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The Cleavable Carboxyl-Terminus of the Small Coat Protein of Cowpea Mosaic Virus Is Involved in RNA Encapsidation

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The site of cleavage of the small coat protein of cowpea mosaic virus has been precisely mapped and the proteolysis has been shown to result in the loss of 24 amino acids from the carboxyl-terminus of the protein. A series of premature termination and deletion mutants was constructed to investigate the role or roles of these carboxyl-terminal amino acids in the viral replication cycle. Mutants containing premature termination codons at or downstream of the cleavage site were viable but reverted to wild-type after a single passage through cowpea plants, indicating that the carboxyl-terminal amino acids are important. Mutants with the equivalent deletions were genetically stable and shown to be debilitated with respect to virus accumulation. The specific infectivity of preparations of a deletion mutant (DM4) lacking all 24 amino acids was 6-fold less than that of a wild-type preparation. This was shown to be a result of DM4 preparations containing a much increased percentage (73%) of empty (RNA-free) particles, a finding that implicates the cleavable carboxyl-terminal residues in the packaging of the virion RNAs.

INTRODUCTION

A crucial step in the assembly of all viruses is the inclusion of the nucleic acid into the viral capsid. For most icosahedral viruses, little is known about the mechanism or mechanisms by which this occurs. The picornaviruses, comoviruses, fabaviruses, and nepoviruses, members of the picorna-like supergroup of RNA viruses, produce, in addition to the mature, RNA-containing virions, empty capsids (sometimes referred to as "provirions") that lack the viral RNA. Although much is known about the assembly of poliovirus, the mechanism of insertion of the RNA into particles is poorly understood. In particular, the significance of the provirion has been the subject of debate, and it has been proposed either to be the immediate precursor of the mature virion into which the RNA is inserted or simply a byproduct of the assembly process (Ansardi et al., 1996).

Cowpea mosaic virus (CPMV) is the type member of the comovirus group of plant viruses and has a single-stranded, positive-sense, bipartite RNA genome. The two RNA molecules (RNA-1 and RNA-2) are separately encapsidated within icosahedral particles, the structure of which is known to atomic resolution (Lomonossoff and Johnson, 1991; Stauffacher *et al.,* 1987). Virus preparations can be separated into three components, designated top (T), middle (M), and bottom (B), by centrifugation on density gradients. The three components have identical protein compositions and contain 60 copies each of a large (L) and small (S) coat protein. T component is devoid of RNA, M component contains a single molecule of RNA-2, and B component contains a single molecule of RNA-1 (Bruening and Agrawal, 1967). The proportion of T component in a wild-type virus preparation is relatively small (<20%). The high degree of similarity in structure and genome organisation between the picornaviruses and the comoviruses (King *et al.,* 1991) suggests that their assembly may proceed by similar mechanisms, with the T component being the equivalent of the provirion.

CPMV virions can be separated into two forms, fast (f) and slow (s), according to their rate of migration toward the anode during electrophoresis (Agrawal, 1964). Both electrophoretic forms contain all three centrifugal components (T, M, and B), and thus the difference in mobility is unrelated to the RNA content of the particles (Semancik, 1966). The occurrence of different electrophoretic forms is not restricted to CPMV but appears to be a general property of comoviruses (Lomonossoff and Johnson, 1991). In the case of CPMV, the conversion from the s to the f form was shown to be accompanied by a loss of 20–30 amino acids from the carboxyl-terminus of the S subunits (Geelen *et al.,* 1972; Kridl and Bruening, 1983; Niblett and Semancik, 1969). This loss of carboxyl-terminal amino acids occurs naturally during infection in plants, with virus purified at a late stage in infection containing a higher proportion of particles containing

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carboxyl-terminally processed S-protein (Geelen *et al.,* 1972; Niblett and Semancik, 1969).

Attempts to precisely map the processing site have yielded different conclusions. On the basis of digestion with carboxypeptidase Y, Franssen *et al.* (1986) concluded that the processing event occurs after Leu190, resulting in the loss of 23 amino acids from the S-protein. However, x-ray crystallography of particles containing a mixture of the two electrophoretic forms showed that the carboxyl-terminus emerges onto the surface of the particle and that the electron density ends abruptly at Leu189, suggesting that the processing event occurs one residue upstream, resulting in the loss of 24 amino acids (Lomonossoff and Johnson, 1991). The biological significance of the two electrophoretic forms has been unclear. The loss of carboxyl-terminal amino acids from the S subunits appears not to affect the integrity of particles; f and s form particles have identical specific infectivities (Geelen *et al.,* 1973; Theuri *et al.,* 1996), and RNA isolated from both forms is structurally intact (Theuri *et al.,* 1996).

To investigate the role or roles of the carboxyl-terminal amino acids of the S-protein of CPMV in the viral replication cycle, the site of cleavage has been precisely mapped and a series of site-directed and deletion mutants have been produced. Analysis of the properties of these mutants indicates that the carboxyl-terminal amino acids, although not essential for virus infectivity, affect the rate of virus accumulation and spread and are involved in the efficient encapsidation of the viral RNAs.

