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In Vitro Assembly of Cowpea Chlorotic Mottle Virus from Coat Protein Expressed in *Escherichia coli* and *in Vitro*-Transcribed Viral cDNA

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The small spherical plant virus, cowpea chlorotic mottle virus (CCMV), provides an ideal system to examine spherical virus assembly. We have modified the CCMV *in vitro* assembly system to produce virions from coat protein expressed in *Escherichia coli* and viral RNA transcribed *in vitro* from full-length cDNAs. Examination of the *in vitro*-assembled particles with cryoelectron microscopy and image reconstruction techniques demonstrates that the particles are indistinguishable from plant purified particles at 2.5 nm resolution. Mutational analysis of the coat protein N- and C-terminal extensions demonstrate their respective roles in virus assembly. The N-terminus is required for assembly of RNA containing particles but not for the assembly of empty virions. The C-terminus is essential for coat protein dimer formation and particle assembly.
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INTRODUCTION

The assembly of spherical viruses remains a poorly understood process. The bromoviruses, and cowpea chlorotic mottle virus (CCMV) in particular, provide an ideal model system for examining icosahedral virus assembly. Bromoviruses are members of the Bromoviridae virus family (alphavirus-like superfamily). The 28-nm icosahedral virus particles encapsidate four (+) single-stranded viral RNA's (for reviews see Ahlquist, 1992; Bancroft and Horne, 1977; Lane, 1981). The RNA genome is packaged separately in three virus particles, all with similar or identical capsid structures. RNA 1 and RNA 2, which encode proteins involved in RNA-dependent replication, are each packaged in separate particles. RNA 3 (a mRNA for the 32-kDa viral movement protein) and RNA 4 (a subgenomic RNA expressed from RNA 3 which serves as a mRNA for the 20-kDa coat protein) are copackaged into a third particle in a 1:1 molar ratio. Virus particles purified from infected plant cells contain only viral RNAs. Empty particles are not detected *in vivo*.

The assembly of CCMV has been extensively studied both *in vitro* and *in vivo*. CCMV was the first spherical virus to be assembled *in vitro* into an infectious form from its purified components (Bancroft *et al.*, 1969, 1968; Bancroft and Hiebert, 1967; Hiebert and Bancroft, 1969; Hiebert *et al.*, 1968). The *in vitro* assembly studies established that changes in ionic strength and pH alter the type of particle formed, presumably by altering RNA–protein and protein–protein interactions. Under low pH (<6.0) and low ionic strength (i = 0.2) conditions, T = 3 particles devoid of RNA (empty) self assemble. At higher pH (>7.0) and in the presence of viral RNA, RNA-containing capsids form. Under appropriate *in vitro* assembly conditions, CCMV coat protein will preferentially bind its own RNA out of a mixture of nonviral RNAs (Fox *et al.*, 1994; Fox and Young, 1993). Infectious particles assemble *in vitro* in seconds (Hiebert and Bancroft, 1969). Possible CCMV assembly and disassembly intermediates have been suggested but not characterized (Adolph, 1975; Fox and Young, 1993). The native coat protein purified from virus particles forms a noncovalent dimer in solution (Adolph and Butler, 1977; Adolph, 1975). The high resolution structure of CCMV suggests that C-terminal "arms" extending away from the central coat protein core are involved in dimer formation (Speir *et al.*, 1994). The first 25 N-terminal residues of the coat protein contain a high proportion of basic amino acids which have been modeled to interact with viral RNA on the interior of the virus particle (Verduin *et al.*, 1984; Vriend, 1983; Vriend *et al.*, 1986). Deletion of this region eliminates the ability of the virus to assemble RNA-containing particles but not empty particles *in vitro* (Vriend *et al.*, 1981). Deletion of the N-terminal 25 amino acids in the closely related brome mosaic virus eliminates viral assembly *in vivo* without elimination of viral RNA replication (Sacher and Ahlquist, 1989).

We are using CCMV as a model system to examine the role of RNA–protein and protein–protein interactions in regulating icosahedral virus assembly. We report here the development of a CCMV *in vitro* assembly system which uses coat protein expression in *Escherichia coli* and viral RNAs *in vitro* transcribed from full-length cDNA clones. The utility of this assembly system is demon-

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strated by the characterization of N- and C-terminal coat protein deletion mutants to form dimers in solution and to assemble into virus particles.

