

Maize streak virus coat protein binds single- and double-stranded DNA *in vitro*

Huanting Liu, Margaret I. Boulton and Jeffrey W. Davies

Department of Virus Research, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

Maize streak virus (MSV) coat protein (CP) is required for virus movement within the plant. Deletion or mutation of MSV CP does not prevent virus replication in single cells or protoplasts but leads to a loss of infectivity in the inoculated plant. The mechanism by which MSV CP mediates the transfer of MSV DNA from cell to cell and through the vascular bundle is still unknown. Towards understanding the role of MSV CP in virus movement, the interaction of the CP with viral DNA was investigated using the 'south-western' assay. Wild-type and truncated MSV CPs were expressed in *E. coli* and the

expressed CPs were used to investigate interactions with single-stranded (ss) and double-stranded (ds) DNA. The results showed that MSV CP bound ss and ds viral and plasmid DNA in a sequence non-specific manner. The binding domain was mapped to within the 104 N-terminal amino acids of the MSV CP. We propose that the binding of CP to MSV DNA is involved in viral DNA nuclear transport as well as encapsidation and thus may have a role in intra- and inter-cellular movement as well as systemic infection.

Introduction

Maize streak virus (MSV) is the type member of genus (subgroup) I of the family *Geminiviridae* (Murphy *et al.*, 1995). It has a monopartite genome of single-stranded (ss) circular DNA (Mullineaux *et al.*, 1984; Howell, 1985; Lazarowitz, 1988) and is transmitted by a leafhopper (*Cicadulina mbila*). Virus replication is via a double-stranded (ds) DNA replicative form, which is bidirectionally transcribed (Morris-Krsinich *et al.*, 1985). Four genes are expressed (Mullineaux *et al.*, 1984; Lazarowitz, 1992): C1 and C2, encoded by the complementary (C) sense transcripts, are required for virus replication; V1 and V2, encoded by the virion (V) sense transcripts, are required for systemic infection and subsequent disease development (Boulton *et al.*, 1989, 1993; Lazarowitz *et al.*, 1989). The MSV V1 gene encodes a 10.9 kDa protein that can be detected *in vivo* (Mullineaux *et al.*, 1988), and mutagenesis studies of this gene have shown that it encodes a movement protein (MP; Boulton *et al.*, 1993). Gene V2 encodes the coat protein (CP), mutation of which prevents the accumulation of viral ssDNA in protoplasts and around the inoculation site in plants (Boulton *et al.*, 1989, 1993).

The mechanism by which the two virion-sense proteins of the subgroup I geminiviruses mediate systemic infection is not

known, but the requirements for movement of bipartite (subgroup III) geminiviruses have been studied more thoroughly. The bipartite geminivirus DNA B component encodes two MPs, BR1 and BL1 (recently renamed BV1 and BCI, respectively), which are required for virus movement (reviewed by Lazarowitz, 1992). The BL1 protein has been shown to increase the size exclusion limit of plasmodesmata (Noueiry *et al.*, 1994) whereas the BR1 protein traffics ss (Pascal *et al.*, 1994) or ds (Noueiry *et al.*, 1994) viral DNA into and out of the nucleus. Thus BL1 and BR1 have distinct and essential roles in cell-to-cell movement (Noueiry *et al.*, 1994; Pascal *et al.*, 1994). Both also have a role, and may act in combination with CP, in systemic movement (Ingham *et al.*, 1995; Jeffrey *et al.*, 1996; Pooma *et al.*, 1996). Although MSV has no B component, the requirements for virus movement are likely to be similar, and MSV V1, or V1 and V2, may share the functions of BR1 and BL1. To investigate the functions of the MSV CP in virus movement and encapsidation, binding of intact and truncated CPs with viral ss and dsDNA was analysed.