RESULTS

Identification of processing site in the S-protein by electrospray mass spectrometry

To determine the point of processing in the S-protein, a sample of virus in which processing was complete as judged by SDS–PAGE was reacted with iodoacetamide under denaturing conditions and the S-protein was purified through HPLC. Electrospray mass spectrometry of the iodoacetamide-treated processed S-protein gave a molecular weight of 21121.8, which is in close agreement with the predicted weight of 21119.84 for modified S-protein (with three carboxamido-methyl groups), where the last residue is Leu189. Therefore, proteolytic processing results in the loss of the 24 residues from the S-protein.

Mutants containing premature stop codons rapidly revert

In initial experiments to investigate the role of the carboxyl-terminus of the S-protein in the replication cycle of CPMV, premature stop codons were introduced into RNA-2 either upstream of (pCP-SM2, pCP-SM3), precisely at (pCP-SM4), or downstream of (pCP-SM5; pCP-SM6) the region encoding the identified cleavage site (Fig. 1). Throughout this report, the designation pCP-SMx refers to the plasmid construct, whereas SMx refers to the resultant mutant virus. The premature stop codons in pCP-SM2 and pCP-SM3 were designed to give rise to S-proteins lacking features believed to be important for either the correct tertiary folding of the S-protein (pCP-SM2) or the interactions between the S and L proteins in the assembled virions (pCP-SM3). pCP-SM4 was designed to produce a S-protein exactly the size of the S-protein found in fast-form particles (i.e., lacking the carboxyl-terminal 24 amino acids), whereas pCP-SM5 and pCP-SM6 were designed to encode S-proteins lacking the carboxyl-terminal 16 or 7 amino acids, respectively.

Although the constructs truncated upstream of the natural cleavage site (pCP-SM2 and pCP-SM3) failed to produce an infection when inoculated onto cowpea plants, the constructs with truncations at (pCP-SM4) or downstream of (pCP-SM5 and pCP-SM6) the cleavage site produced symptoms on primary leaves. All three infectious constructs gave lesions on the inoculated leaves that were distinctly smaller than those produced by wild-type virus, and in the case of pCP-SM4, the lesions were surrounded by red, necrotic rings. However, none of the plants inoculated with the mutants developed systemic symptoms during the period of the experiment (21 days), whereas plants inoculated with wild-type virus all developed a distinct mosaic on their upper leaves by 12 days postinfection.

When sap was extracted from leaves infected with pCP-SM4, pCP-SM5, or pCP-SM6 DNA and used to inoculate healthy cowpea plants, all the plants developed symptoms characteristic of an infection with wild-type CPMV (i.e., large chlorotic lesions on the inoculated leaves and a systemic mosaic). Approximately wild-type yields of particles could be purified from both the inoculated and systemically infected leaves of such first-passage plants. For all three mutants, SDS–PAGE analysis of virions isolated from the trifoliate leaves of these first-passage plants gave a protein profile identical to that obtained with wild-type virus (data not shown), strongly suggesting that reversion had occurred. Sequence analysis of RT-PCR products derived from the RNA extracted from such particles confirmed that the premature stop codons had completely reverted to the wild-type codon in the case of SM4 and SM5. RNA extracted from a SM6 preparation contained two sequences in approximately equal amounts. One corresponded to the retention of the introduced termination codon, and the other represented pseudoreversion in which the stop codon had been changed to UGG (Trp) rather than the wild-type GGA (Gly).

Deletion mutants are genetically stable and have attenuated phenotypes

To produce genetically stable mutants with truncations in the carboxyl-terminal region of the S-protein, a series of mutants was produced in which deletions were made

