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SHORT COMMUNICATION

N-Terminal Basic Amino Acids of Alfalfa Mosaic Virus Coat Protein Involved in the Initiation of Infection

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Alfalfa mosaic virus coat protein or its messenger RNA is required in the inoculum for virus infection. The N-terminus of the coat protein is required for activity; thus, changes were made in the amino acid sequence of this region. Six coat protein mutants were tested for activity in virus infection assays in protoplasts. A coat protein mutant in which N-terminal residues 3–19 were absent was inactive; whereas, a mutant in which residues 3–11 were absent (CPΔN9) still had 73% of wild-type activity. Substitution of alanine for the basic residues at positions 14, 17, and 18 in full-length coat protein and in CPΔN9 resulted in mutant proteins that were inactive in infection. Thus, one, two, or three of these basic residues in CP are required for activity. © 1995 Academic Press, Inc.

Protein–RNA interactions are important for many regulatory processes, such as protein biosynthesis (*5*), RNA splicing (*12*), and virus replication (*18, 19*). Nine families of RNA binding proteins have been described based upon their binding motifs (*11*). However, there are protein–RNA interactions that involve nonconsensus binding motifs, such as the interaction of alfalfa mosaic virus (AMV) coat protein (CP) and AMV RNA. AMV is a multipartite plus-sense RNA virus. A single type of protein encapsidates three virus genomic RNAs (RNA1, 2, and 3) and one subgenomic RNA (RNA4) to form bacilliform particles (*7*). RNA4, synthesized from RNA3, is the messenger for AMV CP. The CP plays a key role in early and late infection through (i) stability of viral RNAs (*10, 13*), (ii) switching from minus to plus sense RNA synthesis (*17*), (iii) movement of infectious material throughout the plant (*16*), and (iv) encapsidation (*7*).

AMV differs from other viruses in the family *Bromoviridae* because its genomic RNAs are not infectious alone but rather require the presence of CP or RNA4 (*2*). This early function of CP, called genome activation, involves specific protein–RNA interactions. The function(s) involved in genome activation are not fully understood and may involve some of the roles listed above. The CP binds to the 3′ untranslated regions of all AMV RNAs, which have nearly identical sequences for the last 145 nucleotides and similar predicted secondary structures (*8, 14, 20*). The presence of AUGC repeats in the 3′ ends of the RNAs are required for binding (*6, 14*). The N-terminus of the CP was identified as the region involved in interactions with the virus RNAs (*20*) and in genome activation (*7*). Recent studies indicate that peptides, consisting of N-terminal amino acids 2–25 or 2–38, bind to AMV RNA and substitute for CP in the infection of protoplasts (*1*). To more fully understand the specific amino acid–nucleotide interactions in AMV, we investigated the activity of several mutant CPs in infection.

Several N-terminal CP mutants were constructed using the RNA4 cDNA, pSP65A4, previously described (*10*) as a template (Fig. 1). For the construction of deletion mutants, a cDNA was made from pSP65A4 by PCR to abolish the start codon. A 21-nucleotide primer previously described (*10*) and 5′-CCCTGAATTCGTTTTTATTTTTAATTTTCTTTCAATTACTTCCATCTCGAGTTCTTC-3′ were used as first and second strand primers, respectively. An *Eco*RI to *Bam*HI fragment of the cloned PCR product, which contained the mutation plus an introduced *Xho*I site, was exchanged with the wild-type fragment in pSP65A4 to lessen the possibility of PCR introduced mutations to produce pCPΔATG (Fig. 1). Plasmid pCPΔATG was used as the template for the construction of pCPΔN2, pCPΔN9, and pCPΔN17 (Fig. 1). The 21 nucleotide first strand primer (*10*) and 5′-CCATCTCGAGTTCT**ATG**TCACAAAG-3′, 5′-CCATCTCGAGTTCTTCACAAAAGAAAGCTGGTGGGAAA**ATG**AGTGCTGGTAAACC-3′ or 5′ CCATCTCGAGTTCTTCACAAAAGAAAGCTGGTGGGAAAGCTGGTAAACCTACTATACGT**ATG**TCTCAGAAC-3′ second strand primers were used in PCR to reposition the ATG codons (in bold type) and to introduce *Xho*I sites (underlined). The repositioned ATG codons shorten the open reading frame by deletion of the codons