Methods

■ **Virus purification and antiserum preparation.** Virus purification was carried out as described by Bock *et al.* (1974) except that 2 vols (w/v) 0.1 M phosphate buffer (pH 5.6) containing 0.1% thioglycolic acid and 0.5% Triton X-100 and 1 vol. (w/v) chloroform were used during the homogenization of infected tissue. The final pellet was

Author for correspondence: Jeff Davies.

Fax +44 1603 456844. e-mail daviesjw@bbsrc.ac.uk

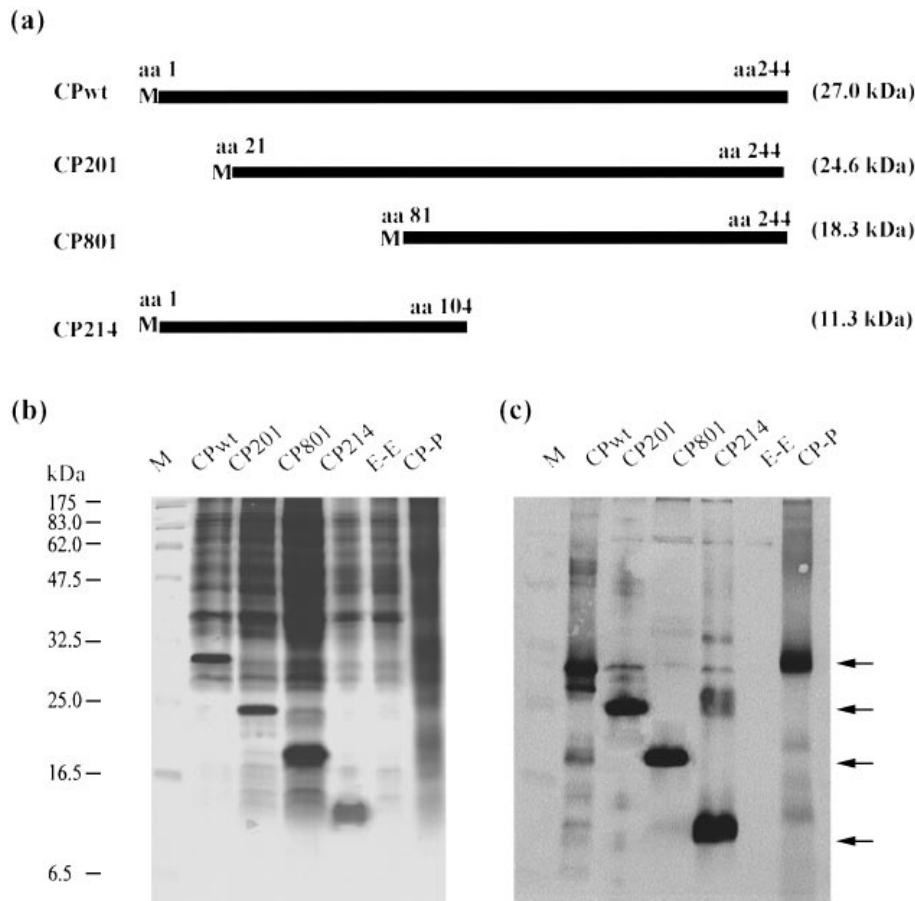


Fig. 1. Accumulation of CPs in *E. coli* BL21 (DE3) transformed with wt and mutant CP genes assessed by SDS-PAGE. (a) Schematic representation of the expressed wt and mutant MSV CPs. The molecular sizes were calculated by computer. (b) Coomassie blue stained gel. (c) Western blot using a polyclonal antiserum raised against purified MSV. Lanes CPwt, CP214, CP201 and CP801 in (b) and (c) were extracts of the cells expressing these proteins respectively (arrows denote the positions of the CP and its derivatives); E-E, extract of *E. coli* cells transformed with pET3a; CP-P, infected plant extract; M, protein molecular size markers.

resuspended in 0.1 M phosphate buffer (pH 5.6). The virus concentration was determined by UV absorption spectrophotometry as described by Brakke (1990).