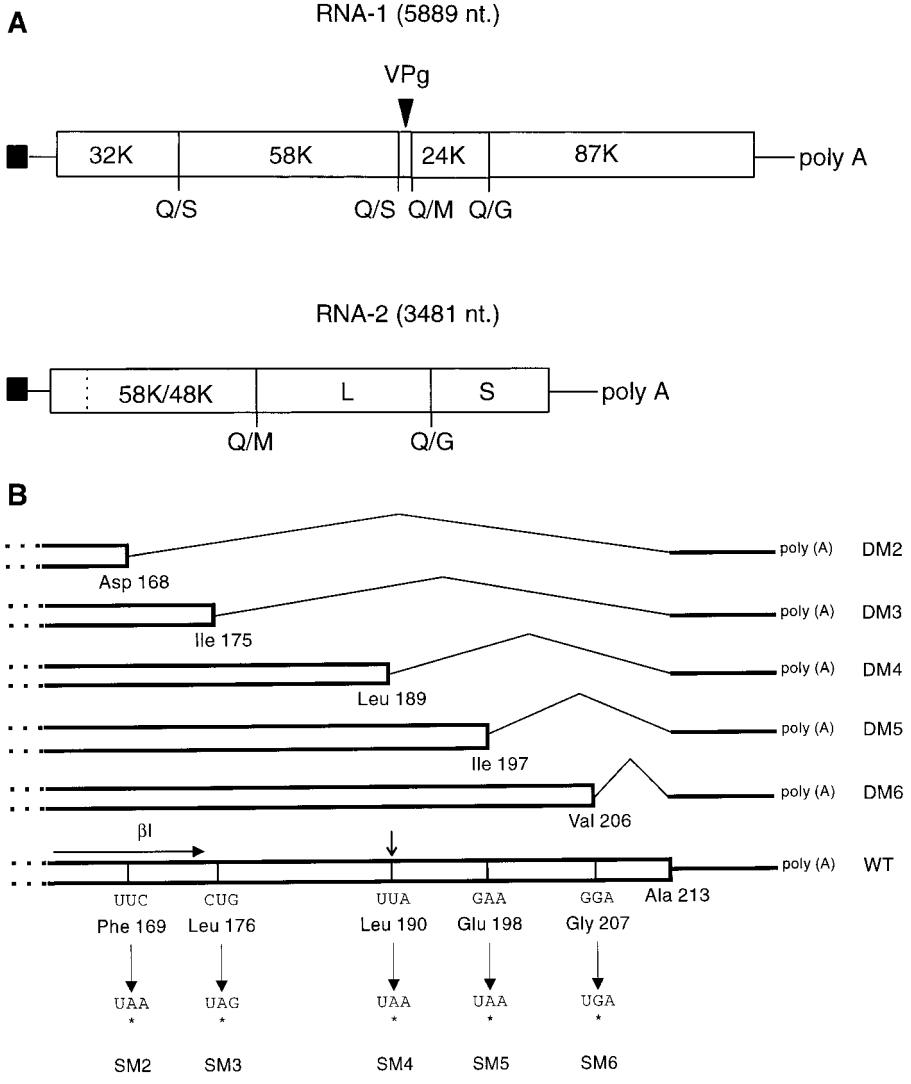

FIG. 1. (A) Organisation of the CPMV genome. The genome consists of two molecules of positive-strand RNA (RNA-1 and RNA-2). The genome-linked protein (VPg) at the 5′ end of both RNAs is indicated by the black square. The single long open reading frame (ORF) on each RNA is shown as an open rectangle. The positions of the polyprotein processing sites are indicated together with the dipeptide sequence that is cleaved (Q/S, Q/M, or Q/G). The identity of each final cleavage product is indicated (e.g., 32K). Both coat proteins (L and S) are encoded by RNA-2. (B) Schematic representation of the mutations introduced into the carboxyl-terminal region of the CPMV S-protein. The open rectangle represents the region of the ORF on RNA-2 that encodes the carboxyl-terminus of the S-protein. The position of the proteolytic cleavage site responsible for conversion from the slow to fast form of the virus is indicated by the bold arrow above the ORF of the wild-type (WT) S-protein. The last major structural element in the S-protein, the βI strand, is also indicated. The 3′ noncoding region of RNA-2 is shown as a solid line. The codons mutated to stop codons in the site-directed mutants SM2–SM6 are shown below the ORF for wild-type virus. The extent of the deletions in mutants DM2–DM6 are shown in the top half of the figure. The new carboxyl-terminal amino acids of the deleted S-proteins are indicated, and all the deletion mutants contained the entire 3′ noncoding region of RNA-2.

in pCP2 so that the S-protein would be truncated in a manner similar to that occurring in pCP-SM2 to pCP-SM6 (Fig. 1). The resulting plasmids were designated pCP-DM2 to pCP-DM6 (as in the case of the site-directed mutants, pCP-DMx refers to the plasmid and DMx the resultant virus). Consistent with the results obtained with pCP-SM2 and pCP-SM3, pCP-DM2 and pCP-DM3 produced no detectable infection when inoculated onto cowpeas, whereas deletion mutants pCP-DM4, pCP-DM5, and pCP-DM6 all produced symptoms on only the primary leaves, the appearance of which was delayed in comparison with wild-type. In addition, as was found for pCP-SM4, pCP-SM5, and pCP-SM6, the lesions were smaller (~1 mm in diameter) than those produced by wild-type CPMV (2–3 mm in diameter), and those observed on pCP-DM4-infected plants were surrounded by red, necrotic rings as previously observed with pCP-SM4 infections (Fig. 2).

When sap from leaves inoculated with pCP-DM4, pCP-DM5, or pCP-DM6 was used to infect further cowpea plants, the small lesion phenotype was maintained. Systemic symptoms eventually could be observed although

