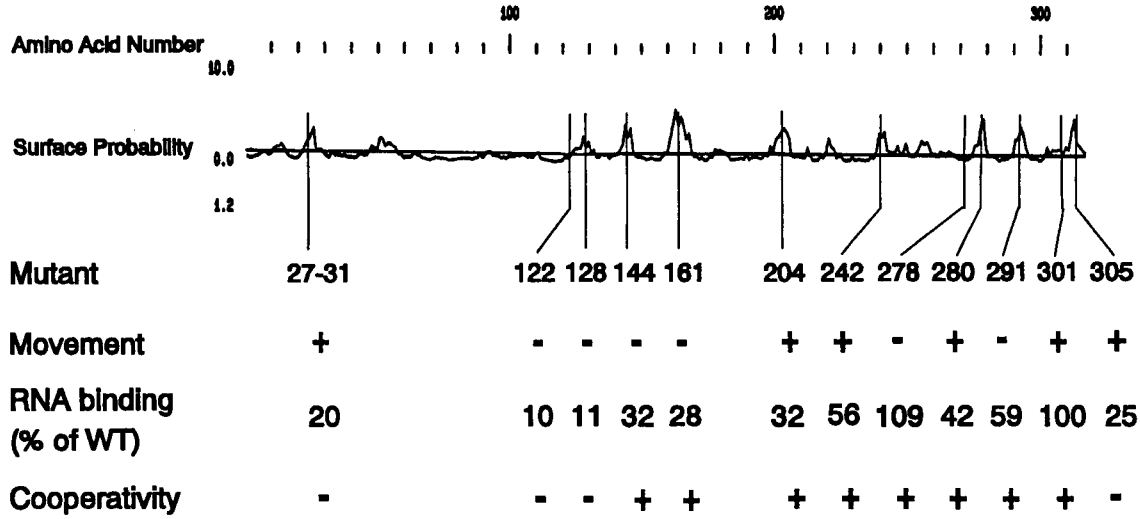

Figure 7. Cell-to-Cell Movement, RNA Binding, and Cooperative RNA Binding Activities for Each RCNMV MP Alanine Scanning Mutant.

A (+) indicates that the activity was detected and a (−) indicates that the activity was not detected. Numerical results were derived from data obtained in quantification of mobility shift assays. The surface probability plot (Emini et al., 1985) illustrates the likelihood that mutagenized residues are localized to the surface of the folded protein. WT, wild type.

the results of Osman et al. (1992), who showed that the RCNMV MP has RNA binding activity in vitro and that this binding is cooperative. One difference between our results and those of Osman et al. (1992) is that the RCNMV isolate that we have characterized (Australian) does not enter the polyacrylamide gel under the electrophoretic conditions common to the two studies (a continuous, native gel system), whereas the MP of the Czechoslovakian or TpM-34 isolate described by Osman et al. (1992) does. Although in both cases the MP clearly binds to the RNA probe, one interpretation of our observations is that the MP may be aggregating or forming partially insoluble complexes with the RNA probe prior to electrophoresis rather than binding RNA in a cooperative manner. However, we do not believe that this is the case. Some of the mutant MPs do migrate into the gel (Figure 2B), for example, and among these, some bind RNA cooperatively (204 and 242) and some do not (128 and 305). In a discontinuous, native gel system with different buffers and pH, the same mutant MPs do not migrate into the gel (Figures 4A and 4B). Consequently, we conclude that the *E. coli*–produced MPs are not aggregating or becoming insoluble, but rather that the electrophoretic properties (pH, pI, net, or effective charge) of the proteins determine whether the MPs migrate into the gel. In addition, the TpM-34 isolate of RCNMV has only 80% amino acid sequence identity with the Australian isolate, and near the C terminus, between amino acids 237 and 288, the proteins are only 44% identical (Osman et al., 1991a). Therefore, it is not surprising that these two RCNMV MPs have different electrophoretic mobilities under the same gel conditions.