Rabbit polyclonal anti-MSV serum was produced using purified virions as the immunogen, essentially as described by Pinner & Markham (1990).

Cloning of the MSV CP gene in an *E. coli* expression vector.

For cloning into the *E. coli* protein expression vector pET3a (Novagen), the MSV CP gene was amplified by PCR. For amplification of the wild-type (wt) CP gene and the CP genes with N-terminal deletions, an infectious full-length tandem dimer clone, pMSV-Ns (Boulton *et al.*, 1991), was used as the template. The 3'-end primer 5' ACGGCGTTGGATCCATTACTG 3' (the *Bam*HI site introduced to facilitate cloning is underlined) and 5'-end primers 5' CATTCACATATGTCCACGTCC 3', 5' GGGTGACTAAGCATATG-CCTTCTT 3' or 5' CACACCAGCCATATGCTGACGTAC 3' (the *Nde*I site introduced for cloning is underlined) were used to synthesize CPwt, CP201 (20 amino acids deleted from the N terminus) or CP801 (80 amino acids deleted from the N terminus), respectively. To produce a C-terminally truncated MSV CP (CP214), mutant pMSV214 (Boulton *et al.*, 1989) was used as the template and primers 5'

CATTCACATATGTCCACGTCC 3' and 5' ACGGCGTTGGATCC-ATTACTG 3' provided the 5' *Nde*I and 3' *Bam*HI sites respectively. In all cases translation was initiated from a methionine codon. The coding capacity of these genes is shown in Fig. 1(a).

■ **Construction of CP expression vectors.** The amplified MSV CP gene products were digested with *Nde*I and *Bam*HI and the expression constructs pETCPwt, pETCP201, pETCP801 or pETCP214 were obtained by ligating the appropriate fragment into a similarly digested pET3a vector. The ligation mixture was transformed into *E. coli* strain DH5 α , and the amplified wt CP gene was sequenced to confirm its integrity.

■ **MSV CP expression.** Recombinant plasmids pETCPwt, pETCP201, pETCP801 or pETCP214 were transformed into *E. coli* strain BL21(DE3), which contains the T7 RNA polymerase gene under the control of the *lac* UV5 promoter (Studier & Moffatt, 1986). The expression of CP was induced by addition of IPTG (final concentration 0.4 mM) to a log-phase culture (OD₆₀₀ 0.25). Cultures were grown for a further 16 h before cells were harvested by centrifugation for protein analysis.

■ **Analysis of the expressed MSV CPs.** Coat protein expression was assessed by SDS-PAGE (Laemmli, 1970) using a stacking gel of 3%

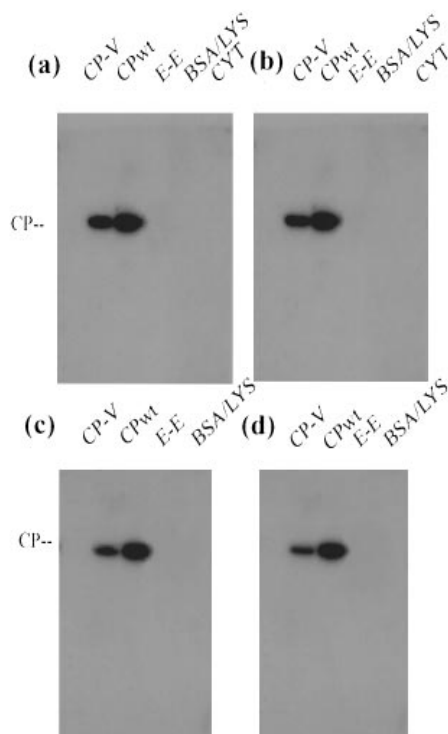


Fig. 2

Fig. 2. 'South-western' assay of MSV CP-DNA binding ability. Autoradiographs were obtained using either MSV dsDNA probe (a), MSV ssDNA probe (b), pUC19 plasmid dsDNA probe (c) or pUC19 plasmid ssDNA probe (d). E-E, CPwt, as described in the legend to Fig. 1. CP-V, purified virus; BSA/LYS, bovine serum albumin and lysozyme; CYT, cytochrome *c*.