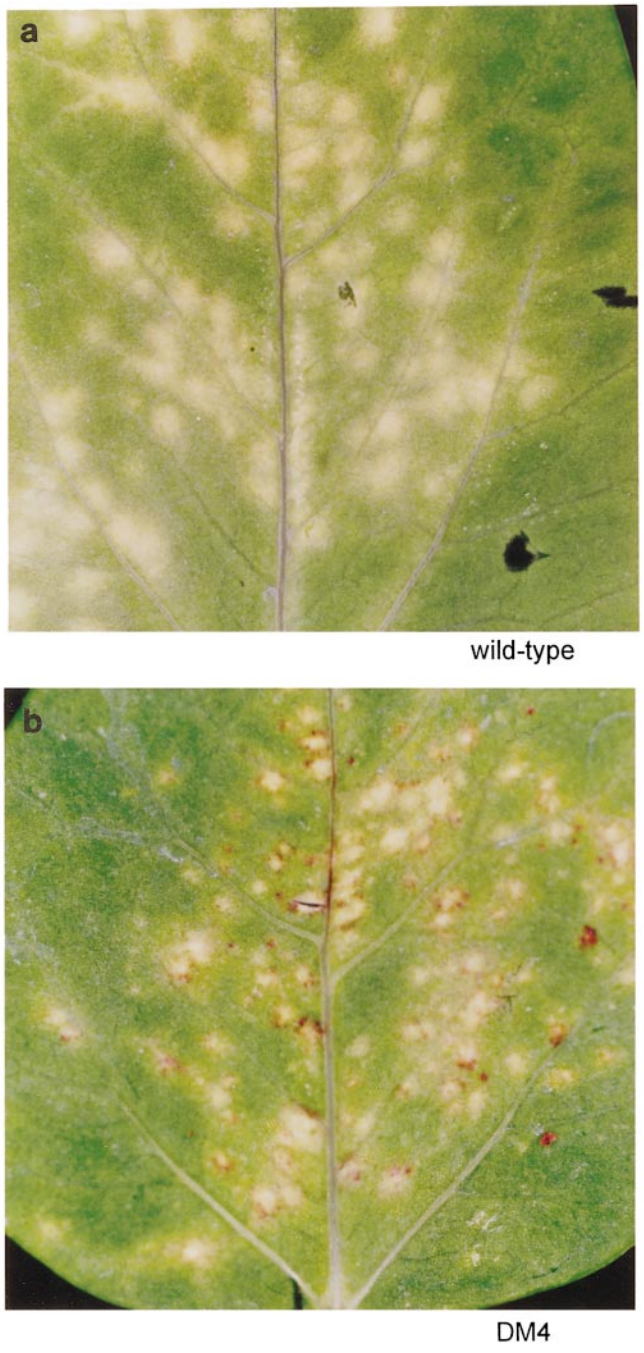

FIG. 2. Symptoms obtained on cowpea (*Vigna unguiculata*) leaves after inoculation with a 10 μg/ml solution (as estimated by $A_{260\ nm}$) of purified wild-type (a) or DM4 (b) virions. The photograph was taken 13 days postinoculation.

they were delayed 10–14 days compared with a wild-type infection. Virus particles could be purified from both the inoculated and systemically infected leaves, although the yields obtained were in each case lower than that obtained from the equivalent leaves infected with wild-type virus (Table 1). Repeated passaging consistently produced the same attenuated phenotype, and the appearance of the necrotic rings in the case of DM4 also was consistently reproducible. SDS–PAGE analysis of the mutant virus preparations indicated that the truncations in the S-protein were maintained through passaging, an observation confirmed by RT-PCR analysis of the RNA contained within the virus particles (data not shown). SDS–PAGE also indicated that cleavage after leucine 189 still occurs in mutants DM5 and DM6. The degree to which the viable deletion mutants are debilitated with respect to yield, as judged by the $A_{260\ nm}$ value of the virus preparations (see below), is roughly correlated with the size of deletion (Table 1).

Preparations of the deletion mutants have a lower specific infectivity than wild-type virus

Opposite half-leaves of the local lesion host *Phaseolus vulgaris* were inoculated with purified wild-type CPMV and virions purified from either DM4-, DM5-, or DM6-infected leaves. Two concentrations, 0.001 or 0.01 mg/ml, as judged on the basis of $A_{260\ nm}$, were used. At 0.001 mg/ml, no lesions were observed on plants inoculated with the DM4 virus preparation, whereas DM5- and DM6-inoculated half-leaves showed approximately half the number of lesions produced by the equivalent amount of wild-type virus (Fig. 3A). At 0.01 mg/ml, no significant difference was observed between the numbers of lesions/half-leaf for DM5 and DM6 in comparison with wild-type virus, suggesting that saturation is being approached at this high level of inoculum (Fig. 3B). However, even at this high level of inoculum, half-leaves inoculated with the DM4 virus preparations still showed on average less than half the number of lesions compared with those inoculated with wild-type.

The dramatically reduced specific infectivity of DM4 virions compared with wild-type virions suggested that the DM4 preparation might contain substantially less infectious viral RNA than normal CPMV preparations. If this were the case, the $E^{0.1\%}_{260nm}$ of the preparation would differ considerably from the value used to calculate the amount of virus applied to the leaves, making an accurate comparison of the relative specific infectivities of

TABLE 1

Yields of Deletion Mutant Viruses

Virus	Yield range (% of wt)	
	Primary leaves	Secondary leaves
Wild-type	100	100
DM4	0.5–3.3	11–45
DM5	8.8–27	15–60
DM6	28–40	65–75

Note. The range of yields of virus purified from the primary and secondary leaves of plants infected with the deletion mutants DM4, DM5, and DM6. The yields are expressed as a percentage of the yield of the wild-type virus obtained from control plants in each experiment.