Using a mobility shift assay, we have shown that RCNMV MP mutations alter its RNA binding profile. Mutations in the N-terminal half of the MP appear to have a greater effect on RNA binding than those in the C-terminal region. Mutations 122 and 128, which reduce the level of RNA binding to 10% of that of the wild-type MP, are in a region of the protein that is either directly or indirectly involved in RNA binding. However, this analysis does not distinguish phenotypically between mutants that may prevent folding of the protein into an active conformation and mutants that fold correctly but have an altered active site. Further, changes in RNA binding activity cannot be correlated directly with the biological activity of the mutant MPs. For example, mutant 278 binds RNA as well as the wild-type MP in vitro but is not capable of facilitating cell-to-cell movement in the systemic host plant *N. benthamiana*. Mutants 27-31 and 305 bind RNA at 20% of the level of the wild-type protein but facilitate rapid systemic infections of *N. benthamiana*.

Assuming that the RNA binding activity characterized in vitro reflects RNA binding in vivo, it is possible that relatively little RNA binding is actually required for cell-to-cell movement of RCNMV (≤20% of wild type). We speculate that the high levels of cooperative RNA binding exhibited by the wild-type MP may be required only under certain conditions. For example, it may be important in systemic hosts other than *N. benthamiana* or under a particular set of environmental conditions. Preliminary evidence in our laboratory from a host range analysis of these alanine scanning mutants suggests that the MP may be involved in long-distance (systemic) movement of the virus out of the inoculated leaves. Indeed, some of the mutants with altered levels of RNA binding, such as 27-31 and 204, appear restricted to the inoculation leaves of *N. edwardsonii* and cowpea (D. Giesman-Cookmeyer and S. A. Lommel,

unpublished observations). Other mutants, such as 242 and 305, appear to facilitate systemic movement more rapidly than the wild-type MP. It is possible, therefore, that the RNA binding properties of the RCNMV MP are important for its role in long-distance movement.

Mutant MPs 27-31, 122, 128, and 305 appear to disrupt a functional domain responsible for cooperative protein–protein interactions during RNA binding. That is, binding of one MP molecule to RNA enhances the binding of additional MP molecules. This domain is genetically distinct from that required for RNA binding, as demonstrated by the isolation of mutant MPs that interact cooperatively but have a reduced level of RNA binding (Figure 7). However, no mutants were isolated that affected cooperative RNA binding without also affecting the absolute levels of RNA binding.

Finally, a third functional domain of the RCNMV MP has been defined by the isolation of mutant 278. This mutant binds RNA as cooperatively and to the same extent as the wild-type MP. However, it is not capable of facilitating RCNMV movement from cell to cell. In addition, three other mutants, 144, 161, and 291, have higher levels of RNA binding and bind more cooperatively to RNA than infectious mutants 27-31 or 305 (Figure 7) but, again, do not facilitate cell-to-cell movement. These mutants may be movement defective because they disrupt a putative third domain of the MP required for cell-to-cell movement. The TMV movement protein has been shown to localize to plasmodesmata and to alter the size exclusion limit of plasmodesmata during infection (Tomenius et al., 1987; Wolf et al., 1989; Atkins et al., 1991). It is possible that the third functional domain of the RCNMV MP may be involved in one or both of these activities, perhaps by targeting the MP to the plasmodesmata or by interacting with host factors to modify the plasmodesmata. Alternatively, this third domain may simply be required for folding or for binding RNA into a functional viral ribonucleoprotein complex that is capable of cell-to-cell movement. Citovsky et al. (1992) suggest that the TMV MP must unwind the viral RNA and relieve its secondary structure for the RNA to move through the plasmodesmata. Mutants such as 278 may bind RNA but be unable to eliminate the secondary structure and/or unwind the RNA.

