The Carboxy-Terminal Two-Thirds of the Cowpea Chlorotic Mottle Bromovirus Capsid Protein Is Incapable of Virion Formation yet Supports Systemic Movement

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Previous investigations into recombination in cowpea chlorotic mottle bromovirus (CCMV) resulted in the recovery of an unusual recombinant virus, 3-57, which caused a symptomless infection of cowpeas but formed no detectable virions. Sequence analysis of cDNA clones derived from 3-57 determined that mutations near the 5′ terminus of the capsid protein gene introduced an early translational termination codon. Further mutations introduced a new in-frame start codon that allowed translation of the 3′ two-thirds of the capsid protein gene. Based on the mutations observed in 3-57, wild-type CCMV clones were modified to determine if the carboxyl two-thirds of the capsid protein functions independently of the complete protein in long-distance movement. Analysis of these mutants determined that while virion formation is not required for systemic infection, the carboxy-terminal two-thirds of the capsid protein is both required and sufficient for systemic movement of viral RNA. This indicates that the CCMV capsid protein is multifunctional, with a distinct long-distance movement function in addition to its role in virion formation.

Plant viruses typically have genomes of limited size which produce as few as four proteins, a surprisingly small number in comparison to the numerous functions required for infection. Multifunctional proteins may provide a mechanism to maximize the use of virally encoded proteins. Previous studies of plant viruses have discovered several multifunctional viral proteins, such as the tobacco etch virus helper component (8), the alfalfa mosaic virus capsid protein (21), and the bromovirus 1a protein (1). Plant virus capsid proteins, in particular, have demonstrated movement functions in addition to their structural roles (3, 11, 15, 18, 23, 26, 27, 29). Within bromoviruses, the 3a gene and the capsid gene are required for systemic movement of cowpea chlorotic mottle virus (CCMV) and brome mosaic virus (3, 23). Mutational analysis of the bromovirus capsid protein demonstrated that various regions of the capsid protein are important for viral movement in different hosts (12). These reports have not attempted to distinguish between the structural role of the bromovirus capsid protein and its role in systemic movement.

While using an in vivo assay to measure recombination in transgenic plants, Greene and Allison (13) recovered several genetically distinct CCMV recombinants with mutations in the capsid protein. One recombinant, 3-57, caused symptomless systemic infections of cowpea (Vigna sinensis) and Nicotiana benthamiana. While Northern blots of total RNA from infected plants showed the normal CCMV genomic RNA banding pattern, no virions were detectable in 3-57-infected plants by use of either standard virion preparations or electron microscopy. Sequence analysis of several 3-57 RNA 3 cDNA clones revealed a single nucleotide deletion nine nucleotides from the capsid protein initiation codon. This deletion caused a frameshift which introduced a termination codon at the ninth codon. In addition, a three-nucleotide insertion, approximately one-third of the way into the capsid protein gene, created a translation initiation codon within the original capsid protein reading frame (14) (Fig. 1).

Although several plant viruses move systemically without forming virions (9, 10, 16, 22, 24, 28), a previous CCMV study (3) indicated that the capsid gene is required for systemic infection. The lack of virions in 3-57 infections posed an interesting question: is CCMV virion formation required for systemic movement, or is a structurally deficient segment of the capsid protein adequate for systemic infection? Sequence data from 3-57 cDNA clones suggested that the complete capsid protein was not produced. This would prevent virion formation in the 3-57 infection, since CCMV ultrastructural data indicate that the amino end of the CCMV capsid protein is necessary for virion formation (25).

The internal initiation codon in recombinant 3-57. The absence of detectable virions in the 3-57 infections did not exclude the possibility that some form of the CCMV capsid protein was required for systemic movement. Comparison of the sequence surrounding the newly inserted initiation codon with functional eukaryotic translation initiation sites (6) suggested that the new codon may be functional (Fig. 1). No other

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FIG. 1. Comparison of mutated regions in recombinant 3-57 (Rec. 3-57) to homologous regions in wild-type (w.t.) CCMV. Deletion of nucleotide 1370 in the 3-57 recombinant introduces an early termination signal. Mutations in the region between nucleotides 1582 and 1593 introduce an in-frame initiation codon (underlined). The A at the −3 position (nucleotide 1585) indicates that the internal initiation codon in recombinant 3-57 is typical of functional eukaryotic initiation codons.
in-frame initiation codons were present upstream of the inserted start codon. To determine if only a portion of the capsid protein enables systemic movement of CCMV, it was necessary to establish whether the 3-57 recombinant was (i) producing a trace amount of full-length capsid protein via a frameshift, (ii) producing a truncated capsid protein incapable of virion formation from the newly inserted interior initiation codon, or (iii) producing no capsid protein at all. If no capsid protein was produced in the 3-57 infection, the capsid protein of CCMV would be dispensable for systemic movement. If the 3-57 mutant was relying on a truncated, virion-deficient capsid protein for systemic movement, the capsid protein of CCMV must be multifunctional. Finally, if a low level of full-length capsid protein was being expressed, the 3-57 virus could move in a manner functionally identical to that of the wild-type virus. These possibilities were tested by using a CCMV capsid gene mutant series (Fig. 2).