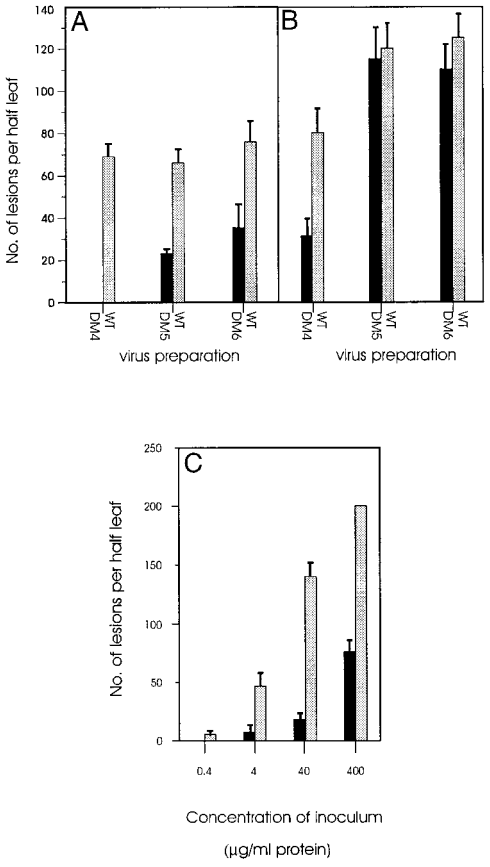

FIG. 3. Specific infectivities of purified preparations of deletion mutants DM4, DM5, and DM6 compared with wild-type (WT) virus. (A and B) Equal weights, as determined by $A_{260\ nm}$, of mutant and wild-type virus were inoculated onto opposite half-leaves of *P. vulgaris*. The number of local lesions obtained after inoculation with 0.001 mg/ml (A) or 0.01 mg/ml (B) of virus is shown. (C) Number of lesions obtained when equal weights of DM4 and wild-type virus, as judged by their protein content, were inoculated in a dilution series. The black bar represents the average number of lesions obtained with a deletion mutant, and the corresponding gray bar represents the number obtained on the opposite half-leaves with wild-type virus. The average number of lesions per half-leaf is shown, and the error bars represent the SEM.

wild-type and DM4 virions impossible. To overcome this problem, the local lesion assay was repeated using DM4 and wild-type virus preparations diluted to the same concentration of protein as judged by the Bradford assay. To this end, DM4 and wild-type virus preparations at protein concentrations of 0.4, 0.04, 0.004, and 0.0004 mg/ml were inoculated onto *P. vulgaris* half-leaves, with each pair of half-leaves being inoculated with DM4 and wild-type virus at the same concentration. The number of lesions/half-leaf is shown in Fig. 3C. The dose-response is probably saturated at 0.4 mg/ml protein (equivalent to ~0.5 mg/ml virus particles for wild-type virions), whereas at lower concentrations, DM4 virus preparations were estimated to contain ~6-fold fewer infectious units in comparison with wild-type CPMV when inoculated on an equal protein basis.

DM4 virus preparations contain a greatly increased proportion of empty capsids

In view of the reduced specific infectivity of the deletion mutants, the component composition of preparations of purified virus was determined. In the first instance, RNA extracted from the purified preparations was examined by electrophoresis on denaturing agarose gels. The relative proportions of RNA-1 and RNA-2 were similar to those found for wild-type virus, and the RNAs were undegraded (data not shown). However, when RNA was extracted from apparently equal amounts (as judged by $A_{260\ nm}$) of DM4 and wild-type virions, the yield of RNA appeared to be much lower in the case of DM4 as judged by the intensity of ethidium bromide staining. Electron microscopy of negatively stained preparations of DM4 indicated that the particles had a morphology typical of wild-type CPMV, although there appeared to be a higher proportion of particles into which the stain could penetrate.

To analyse the component composition of preparations of the deletion mutants, samples of purified virus particles were centrifuged on Nycodenz gradients, and the components were visualised by light scattering. Although preparations of DM5 and DM6 gave patterns similar to that obtained with wild-type CPMV, preparations of DM4 appeared to contain a much higher propor-

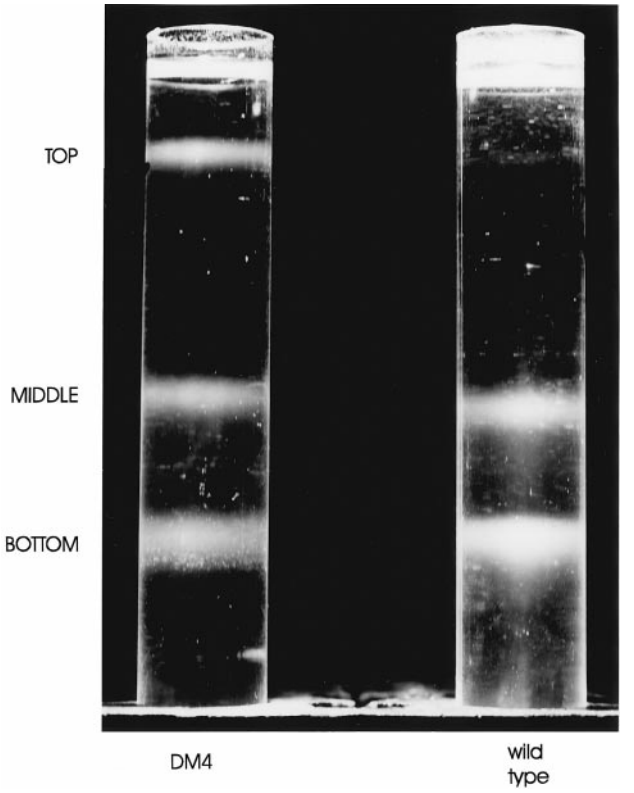

FIG. 4. Component composition of purified preparations of DM4 (right) and wild-type (left) virus. The components were identified by their positions after centrifugation in Nycodenz gradients.