Interestingly, mutations 278 and 291 lie in the C-terminal region of 88 amino acids, which was not absolutely required for cell-to-cell movement in cowpea (Osman et al., 1991b). Yet these mutations are movement defective. Although these data may seem contradictory, it is possible that deletion of the C terminus may not affect the function of the rest of the protein as drastically as the presence of an altered C terminus. For example, the amino acid changes in the MP mutants 278 and 291 alter the net charge of the protein and may affect folding and/or other macromolecular interactions. As mentioned previously, the RCNMV TpM-34 MP studied by Osman et al. (1992) is only 80% homologous to the Australian isolate that we have studied, and the greatest divergence between them is at the C terminus. We have not isolated a deletion mutant larger than 39 amino acids from the C terminus in the Australian isolate of RCNMV that still facilitates cell-to-cell movement (Xiong et al., 1993). A deletion mutant that is lacking amino acids 279 to 317 of the RCNMV MP is only functional in *N. benthamiana*. This particular host has been found in many systems to be unusually permissive for viral infection. We believe that mutant 278 must affect a function of the MP that is distinct from RNA binding and cooperative binding. This function could be folding, targeting to the plasmodesmata, modifying the plasmodesmata, interacting with host proteins, or trafficking through the plasmodesmata.

METHODS

Site-Directed Mutagenesis

Alanine scanning mutants of the red clover necrotic mosaic virus (RCNMV) movement protein (MP) gene were generated by site-directed mutagenesis using oligodeoxynucleotide primers of 30 to 35 nucleotides in length (Kunkel et al., 1987). A total of 12 clusters of charged amino acid residues identified in the RCNMV MP were changed to alanines. Clusters of charged amino acids were identified using the complete nucleotide sequence described by Lommel et al. (1988) and the surface probability algorithm described by Emini et al. (1985). Each mutant was created directly in the vector pRC21G$^{5'}$Nco, from which infectious RNA 2 transcripts could be derived. An NcoI restriction site was engineered at the initiation codon of the RCNMV MP gene in pRC21G (Xiong and Lommel, 1991) by site-directed mutagenesis (Kunkel et al., 1987) to make the vector pRC21G$^{5'}$Nco. This vector was used for all of the site-directed mutagenesis and yields an infectious RNA transcript indistinguishable from the original infectious clone. Reactions were performed as described in the Mutagene Kit (BioRad). CJ236 was the *dut ung Escherichia coli* host used, and DH5αF′ was the wild-type host. Mutant DNA was sequenced using the Sequenase protocol and reagents (U.S. Biochemical Corp.).

MP Expression in *E. coli*

Once the 12 MP mutants were generated, the entire RNA 2 gene from the wild-type and mutagenized vectors was then excised by NcoI restriction and cloned into the NcoI site of the pET3d expression vector (Studier and Moffat, 1986; Rosenberg et al., 1987). The resulting plasmids were transformed into the BL21(DE3)pLysS strain of *E. coli*. For the wild type and each of the 12 mutant proteins, a 100-mL culture of the transformed bacteria was grown to an OD_{600} of 0.6 and then induced with the addition of 400 μL of 100 mM isopropyl β-D-thiogalactopyranoside. After 2 hr of incubation at 37°C, the cells were harvested and resuspended in 2.5 mL of buffer B1 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol, and 200 mM NaCl). Cells were lysed in a French press at 20,000 psi, and cell debris was pelleted and washed twice with high-salt buffer B2 (B1 + 1 M NaCl) and twice with 4 M urea buffer B3 (B1 + 1 M NaCl + 4 M urea). Pellets were resuspended in 1.5 mL of 8 M urea buffer B4 (B1 + 1 M NaCl + 8 M urea); the debris was then pelleted by centrifugation, and the supernatant was dialyzed at room temperature against buffers B3, B2, and B1 for 30 min each. The insoluble debris resulting from the dialysis was pelleted by centrifugation, and the concentrations of the soluble fractions were determined by the modified Bradford assay (Bio-Rad). The

concentration of the wild-type protein was estimated using BSA as a standard. The relative concentrations of the 12 MPs were then determined using the wild-type protein as a standard. Several batches of the MPs were isolated from *E. coli*, including at least four for the wild-type protein and two from some of the poor RNA binding MPs. In each case, the isolated proteins did not vary in function or solubility, as determined by the assays described in the text.