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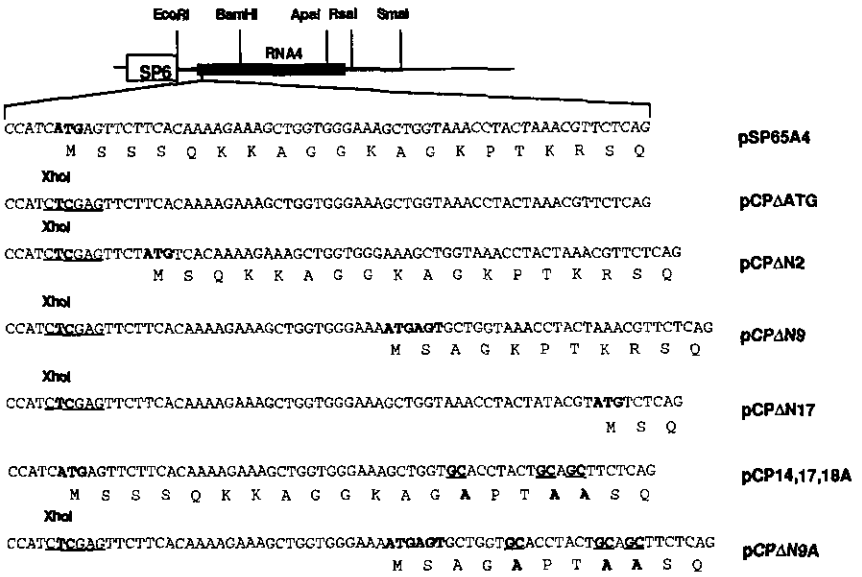

FIG. 1. AMV CP mutants. Plasmid pSP65A4 containing wild-type RNA4 cDNA was used as the template for construction of the CP mutants by PCR. The plasmid pCPΔATG does not contain the original ATG codon. Plasmids pCPΔN2, pCPΔN9, and pCPΔN17 code for AMV CPs with deletions of amino acids 3–4, 3–11, or 3–19, respectively. Two additional mutants, pCP14,17,18A and pCPΔN9A, were constructed to replace basic amino acids with alanine (indicated in bold type). Nucleotide sequences are indicated above the derived amino acid sequences for wild-type and mutant CP genes. The ATG codons and nucleotide changes are in bold type; the nucleotides of the introduced *Xho*I site are underlined.

for amino acids 3 and 4 (pCPΔN2), 3–11 (pCPΔN9), and 3–19 (pCPΔN17). The mutants were cloned into pGEM-T plasmid (Promega Corp., Madison, WI) and analyzed by restriction enzyme digestion. *Xho*I–*Bam*HI fragments (260 bp) containing the changes were exchanged with the *Xho*I–*Bam*HI fragment of pCPΔATG to create full-length mutant RNA4 cDNAs (Fig. 1).

Two mutants were made to change the basic amino acids at positions 14, 17, and 18 in pSP65A4 and in pCPΔN9 to alanine and to introduce an *Nhe*I cloning site (underlined in the following primer sequences). The codon for amino acid 18 was changed to that of alanine in a partial RNA4 cDNA by PCR using the 21 nucleotide first-strand primer (*10*) and 5′-CTAAAGCTAGCCAGAACTATCGTGCC-3′ second strand primer with pSP65A4 as the template. This cDNA lacks 96 nucleotides of the 5′ end of RNA4 cDNA. The codons for amino acids 14 and 17 were changed to those of alanine by PCR using 5′-TTCTGGCTAGCTGCAGTAGGTGCACCAGCTTTCCCACC-3′ as a first strand primer and a 24-nucleotide second strand primer previously described (*10*) using pSP65A4 and pCPΔN9 as templates. The cDNA with a change in codon 18 was combined with the cDNAs containing changes at codons 14 and 17 by ligation at the *Nhe*I site to create full-length mutant RNA4 cDNAs. The regions containing the mutations between the *Eco*RI and *Bam*HI sites were subcloned into pCPΔN9 plasmid to create pCP14,17,18A and pCPΔN9A (Fig. 1). To confirm that the desired changes were made, the plasmids were analyzed by sequencing.

In vitro RNA transcripts from pSP65A4 and the mutant plasmids were made using SP6 polymerase (Epicentre, Madison, WI) as described (*10*). The transcripts were analyzed by *in vitro* translation assays (*4*) to determine the messenger activity of each RNA. Figure 2 shows that transcripts from all constructs directed the synthesis of polypeptides of the expected size except for the CPΔATG transcript which does not contain the original AUG start codon. Translation of the mutant transcripts resulted in similar incorporation of [^{35}S]methionine (data not shown) and the synthesis of similar amounts of protein product (Fig. 2). To determine the biological activity of the mutant CPs, tobacco protoplasts were inoculated with genomic RNA1, 2, and 3 and either wild-type or mutant RNA4 transcripts. Protoplasts were isolated from axenic tobacco plants (*Nicotiana tabacum* var. Xanthi-nc) as described (*10*) and inoculated with transcripts plus genomic RNAs (3 and 0.5 μg per 10^5 proto-