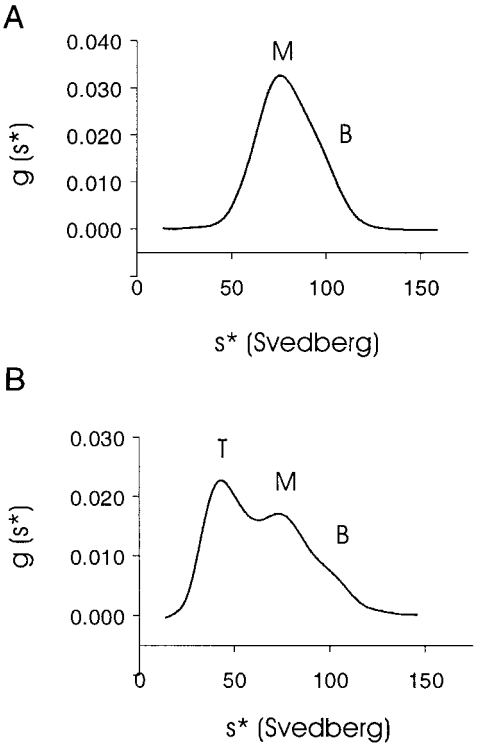

FIG. 5. Analytical ultracentrifuge data for wild-type virus (A) and DM4 (B). The data are shown as g(s*) versus s* plots, which show the amount of a particular component at a particular sedimentation coefficient. The peaks were assigned to a component (T, M, and B) on the basis of their sedimentation behaviour.

tion of material with the density of T component (Fig. 4). For a more detailed, quantitative analysis, virus preparations grown at the same time were analysed by analytical ultracentrifugation under conditions designed to give a good separation between the empty capsids and the nucleoprotein components. Fig. 5 shows the resultant g(s*) versus s* plots for wild-type virus and DM4. These plots show the amount of a given component against its sedimentation coefficient (Stafford, 1994a) and demonstrate that preparations of DM4 do indeed contain far more material sedimenting at the position of T components (empty capsids) than do preparations of wild-type virus. With the analytical ultracentrifugation data, it is possible to quantify the relative amount of each of the three components. The results of such a calculation for all three viable deletion mutants and wild-type virus are shown in Table 2. Such calculations confirm that the DM4 virus preparation contained a much higher proportion of T component (73%) than wild-type virus and that the proportion of M and B components remained roughly equal. Preparations of the other deletion mutants, DM5 and DM6, did not appear to have significantly enhanced levels of T components.

DISCUSSION

The results of the electrospray mass spectrometry analysis of the fully processed CPMV S-protein confirm the prediction based on crystallographic data (Lomonossoff and Johnson, 1991) that the processing results in the loss of 24 rather than 23 amino acids from the carboxyl-terminus of the protein. The fact that only a single species was found indicates that the limit of processing is precise, although it does not rule out the possibility that intermediates in the processing pathway might exist. The viability of mutants containing premature stop codons or deletions up to and including the last residue, which is lost (Leu190), demonstrates that the cleaved sequence is not essential for virus particle formation because mutations that destroy the functionality of the coat proteins are known to abolish infectivity on plants (Wellink and van Kammen, 1989). The necessity of functional coat proteins for infectivity on whole plants is confirmed by the nonviability of constructs pCP-SM2, pCP-SM3, pCP-DM2, and pCP-DM3, which contain mutations that are predicted to affect either the tertiary structure of the S-protein itself (pCP-SM2, pCP-DM2) or the ability of the S-protein to interact with the L-protein (pCP-SM3, pCP-DM3). The small lesion phenotype and the rapid reversion of the premature stop codons in pCP-SM4, pCP-SM5, and pCP-SM6 indicate that the residues cleaved from the carboxyl-terminus of the S-protein, although not essential for virus viability, have a significant role in the viral replication cycle. However, the genetic instability of the viable SM mutants precluded their use in elucidating this role.

The deletion mutants, DM4, DM5, and DM6, all displayed attenuated phenotypes similar to those observed with the primary inoculations of their equivalent site-directed mutants. The major difference was that the phenotypes were consistent over a number of serial passages and, unsurprisingly, no reversion could be detected. All three mutants gave small lesions on the inoculated leaves, delayed systemic spread, and low virus yields, confirming the importance of carboxyl-terminal 24 amino acids of the S-protein. The reduction in virus yield is correlated with the size of the deletion, with DM4, the mutant that encodes a S-protein exactly equivalent to that occurring in the fast electrophoretic form of CPMV, being the most debilitated. The debilitation does not result from impaired RNA replication because experi-

TABLE 2

Proportion of Top, Middle, and Bottom Components

Virus	% T	% M	% B
Wild-type	N.D.	40	60
DM4	73	16	11
DM5	N.D.	38	62
DM6	16	34	50

Note. The percentage of top (T), middle (M), and bottom (B) components in typical preparations of each of the deletion mutants DM4, DM5, and DM6 compared with wild-type virus. N.D., none detected.

ments in protoplasts indicate that the RNA-2 from the deletion mutants can replicate as well as wild-type RNA-2 (P. Bertens and J. Wellink, personal communication). Thus the truncation at the carboxyl-terminus of the S-protein is caused by changes to the protein itself either affecting the ability of particles to assemble and/or reducing the ability of the assembled particles to move through the plant both locally (small lesions on inoculated leaves) and systemically (delayed appearance of symptoms on the upper leaves).

As well as being produced in reduced amounts, the particles produced by mutants pCP-DM4, pCP-DM5, and pCP-DM6 have reduced specific infectivities. This reduction in specific infectivity is not due to a failure of the deleted RNA-2 molecules to be encapsidated. Because the RNA-1 molecules of the deletion mutants are wild-type, any defective encapsidation of the modified RNA-2 molecules would be expected to result in preparations containing an aberrantly high proportion of RNA-1-containing particles, something that was not found. In the case of DM5 and DM6, the reduction in specific infectivity is modest and its significance is unclear. However, in the case of DM4, the effect is far more dramatic and was shown by density gradient and analytical ultracentrifugation, to be accompanied by a great increase in the proportion of nucleic acid-free capsids (T component).