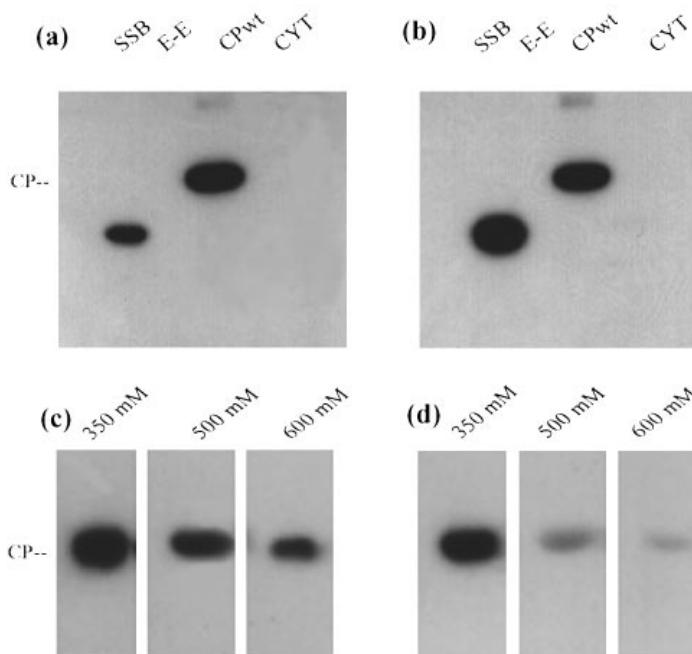


Fig. 3

Fig. 3. 'South-western' assay of MSV CPwt ss and dsDNA binding. Autoradiographs show (a) CP binding to MSV dsDNA probe or (b) ssDNA probe at 250 mM KCl concentration. CPwt binding to MSV dsDNA probe (c) or ssDNA (d) at 350, 500 or 600 mM KCl. CPwt, E-E, as described in the legend to Fig. 1; CYT, as for Fig. 2; SSB, *E. coli* ssDNA binding protein.

and a separating gel of 15%. Cells from 50 μ l culture were collected by centrifugation and boiled for 10 min with 100 μ l 1 \times loading buffer. The supernatant was collected after brief centrifugation and 15 μ l loaded onto the gel. An extract of MSV infected maize was loaded to provide a CP control. Molecular sizes of the CPs were determined by comparison with prestained protein markers (SeeBlue, Novex) on either Coomassie blue stained gels or western blots. For western analysis, proteins were electroblotted onto nitrocellulose membrane using a semi-dry transfer cell (Bio-Rad) at 12 V for 40 min and detected using MSV CP antiserum essentially as described by Towbin *et al.* (1979).

■ **Synthesis of radioactive probes.** A 2.7 kb *Bam*HI fragment from pMSV-Ns was used as the template for synthesis of the MSV probe; for non-MSV probes, linearized pUC19 plasmid DNA, or a 1.8 kbp *uidA* (*gus*) gene fragment (excised from pJIT166; Guerineau *et al.*, 1992) were used. Probes were synthesized using a DNA labelling kit ('Ready-To-Go', Pharmacia) and separated from unincorporated nucleotides using a Sephadex G-25 mini column. The probe was used directly for the dsDNA probe and the ssDNA probe was achieved by boiling the probe for 3 min followed by rapid dilution and incubation on ice as described by Pascal *et al.* (1994). For comparative studies, the same amounts of ss and ds probes were used.