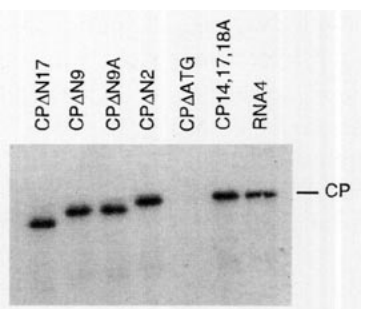

FIG. 2. Electrophoretic profile of AMV CP mutants synthesized *in vitro*. Proteins were labeled with [^{35}S]Met in a wheat germ cell-free translation system and separated by electrophoresis in a 13% SDS–polyacrylamide gel followed by autoradiography (*15*). Equal volumes of individual translation reactions were analyzed. The lanes of the gel are labeled with the RNA transcript used for translation. The position of wild-type CP is indicated to the right.

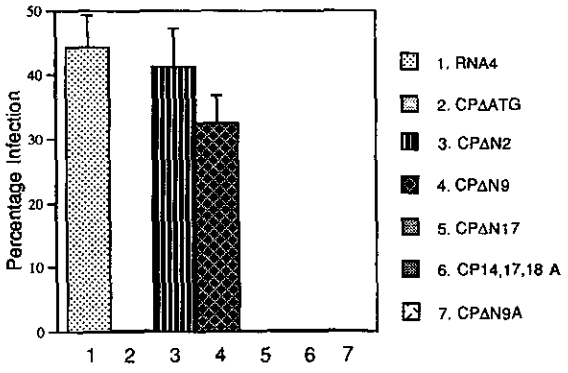

FIG. 3. Genome activation by wild-type and mutant AMV CP messenger RNAs. AMV RNAs 1,2,3 (0.5 μg per 10^5 protoplasts) were mixed with wild-type or mutant transcripts (3 μg per 10^5 protoplasts) and inoculated to tobacco protoplasts (*N. tabacum* var. Xanthi-nc). Protoplasts were collected at 24 hr after inoculation and assayed by immunofluorescence to determine the number of infected protoplasts. The legend to the right indicates the specific RNA transcript used to inoculate the protoplasts. The bars indicate the average percentage of protoplasts that were infected in at least five experiments; the standard deviation for each sample is indicated.

plasts, respectively) using a polyethylene glycol procedure (*15*). The percentage of infected protoplasts was determined at 24 hr after inoculation using an immunoassay (*9*). Figure 3 shows that inoculum containing wild-type transcripts (RNA4, lane 1) resulted in infection of 45% of the protoplasts, whereas, inoculum containing CPΔATG transcripts did not result in infection (Fig. 3, bar 2). This was expected because, without CP accumulation, infection does not occur (*2*). CPΔN2 transcripts (bar 3) were nearly as active as wild-type transcripts; CPΔN9 (bar 4) transcripts were only slightly less active. Therefore, up to nine amino acids can be deleted from the N-terminus of AMV CP with retention of 73% of the activity in genome activation. CPΔN17 transcripts (Fig. 1, bar 5) were inactive. Thus, all or some of the amino acids from position 12 to 19, which are absent in the CPΔN17 mutant, are important for activity.

It is thought that basic amino acids are responsible for the ability of the N-terminus to bind to AMV RNA and activate infection. There are three basic N-terminal amino acids in CPΔN9 that are absent in CPΔN17, therefore we created mutants that contained amino acid substitutions at these three positions (CP14,17,18A and CPΔN9A, Fig. 1). Figure 3, bar 7, shows that substitution of alanine for lysine at positions 14 and 17 and for arginine at position 18 in CPΔN9 abolished the activity of the mutant CP. The same substitutions in wild-type coat protein, CP14,17,18A (Fig. 3, bar 6) also abolished the activity of the full-length mutant protein. Therefore, all three of these amino acids or a subset of them are required for the activity of CP in infection.

The results presented here indicate that one, two, or all three of the N-terminal amino acids at positions 14 (lysine), 17 (lysine), and 18 (arginine) are required for AMV genome activation. It is likely that these three amino acids are part of the RNA binding motif of CP which could be as short as five amino acids with the sequence, KPTKR. The RNA binding motif of the Tat peptide of human immunodeficiency virus is only nine amino acids long (*3*). A single arginine located in a basic environment was critical for the binding of Tat to RNA. Additional experiments are required to determine the contribution of each of the three basic amino acids in genome activation. The data presented here strengthen the conclusion that the N-terminus of AMV CP is necessary and sufficient for one of the early steps in infection.

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