Within the carboxyl-terminal 24 amino acids of the CPMV S-protein, there are four arginine and two lysine residues, giving this sequence an overall positive charge. None of these basic amino acids are removed in DM6, and two arginine and one lysine are removed in DM5, whereas DM4 lacks all these residues. Thus it is possible that the carboxyl-terminus is involved in the encapsidation of viral RNA through the binding of these basic residues to the phosphodiester backbone of the viral RNA. Because the effect on yield and proportion of T components is most dramatic for DM4, the results suggest that it is the three basic amino acids (Lys191, Arg193, and Arg195) present in DM5 but not in DM4 that are most important in this role. The equivalent carboxyl-terminal regions of the S-proteins of two other comoviruses for which structural information is available also contain basic residues: three lysines in the case of red clover mottle virus and a single arginine in the case of bean pod mottle virus. It is possible that only a single residue could be involved in RNA binding because deletion of a single lysine residue at the amino-terminus of the brome mosaic virus coat protein prevented virion assembly (Rao and Grantham, 1995).

The mechanism by which the carboxyl-terminus of the S-protein promotes RNA encapsidation is uncertain. The sequence clearly is not absolutely required because a proportion of the capsids in a DM4 virus preparation do contain nucleic acid. Thus other regions of the capsid protein must be able to interact with the viral RNA. It seems, rather, that the carboxyl-terminal region of the S-protein acts in such a way as to enhance RNA encapsidation. At first sight, the location of the carboxyl-terminal sequence on the external surface of the virion particle would seem to argue against it having such a role. However, it is possible that the topology of the capsid could change during the assembly process. Changes in the topology of the coat proteins of icosahedral virus particles have been described in the case of poliovirus (Fricks and Hogel, 1990; Roivainen *et al.,* 1991) and flock house virus (Bothner *et al.,* 1998), although such changes have not, as yet, been associated with RNA encapsidation. The positive charges on the exposed carboxyl-terminus of the S-protein could direct viral RNA toward an "assembly complex," after which further specific interactions would occur, resulting in the RNA being internalised in the mature particle. The hypothesis of a role for the carboxyl-terminus only in the initial stages of assembly is compatible with its dispensability on the mature particles. Such an initial role would also indicate that empty capsids are not precursors of mature virions but rather a byproduct of assembly.

An intriguing observation concerns the fact that mutants SM4 and DM4 produced a distinctive host response in which red, necrotic rings were observed around the primary lesions. This necrosis was consistently obtained through serial passaging of DM4 and was never seen with the other deletion mutants or with wild-type CPMV. Coat protein mutations that result in conversion to a necrotic phenotype have been observed with other plant viruses. For example, deletion of the seven amino-terminal amino acids of the brome mosaic virus coat protein resulted in a virus that had a necrotic phenotype (Rao and Grantham, 1995). Furthermore, mutations that disrupt the quaternary structure of the coat protein of the U1 strain of tobacco mosaic virus convert the virus from being a nonelicitor of the N′ hypersensitive response to one that produces a hypersensitive response in N′ gene-containing *Nicotiana sylvestris* (Taraporewala *et al.,* 1996). These observations have generally been interpreted as indicating that an elicitor sequence has been unmasked in the mutant coat protein. If this interpretation is correct, it suggests that an additional role of the carboxyl-terminus of the S-protein may be to mask a potential elicitor of a host defence mechanism. However, because the induction of necrosis is observed only with DM4, the additional eight amino acids present in DM5 must be sufficient for any such masking property. An alternative hypothesis to account for the induction of necrosis is that the mutant coat proteins encapsidate the virion RNA with reduced efficiency and that it is the subsequently elevated levels of unencapsidated viral RNA rather than the modified protein itself that stimulate the necrogenic response. This alternative explanation is particularly attractive in the case of DM4 because this mutant has been shown to have a reduced ability to encapsidate the virion RNAs but

is predicted to produce capsids identical in structure to those found during an infection with wild-type virus.

MATERIALS AND METHODS

Mass spectrometry

S-protein samples were prepared from a fully cleaved CPMV preparation by the addition of iodoacetamide to 20 mM, degassing, and then the addition of an equal volume of 5.3 M guanidinium chloride, 3.3 M LiCl, 0.02 M boric acid, and 0.01 M NaOH. The reaction was allowed to proceed overnight at 0°C before centrifugation (~12,000*g* for 10 min) and collection of the supernatant. The pellet was washed twice with 1/10th the original volume of this solution diluted 1:1 with 20 mM iodoacetamide and the supernatants combined. Additional iodoacetamide was added (10 mM), and then solid guanidinium chloride was added to 5 M. The reaction was continued at 0°C for 2 h and then terminated by the addition of 2-mercaptoethanol to 50 mM.

The modified S-protein was purified by HPLC with a trifluoroacetic acid/acetonitrile gradient and gave a single peak, corresponding in an SDS–polyacrylamide gel to the cleaved S-protein. A sample from this peak was injected directly into an electrospray ionisation spectrometer (Sciex API III Plus triple quadrapole mass spectrometer; Perkin–Elmer Cetus).