MATERIALS AND METHODS

Propagation and purification of CCMV coat protein from plants

CCMV (Bancroft, 1971) was propagated in cowpea plants (*Vigna unguiculata* (L.) var. California Blackeye) and purified by methods previously described (Bancroft and Hiebert, 1967). Purified CCMV was disassembled by dialysis against buffer A (0.05 *M* Tris–HCl, pH 7.5, 0.5 *M* $CaCl_2$, 0.001 *M* dithiothreitol (DTT), 0.0002 *M* phenylmethylsulfonyl fluoride (PMSF) for 16–20 hr at 4°. The disassembled virus was centrifuged at 14,000 rpm for 15 min at 4° to pellet the viral RNA. The remaining supernatant was dialyzed against buffer B (0.02 *M* Tris–HCl, pH 7.5, 1.0 *M* NaCl, 0.001 *M* DTT, 0.0002 *M* PMSF) for 2 hr at 4°. The coat protein was further purified by FPLC Superose 12 size exclusion chromatography (Pharmacia Biotech Inc., Piscataway, NJ).

In vitro RNA transcription of CCMV cDNA

CCMV RNA 2 was synthesized by *in vitro* transcription from *Xba*I-linearized pCC1TP2 (kindly provided by P. Ahlquist, University of Wisconsin, Madison, (Allison *et al.,* 1988)) with T7 Megascript (Ambion, Austin, TX). RNA quantitation and integrity were determined by uv spectrophotometry and visualization on denaturing agarose gels (Sambrook *et al.,* 1989).

Construction, expression, and purification of CCMV coat protein from *E. coli*

All DNA and RNA manipulations were carried out with previously described methods (Sambrook *et al.,* 1989) unless stated otherwise.

A CCMV RNA 4 cDNA, pCC4JF14, was constructed by cloning the *Apo*I–*Xba*I fragment (RNA 3 coordinates 1352–2173) from pCC3TP4 (kindly provided by P. Ahlquist (Allison *et al.,* 1988)) into the *Eco*RI site of pBluescript SK+ (Stratagene, La Jolla, CA). Polymerase chain reaction amplification with pCC4JF14 and the deoxyoligonucleotide primers GTAATTCATATGTCTACAGTCG (an introduced *Nde*I endonuclease restriction site at RNA 3 genomic coordinate 1357–1362 is underlined) and AACAGCTATGACCATG (a primer to the T3 RNA polymerase promoter located in pCC4JF14) was digested with *Nde*I and *Bam*HI (located in the polylinker of pCC4JF14) and cloned into the *Nde*I and *Bam*HI sites of the *E. coli* expression vector pET 23a (Novagen, Madison, WI). The resulting full-length coat protein cDNA clone was termed

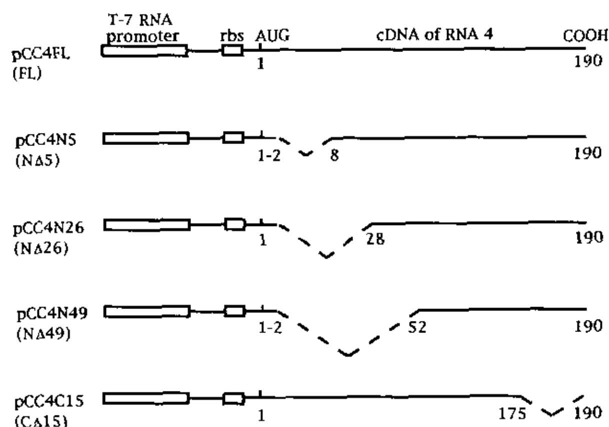

FIG. 1. Schematic representation of the full-length and deletion forms of the CCMV coat protein cDNAs. All cDNAs are cloned downstream of the T7 RNA polymerase promoter and ribosomal binding site (rbs) provided in the pET expression vectors (Novagen, Madison, WI). The designation of each construct and corresponding protein is indicated. The numbers indicate coat protein amino acid positions. Dashed lines represent deleted regions of the coat protein.