■ **MSV CP binding by 'south-western' analysis.** To assess CP-DNA binding, the 'south-western' method was used as described by Sukegawa & Blobel (1993), except that the reaction buffer contained

250 mM KCl. The proteins were fractionated by SDS-PAGE and then electrotransferred onto a nitrocellulose membrane. After renaturation, the proteins were probed with a radioactive DNA probe. In this experiment, cells from 15 μ l culture were boiled for 10 min with 30 μ l 2 \times loading buffer and subjected to SDS-PAGE and electroblotting. In all cases, extracts from *E. coli* cells transformed with pET3a were also loaded. To assess non-specific binding to large amounts of protein, 4 μ g BSA, lysozyme or cytochrome *c* were also loaded.

To analyse the relative strength of CP binding to ss or dsDNA, the binding assay was carried out as before except that the blots were washed in buffer containing 250, 350, 500 or 600 mM KCl. As a control, 0.2 μ g *E. coli* ssDNA binding protein (SSB, Sigal *et al.*, 1972; purchased from Promega) was used at the lowest salt concentration.

To map the DNA binding domain, approximately equal amounts of CP and its truncated derivatives (CPwt, CP201, CP801 or CP214, Fig. 1a) were used for 'south-western' analysis. After autoradiography, the membrane was washed with 500 mM KCl, 0.1% Triton X-100 to facilitate immunolocalization of the blotted proteins.

■ **Computer-assisted analysis of the N-terminal peptide sequence.** The first 104 amino acids of the CP were subjected to computer-assisted analysis to search for recognized DNA binding and functional motifs. The protein analysis programs used were accessed through the NCSA Biology Workbench web site at the National Center for Supercomputing Applications, Urbana, Illinois, USA (<http://biology.ncsa.uiuc.edu>) and included FASTA, HTH and ZIPSCAN; the

PSORT program, coded by K. Nakai, was accessed at <http://psort.nibb.ac.jp/form.html>.

Results

Expression of MSV CP and its truncated derivatives in *E. coli*

Coomassie blue staining showed that polypeptides of the predicted sizes were produced by *E. coli* cells transformed with each of the CP-based pET constructs following induction by IPTG (Fig. 1*b*). None of these polypeptides was seen in uninduced cell extracts (data not shown). The induction conditions and sampling time were optimized to give the best yield of CP. The highest accumulation was achieved when IPTG was added at cell density $OD_{600} = 0.25$ and sampling was done at 16 h post-induction (data not shown).

The expressed wt and truncated CP had mobilities consistent with CP seen in the infected plant extract (Fig. 1*b, c*), or expected from the molecular masses predicted by computer analysis (Fig. 1*a*). In all cases, the polypeptides were prominent on Coomassie blue stained gels and all were recognized by MSV CP antiserum on western blots (Fig. 1*c*). No signal was seen with the *E. coli* cells transformed with the pET3a vector. Fractionation of *E. coli* extracts using the method of Citovsky *et al.* (1991) showed the CP to be present in the soluble fraction. However, when the purified protein was electrophoresed through non-denaturing gels a discrete band was not formed, suggesting that, at high concentration, the protein has a tendency to aggregate (data not shown). This precluded its use in a gel shift assay.

DNA binding properties of the MSV CP

To determine whether MSV CP bound to ss or dsDNA, purified virus or *E. coli* cell extracts were used in the 'south-western' assay. The resulting autoradiograph showed that CP from both *E. coli* and virions bound the MSV dsDNA (Fig. 2*a*), and ssDNA (Fig. 2*b*) probes equally well. No binding was seen with the cell extract from *E. coli* transformed only with pET3a, BSA, lysozyme or the highly basic cytochrome *c*. However, binding of some *E. coli* proteins to DNA could be seen if the KCl concentration was lower than 100 mM, or if the level of an expressed target protein was low, requiring large amounts of cell extract and a long autoradiograph exposure time (data not shown). The CP also bound pUC19 plasmid (Fig. 2*c, d*) and *gus* (data not shown) ds and ssDNA probes at 250 mM KCl. Thus the MSV CP binds both ss and dsDNA in a sequence non-specific manner.