Mobility Shift Assay

The infectious transcript vector pRC21G$^{5'}$Nco was restricted with NdeI and the first 200 nucleotides of the RCNMV MP gene were uniformly labeled with α-^{33}P-UTP in a transcription reaction (Xiong and Lommel, 1991). The resulting RNA transcript was purified over a G50 Sephadex column, extracted with phenol and chloroform, and precipitated with ethanol. Typically, 5 ng of transcript and 0.01 to 4 μg of protein were used in a binding reaction. Reactions were conducted for 20 min on ice in buffer B1, and the reaction products were electrophoresed at 100 V in a continuous, nondenaturing (native) 4% (19% T 1% C) polyacrylamide gel in 1 × Tris-buffered EDTA at 4°C. A discontinuous, native gel system was also used in some experiments. For this system, a stacking gel of 3.125% polyacrylamide (6.25% T 20% C), pH 6.9, and a separating gel of 4% (19% T 1% C) polyacrylamide, pH 8.48, were used. The gel was electrophoresed at 100 V with an upper tank buffer composed of 37.6 mM Tris-HCl and 40 mM glycine, pH 8.89 and a lower tank buffer composed of 63 mM Tris-HCl, pH 7.47. Both types of gel were then dried and autoradiographed from 2 to 24 hr at room temperature. RNA binding was quantified by radioanalytic imaging of the dried gel on an Ambis scanner (San Diego, CA). The percent of RNA bound was determined by dividing the radioactivity measured in the retarded species by the total input radioactivity (total counts present in each lane). The percent bound is in every case less than 100% for two reasons. First, some of the input radioactivity is in the form of unincorporated nucleotides, despite column purification of the radiolabeled RNA probe. The unincorporated nucleotides are easily distinguished from the probe in the discontinuous gel system and can be quantified. Second, the RNA probe is often degraded when incubated with MP mutants that do not bind RNA well, especially at higher concentrations. This can be quantified by measuring the amount of radioactivity that migrates with unincorporated nucleotides in the presence and absence of MP. The degree of protection from degradation is, in fact, another measure of the ability of the mutant MP to bind RNA. In the case of mutant 278, for example, which binds RNA slightly better than the wild-type MP, we found that the amount of RNA degraded by this mutant MP was lower than for the wild-type MP. Conversely, most of the other mutants exhibited higher levels of RNA degradation.

Plant Inoculations and Phenotype Analysis of the MP Mutants

The mutant and wild-type RCNMV RNA 2 cDNA clones were linearized with SmaI prior to in vitro transcription with bacteriophage T7 RNA polymerase. Capped transcripts from a 50-μL reaction (~10 μg RNA) were resuspended in GKP buffer (50 mM glycine, 30 mM K_2HPO_4, pH 9.2, 1% bentonite, 1% celite) and inoculated into a total of four leaves on two *N. benthamiana* plants, as described previously (Xiong and Lommel, 1991). A pair of the next to the lowest set of leaves were inoculated when the plants were at the six-to-eight leaf stage. Mutant RNA 2 transcripts were coinoculated with the wild-type RNA 1 transcript. Each complete set of mutants was inoculated and assayed a minimum of three times. Plants were maintained under normal glasshouse conditions.

The accumulation of viral genomic RNA with mutations in the MP gene in the inoculated leaves of *N. benthamiana* was determined by RNA gel blot analysis. Nucleic acids were phenol extracted from inoculated leaves 7 days postinoculation. The presence of viral RNA in the 2 M LiCl insoluble fraction was determined as described by Carrington and Morris (1984). Gel blots were hybridized with the full-length RNA 2 cDNA and nick translated in the presence of α-^{32}P-dCTP (Maniatis et al., 1982).

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