pCC4FL (Fig. 1). A CCMV coat protein cDNA lacking sequences encoding for the N-terminal five amino acids was generated by cloning the 822-bp *Hpa*I (RNA 3 genomic coordinate 1381) and *Xba*I (located in the polylinker of pCC4JF14 after the 3′ end of RNA 3 cDNA) fragment of pCC4JF14 into the *Nde*I (filled by Klenow fragment of DNA polymerase I to create a blunt end) and *Xba*I sites of pET11a (Novagen, Madison, WI) and designated pCC4N5 (Fig. 1). A CCMV coat protein cDNA lacking sequences encoding for the N-terminal 26 amino acids (RNA 3 genomic coordinates 1361–1442) was generated by PCR amplification from pCC4JF14 with deoxyoligonucleotide primers ACTCCCATGGTCCAACCTG (an introduced *Nco*I site corresponding to RNA 3 genomic coordinate 1357–1362 is underlined) and AACAGCTATGACCATG (T3 primer). The PCR product was subsequently digested with *Nco*I and *Bam*HI (located in polylinker of pCC4JF14), cloned into the *Nco*I and *Bam*HI sites of pET11d and termed pCC4N26 (Fig. 1). A coat protein cDNA lacking sequences encoding for N-terminal amino acids 3–51 was created by deoxyoligonucleotide mutagenesis (Kunkel, 1987) with the single-stranded template generated from pCC4FL and the deoxyoligonucleotide GGCGGTCCACTTCGATACGTAGACATATGTATATCTCC. The resulting construct, pCC4N49 (Fig. 1), deletes the 49 amino acids encoded by RNA 3 genomic coordinates 1368–1513. Likewise, a coat protein cDNA lacking sequences encoding for the C-terminal 15 amino acids was created by deoxyoligonucleotide directed mutagenesis with the deoxyoligonucleotide CGCTCTTCAGCGGGCACTAAACCTCCAAATGCAC which introduces a stop codon (underlined) at RNA 3 genomic coordinates 1931–1933 and deletes 45 nucleotides (genomic RNA 3 coordinates 1885–1929) encoding for the last C-terminal 15 amino acids. This construct was designated pCC4C15

(Fig. 1). All coat protein–pET vector constructs were confirmed by restriction endonuclease mapping and DNA sequencing.

Expression of full-length and deletion forms of the CCMV coat protein in *E. coli* was carried out by established protocols (Studier and Moffatt, 1986; Studier *et al.*, 1990). Briefly outlined, the plasmids containing the coat protein constructs were transformed into *E. coli* strain BL21 (Grodberg and Dunn, 1988) which contains a chromosomal copy of the T-7 polymerase gene under control of *lac* UV5 promoter (Studier *et al.*, 1990). Liquid cultures were grown to an OD_{600} of 0.4–0.6 at which time coat protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 m*M*. The cultures were incubated for an additional 4–6 hr and the cells collected by centrifugation. To partially purify the coat protein, cells were disrupted by sonication in lysis buffer (1× TBS, pH 7.5, 1.0% Triton X-100, 1 m*M* DTT, 1 m*M* PMSF). The insoluble cellular fraction containing the coat protein was resuspended in buffer B containing urea (8.0 *M*) at 4°. The urea concentration was slowly decreased by dilution in 0.25 *M* increments with buffer B to 2.0 *M* urea. Polyethylenimine (PEI, Sigma Chemical Co., St. Louis) was added to a final concentration of 0.033%, incubated on ice for 10 min and centrifuged to remove contaminating nucleic acids. The supernatant was dialyzed against buffer B to remove the remaining urea. The coat protein was selectively precipitated by addition of $(NH_4)_2SO_4$ to a final concentration of 33%, collected by centrifugation, and the protein pellet resuspended and dialyzed against multiple changes of buffer B. The protein preparations were further purified by FPLC Superose 12 (Pharmacia, Uppsala, Sweden) size exclusion chromatography. The FPLC partially purified coat protein was analyzed by SDS–PAGE, Western blotting with polyclonal antibodies produced against purified CCMV, and by spectrophotometry.