To determine the relative strength of ss versus dsDNA binding of the CP, membranes were washed in buffers containing increasing concentrations of KCl. As previously observed, the MSV CP bound both ds or ssDNA equally well in 250 mM KCl (Fig. 3*a, b*), and a similar result was obtained at 350 mM (Fig. 3*c, d*). However, the binding of MSV CP to both ss and dsDNA decreased with higher salt concentrations,

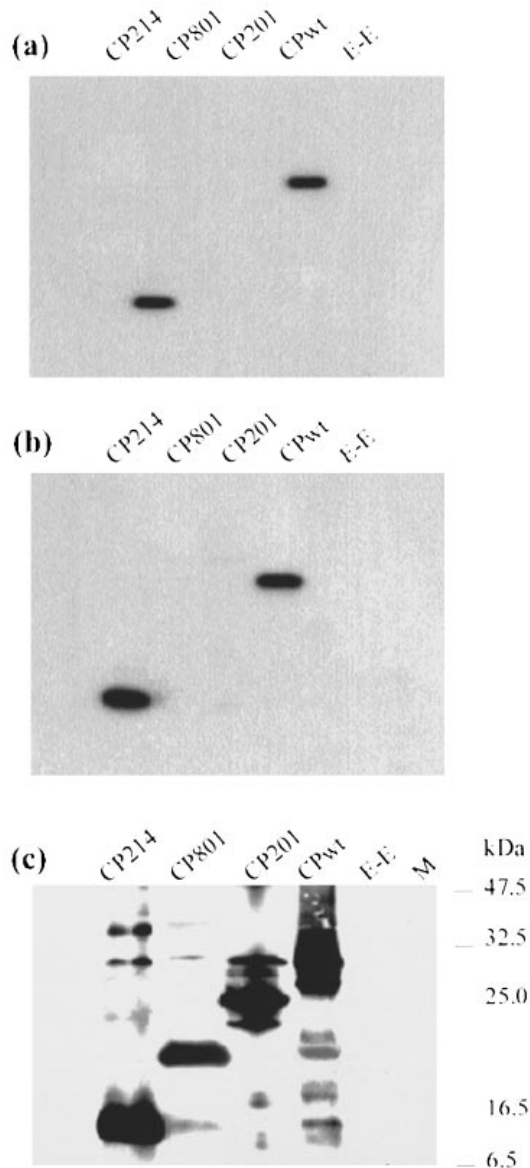


Fig. 4. Mapping the CP-DNA binding domain using truncated MSV CPs in 'south-western' assays. Autoradiographs were obtained using (a) MSV ssDNA or (b) dsDNA probe. Western immunoblot detection of CP and the truncated CPs after autoradiography is shown in (c). E-E, CPwt, CP201, CP801 and CP214, as described in the legend to Fig. 1. M, protein molecular size markers.

with a more marked reduction in the binding of ssDNA at 500 mM KCl. At 600 mM KCl, ssDNA binding was almost completely lost.

Location of the binding domain of the MSV CP

To map the binding domain of the MSV CP, binding to full-length (CPwt) and truncated MSV CPs (CP201, CP801 and CP214) was assayed. As shown in Fig. 4, CP214 bound both ss and dsDNA equally as well as the wt protein showing that the

C-terminal sequence of MSV CP is not critical for DNA binding. However, N-terminal deletions of the MSV CP sequence affected the DNA binding capacity of MSV. Deletion of the 20 (CP201) or 80 (CP801) N-terminal amino acids abolished both ss and dsDNA binding although the proteins were detected readily by immunostaining of the membrane (Fig. 4c).

Computer-assisted analysis of the N-terminal 104 amino acids failed to identify a consensus DNA binding motif, but a domain (residues 5–22) containing nine basic amino acids possesses characteristics appropriate for DNA binding (Varagona & Raikhel, 1994); the remaining 86 residues contained only 11 additional basic amino acids. The basic domain was predicted to contain a bipartite nuclear localization sequence (NLS; according to the criteria of Robbins *et al.*, 1991) by the PSORT program and similar signals were identified in the N-terminal regions of all other subgroup I geminiviral CPs (not shown).