Construction of mutants

Two series of mutants with premature termination codons or deletions in the carboxyl-terminus of the S-protein of CPMV were produced by site-directed mutagenesis (Kunkel *et al.,* 1987). The template for mutagenesis was a M13mp18 derivative containing the 2-kb *Bam*HI/*Eco*RI fragment from pCP2, an infectious cDNA clone of CPMV RNA-2 (Dessens and Lomonossoff, 1993). This restriction fragment corresponds to the region of RNA-2, which encodes both viral coat proteins. The oligonucleotides used to introduce premature termination codons were 5′-GATCCTAATTAAAGGGTTGCC-3′ (pCP-SM2), 5′-GGCAATATCTAGATGCCCCCA-3′ (pCP-SM3), 5′-CCACCGTTATAAAAGTTTAGG-3′ (pCP-SM4), 5′-CGGGATATTTAACGCTCCAAG-3′ (pCP-SM5), and 5′-GTTATGGTTTGACACACTGCT-3′ (pCP-SM6), where the premature stop codons are underlined.

The oligonucleotides used to create the deletion mutants consisted of a common 3′ arm, 5′-TAACTCTGGTTTCATTAAATTTTC-3′, and the following 5′ arms: 5′-CATGCGCTTCGATCCTAAT-3′ (pCP-DM2), 5′-TCAGGGTTGCCGGCAATATT-3′ (pCP-DM3), 5′-CAACGGAAACTCCACCGTTA-3′ (pCP-DM4), 5′-AAGTTTAGGTTTCGGGATATT-3′ (pCP-DM5), and 5′-CCAAGCGTAGTGTTATGGTT-3′ (pCP-DM6).

Introduction of the desired mutation was confirmed by DNA sequence analysis, and the modified *Bam*HI/*Eco*RI fragments were ligated back into *Bam*HI/*Eco*RI-digested pCP2.

Virus propagation and purification

The pCP-2-based plasmids were linearised with *Eco*RI and mixed with *Mlu*1-digested pCP1 (an infectious clone of CPMV RNA-1), and the DNA was used to inoculate cowpea plants as previously described (Dessens and Lomonossoff, 1993). The plants were subsequently maintained in a greenhouse at 25°C. For passaging experiments, either purified virus or sap extracted from infected plants was used as an inoculum.

Virus particles were purified as described by van Kammen and de Jager (1978) and stored at 4°C in 10 mM sodium phosphate, pH 7.0, containing 0.05% (w/v) sodium azide. Virus concentration was measured spectrophotometrically using the relationship $E^{0.1\%}_{260nm} = 8.1$ for purified virus containing a natural mixture of components. The protein concentration of virus preparations was determined according to the method of Bradford (1976) using the Bio-Rad protein assay kit according to the manufacturer's instructions with γ-globulin as a standard.

RT-PCR analysis of viral RNA

RNA was extracted from virus preparations as previously described (Lomonossoff *et al.,* 1982). First-strand cDNA was synthesised after annealing a portion of the purified RNA to an oligo(dT)-based primer [5′-GACTCGAGTCGACATCGA$(T)_{17}$-3′]. Synthesis was performed using the Superscript synthesis kit (GIBCO BRL) according to the manufacturer's instructions. PCR was performed using varying amounts of cDNA and 100 ng of a primer (5′-TGTGTTGCTACCAATCCCAG-3′) corresponding to bases 3101–3120 of RNA-2 and 100 ng of a primer (5′-CTTGCTGAAGGGACGACCTGC-3′) that is complementary to bases 3436–3456 of RNA-2. Reactions were carried out using Amplitaq DNA polymerase (Perkin–Elmer Cetus) in the buffer supplied with the enzyme. DNA was amplified by 30 cycles of incubation at 95°C for 1 min, 40°C for 2 min, and 72°C for 3 min followed by a final incubation step at 72°C for 10 min. Amplified DNA was analysed by electrophoresis through 2% (w/v) agarose gels. PCR products were directly sequenced using the T7 sequencing kit with a primer corresponding to bases 3120–3140 of RNA-2 (5′-GACAAATACAGCAATTTGAGG-3′).

Specific infectivity assay

Opposite half-leaves of the local lesion host *P. vulgaris* were inoculated with a mutant and a wild-type virus preparation, and the plants were grown as described above. Five half-leaves were inoculated with each mutant under investigation. The number of lesions per half-leaf was counted at 14 days postinoculation.

Density gradient analysis of virus preparations

Samples of purified virus particles were layered on top of 30–60% (w/v) Nycodenz (Nycomed, Oslo, Norway) gradients buffered with 10 mM sodium phosphate, pH 7.0. The gradients were centrifuged at 36,000 rpm for 20 h at 4°C in a Beckman SW41 rotor. Bands corresponding to T, M, and B components were visualised by shining light down the length of the tubes.

Analytical ultracentrifugation

Samples of 0.4 ml of virus with an $A_{280\ nm}$ value of 1.0–2.5 were analysed on a Beckmann Optima XLA ultracentrifuge (Giebeler, 1994) at 25,000 rpm with an An 60 Ti rotor at 5°C. Cells were scanned at 280 nm. The distribution of sedimentation coefficients was analysed using plots of g(s*) against s* (Stafford, 1992, 1994a, 1994b) using the software supplied with the instrument. The proportions of T, M, and B components were calculated from scans when the boundaries were well separated, correcting for radial dilution according to Schachman (1959) and allowing for the differential absorbance of the components (van Kammen and de Jager, 1978).

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