Glutaraldehyde cross-linking and FPLC analysis of *E. coli* over expressed proteins

Glutaraldehyde cross-linking of partially purified coat protein was carried out for 1 hr at 25° in the dark in 0.1 *M* phosphate buffer (pH 7.5) containing 1.0 *M* NaCl, 1.0 m*M* DTT, and 0.2 m*M* PMSF. Cross-linking conditions were first optimized by varying the glutaraldehyde concentration from .80 m*M* to 0.17 m*M* at a fixed coat protein concentration of 0.5 mg/ml (in a reaction volume of 20 μl). In the second set of cross-linking reactions, dilutions of the coat protein from 1 mg/ml to 0.01 mg/ml were reacted at the optimized glutaraldehyde concentration of 0.32 m*M*. Both sets of cross-linking experiments were carried out separately for the *E. coli*-expressed coat protein full-length and deletion mutants, as well as controls of native coat protein purified from plants. Cross-linking products were analyzed on SDS–PAGE gels and confirmed by Western blotting.

FPLC size exclusion chromatography was used to compare the oligomeric state of the *E. coli*-expressed coat protein with native coat protein in solution. Coat protein samples were loaded on a Superose 12 column (Pharmacia, Uppsala, Sweden) and eluted with buffer B. Eluted fractions were analyzed by SDS–PAGE gel and confirmed by Western blotting. The column was calibrated with protein gel filtration standards ranging from 6 to 2000 kDa (Sigma Chemical Co., St. Louis, MO).

In vitro reassembly

Partially purified *E. coli* expressed coat protein or native coat protein suspended in buffer B was added to *in vitro*-transcribed CCMV RNA 2 (in H_2O in a 4:1 (wt:wt) ratio (190:1 molar ratio) in a total volume of 100 μl. The reassembly mixture was dialyzed against buffer C (50 m*M* NaOAc, pH 7.0, 50 m*M* NaCl, 10 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, 0.0002 *M* PMSF) for 2.5 hr at 4°. Empty particles were assembled by dialysis of the coat protein suspension against buffer D (0.1 *M* NaOAc, pH 4.8, 0.1 *M* NaCl, 0.0002 *M* PMSF) for 10–12 hr at 4°. The reassembly mixtures were loaded on a 10–40% sucrose gradient and centrifuged at 38,000 rpm, 4°, for 2.0 hr in a Beckman SW41ti rotor. The gradients were fractionated on an ISCO model 640 gradient fractionator while monitoring the absorbance at 250 nm. The desired fractions were collected and dialyzed against virus buffer (0.1 *M* NaOAc, pH 4.8, 0.001 *M* EDTA) for 2 hr at 4°. The virus particles were concentrated using Centricon-100 microconcentrators (Amicon, Beverley, MA).

Electron microscopy

The reassembly reactions were monitored by examining the preparations, stained with 1% uranyl acetate, on a Philips EM300 (Philips Electronic Instruments, Mahwah, NJ) transmission electron microscope.

Cryoelectron microscopy and image reconstruction

The cryoelectron microscopy and image processing procedures used to obtain three-dimensional reconstructions of CCMV particles have been previously described (Baker *et al.*, 1988; Cheng *et al.*, 1994a, 1994b). The *E. coli*-expressed sample was examined in a Philips EM420 (Philips Electronic Instruments, Mahwah, NJ) transmission electron microscope and was maintained at near liquid nitrogen temperature in a Gatan cryotransfer stage (Gatan Inc., Warrendale, PA). The vitrified, native sample was examined in a Philips CM12 electron microscope and micrographs were recorded with a spotscan procedure (Downing and Glaeser, 1986). The micrograph of the *E. coli*-expressed sample chosen for image processing was recorded under minimal dose conditions (~2000 e^-/nm^2), at an instrument magnification setting of 49,000×, at 80 kV, and at an objective lens defocus of 0.9 μm. The orientations and phase origins (particle