Discussion

Expression of proteins in *E. coli* has proven useful for functional studies of many plant virus proteins. We therefore expressed both full-length and truncated MSV CPs in the pET expression system. The expressed wt and truncated proteins had the expected molecular masses as calculated by computer analysis. As the protein was found to be unsuitable for gel-shift assays, its binding to ss or dsDNA was analysed using 'south-western' analysis of total *E. coli* cell extracts. MSV CP is a basic protein with a pI of 10.8. It was therefore possible that the binding of MSV CP to both ss and dsDNA was due only to its charge. However, the lack of binding by two basic proteins, lysozyme and cytochrome *c* (pIs of 9.7 and 10.4, respectively) suggests that this is not the case. Also, CP binding is not due to the presence of a large amount of protein as all three control proteins were overloaded in comparison with the MSV CP.

The DNA binding domain was mapped within the first 104 amino acids of the MSV CP. Disruption of DNA binding following deletion of the 20 N-terminal amino acids of the CP may reflect the position of the binding site. Alternatively, the modification could affect the refolding of the protein following transfer to the membrane, which could reduce its ability to bind DNA. However, the identification of a motif predicted as a NLS and possible nucleic acid binding domain between residues 5 and 22 suggests that this region could be involved both in localizing geminivirus CPs in the nucleus as shown to occur by immuno-electron microscopy (e.g. Pinner *et al.*, 1993) and for nuclear shuttling of viral DNA.

Geminiviruses are believed to replicate in the nucleus (Goodman, 1981; Davies *et al.*, 1987). Therefore, for infection of plants, nuclear shuttling of viral DNA is required in addition to movement across cell boundaries. Unlike the bipartite geminiviruses, in which BR1, BL1 and CP are involved in movement (Jeffrey *et al.*, 1996), MSV encodes only one MP

(PVI) in addition to the CP, which is also required for infection of maize (Boulton *et al.*, 1989, 1993; Lazarowitz *et al.*, 1989). The MPs of both RNA and DNA plant viruses have been shown to bind nucleic acids in a sequence non-specific manner (reviewed in Gilbertson & Lucas, 1996); for squash leaf curl (SqLCV) and bean dwarf mosaic (BDMV) geminiviruses, the BR1 proteins have been shown to have this property. Furthermore, the BR1 proteins are involved in nuclear trafficking of viral DNA. It is therefore tempting to speculate that the MSV CP performs analogous functions in the plant. For example, the CP may bind the viral ssDNA in nucleoprotein form to facilitate nuclear shuttling and cell-to-cell movement, as suggested for SqLCV BR1 protein (Pascal *et al.*, 1994; Sanderfoot *et al.*, 1996). Presumably, CP also has a binding function necessary for the encapsidation of viral (ss) DNA. Although the importance of encapsidation for virus movement is not known, no evidence has, as yet, been provided for either the cell-to-cell or long-distance movement of MSV as virions. The high affinity of the MSV CP for dsDNA may reflect an alternative mechanism for viral DNA transport, with the protein facilitating nuclear shuttling of MSV dsDNA as was suggested for BDMV (Noueiry *et al.*, 1994). Since the MSV MP has been shown to be associated with plasmodesmata (Dickinson *et al.*, 1996), it is likely that CP, complexed with ss or dsDNA, will interact with the MP to accomplish intra- and inter-cellular movement, perhaps in a fashion similar to that suggested for BR1 and BL1 of SqLCV (Pascal *et al.*, 1994).

We thank Dr John Stanley for critical review of the manuscript. H.L. was supported by a studentship from the John Innes Foundation. The John Innes Centre is supported in part by a Grant-in-Aid from the Biotechnology and Biological Research Council. The work was carried out under MAFF licence PHF 1419a/951/24.

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Received 21 November 1996; Accepted 4 March 1997