**Inoculation of cowpea and *N. benthamiana* with AW mutant transcripts.** Three clones were constructed to determine how 3-57 moves systemically (Fig. 2). pCC3AW1 was constructed by making a single nucleotide deletion of the 12th nucleotide of the capsid protein gene in the infectious CCMV RNA 3 clone (2) by PCR mutagenesis. pCC3AW2 added an in-frame initiation codon to pCC3AW1 at the same location described for the recombinant 3-57. To create pCC3AW3, the original initiation codon (nucleotides 1359 to 1362) present in pCC3AW2 was changed from ATG to TTG. All mutations were verified by sequencing before being used as templates for in vitro transcription (2). Wild-type CCMV RNA 1 and 2 transcripts (C1 and C2) were used in combination with either AW1, AW2, AW3, or C3 transcripts to inoculate cowpea and *N. benthamiana* plants. All transcript combinations were also tested on a local-lesion host, *Chenopodium hybridum*, to demonstrate replication. Inoculated plants were tested for the presence of virus by probing dot blots of total RNA with a CCMV-specific probe, RA518 (3).

The AW1 construct, which expresses no capsid protein, was designed to establish that expression of the capsid gene was required for systemic movement. In numerous attempts, transcripts from C1, C2, and AW1 infected neither cowpea nor *N. benthamiana* despite the fact that they caused local lesions on *C. hybridum* (Table 1). The capsid protein requirements for systemic movement were tested by using the AW2 and AW3 constructs, which were designed to express the carboxy-terminal two-thirds of the capsid protein. The only difference between these two constructs was the deletion of the original capsid protein initiation codon in AW3. If 3-57 moved systematically by using a small amount of full-length capsid protein made by a frameshift during translation, AW2 would be infectious and AW3 would not. Both AW2 and AW3 transcripts, in combination with C1 and C2, infected *N. benthamiana* with an efficiency equal to that of wild-type transcripts, while the percentage of cowpeas infected by AW2 and AW3 was slightly reduced. Development of systemic infections by AW2 and AW3 progressed more slowly than that of wild-type infections, taking 7 to 14 days longer (Table 1). In addition, cowpea infections caused by AW2 and AW3 were symptomless, as was the infection caused by the 3-57 recombinant.

Attempts to recover virions from AW2- and AW3-infected plants were unsuccessful, despite the recovery of virions from wild-type CCMV-infected plants by the same techniques (data not shown). In addition, no virions were observed by electron microscope analysis of infected leaf sap (data not shown). Extracts from the same AW2- and AW3-infected plants used for microscopic analysis infected both cowpea and *N. benthamiana*. Total RNA was isolated from infected plants 28 days postinoculation (20), separated on a denaturing agarose gel, transferred to a nylon membrane (19), and probed with RA518. Repeated Northern analyses of total RNA extracted from AW2- and AW3-infected plants consistently confirmed the presence of all four viral RNAs, which accumulated to levels roughly equal to those of wild-type infections, taking 7 to 14 days longer (Table 1). In addition, cowpea infections caused by AW2 and AW3 were symptomless, as was the infection caused by the 3-57 recombinant.

**Expression of the truncated capsid protein.** Polyclonal CCMV antiserum raised against CCMV virions in a rabbit was

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**TABLE 1. Results of inoculation tests with C1, C2, and either AW1, AW2, AW3, or C3 transcripts**

<table>
<thead>
<tr>
<th>Host</th>
<th>Day postinoculation</th>
<th>No. of plants infected/no. inoculated with:</th>
<th>AW1</th>
<th>AW2</th>
<th>AW3</th>
<th>Wild-type CCMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cowpea</td>
<td>14</td>
<td>0/25</td>
<td>3/25</td>
<td>0/22</td>
<td></td>
<td>10/10</td>
</tr>
<tr>
<td>Cowpea</td>
<td>28</td>
<td>0/12</td>
<td>12/12</td>
<td>16/16</td>
<td>10/10</td>
<td></td>
</tr>
<tr>
<td><em>N. benthamiana</em></td>
<td>14</td>
<td>0/22</td>
<td>2/20</td>
<td>10/15</td>
<td></td>
<td>10/10</td>
</tr>
<tr>
<td><em>N. benthamiana</em></td>
<td>28</td>
<td>0/14</td>
<td>13/16</td>
<td>10/15</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. hybridum</em></td>
<td>14</td>
<td>Local lesions</td>
<td>Local lesions</td>
<td>Local lesions</td>
<td>Local lesions</td>
<td></td>
</tr>
</tbody>
</table>

*The number of plants systemically infected is compared to the total number of plants inoculated. Systemic infections were determined by blotting total RNA of inoculated plants at either 14 or 28 days postinoculation. All transcript combinations induced local lesions on *C. hybridum*, a CCMV local-lesion host.*
Conclusions. This mutational analysis of CCMV indicates that the capsid protein plays a multifunctional role in the virus life cycle. Both the RNA and protein data indicate that the truncated capsid protein in AW2 and AW3 infections is both sufficient and required for systemic spread of CCMV. Thus, while the full-length capsid protein is responsible for virion formation, the carboxy-terminal two-thirds of this protein has a distinct movement function, which is fulfilled by the full-length capsid protein in wild-type infections. Since AW2 and AW3 infections proceed at a slower rate than wild-type CCMV infections, either the full-length capsid protein or the virion itself affects systemic movement of the virus, perhaps by stabilizing viral RNAs. Additionally, this demonstrates that virion formation is not required for systemic movement of CCMV and that viral RNA travels within the plant in a nonencapsidated form. This suggests that the carboxy-terminal portion of the CCMV capsid protein must either physically protect viral RNA during movement or actively facilitate long-distance viral RNA movement through host barriers by interactions with host or other viral proteins.

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REFERENCES