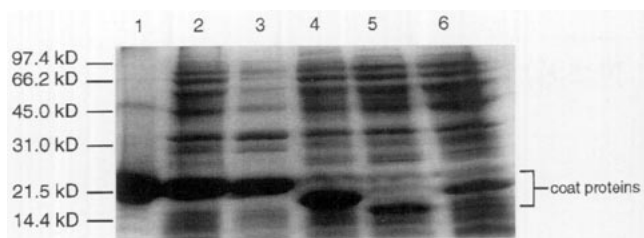

FIG. 2. SDS–PAGE analysis of CCMV full-length and deletion forms of the coat proteins expressed in *E. coli*. Lane 1, coat protein isolated from virions purified from infected plant material; lanes 2–6, coat protein constructs expressed in *E. coli*. Lane 2, FL; lane 3, NΔ5; lane 4, NΔ26; lane 5, NΔ49; and lane 6, CΔ15. Molecular weight standards are indicated.

centers) of the particle images were determined by use of a model-based approach which utilizes a reconstruction of native CCMV as the starting model (Cheng *et al.*, 1994a; Speir *et al.*, 1994). Fifty-seven particle images of the *E. coli*-expressed sample were used to calculate the three-dimensional reconstruction out to an effective resolution of 2.5 nm.

RESULTS

Expression and purification of full-length and mutant form of CCMV coat protein from *E. coli*

Both the full-length and deletion mutant forms of the CCMV coat protein were expressed in *E. coli*. The CCMV coat protein was identified in *E. coli* extracts by migration in SDS–PAGE gels (Fig. 2) and Western blot analysis (data not shown). As expected, the full-length coat protein expressed in *E. coli* and the native coat protein isolated from virus migrated at equivalent positions in gels corresponding to a molecular mass of 22 kDa (Fig. 2, lanes 1, 2). Expression from N(pCC4N5, pCC4N26, and pCC4N49)- and C(pCC4C15)-terminal coat protein deletion constructions resulted in the accumulation of proteins which migrated with a mobility shift corresponding to the extent of the expected deletion (Fig. 2, lanes 3–6). Western blot analysis with CCMV polyclonal antibodies confirmed that the induced proteins were the expected coat protein products (data not shown). The maximum accumulation of coat protein in induced *E. coli* was approximately 10–30% of the total cellular protein as estimated by Coomassie staining of SDS–PAGE gels. As expected, uninduced *E. coli* did not express coat protein.

Both the full-length and mutant forms of the coat protein were localized in the insoluble inclusion body fraction of *E. coli* lysates. Partial purification of the coat protein required resolubilization in 8 *M* urea, followed by a stepwise dialysis with a high salt buffer to remove the urea and renature the protein. A PEI precipitation step was also required to remove RNA that copurified with the coat protein in the presence of urea, presumably due to electrostatic interactions with the charged N-terminus. The observation that RNA does not copurify with coat protein expressed from pCC4N26 or pCC4N49 which lack the charged N-terminus supports this conclusion. The renatured protein was further purified by selective ammonium sulfate precipitation and by FPLC size exclusion chromatography. The final soluble coat protein used in the assembly experiments was 50–80% pure. The final yield of the partially purified protein was estimated from spectrophotometric and Coomassie staining of SDS–PAGE gels to be 75–100 mg/l of induced culture media.

E. coli-expressed coat protein can form dimers in solution

The biological activity of partially purified *E. coli*-expressed coat protein was determined by the ability to form dimers in solution. Glutaraldehyde cross-linking (Fig. 3) and FPLC size exclusion chromatography (Fig. 4) were used as two independent assays to determine the predominant coat protein oligomeric state in solution. The partially purified full-length coat protein (pCC4FL), and all N-terminal deletions (pCC4N5, pCC4N26, pCC4N49) formed noncovalent homodimers in solution under conditions similar to those in which dimers form from native coat protein isolated from virions (Fig. 3). Both the *E. coli*-expressed coat protein and native coat protein can be cross-linked with glutaraldehyde at protein and glutaraldehyde concentrations ranging from 0.5 to 0.01 $\mu g/\mu$ and from 80 to 0.17 m*M*, respectively (Fig. 3A, lanes 1–6). Likewise, the *E. coli*-expressed coat protein and the native coat protein reproducibly eluted at the position appropriate for a dimer (40 kDa) as estimated by FPLC size exclusion chromatography (Fig. 4).

Coat protein C-terminal deletions cannot form dimers in solution

In contrast to full-length and N-terminal deletion forms of the coat protein, dimer formation was not detected by either glutaraldehyde cross-linking (Fig. 3B, lanes 7–9) or FPLC chromatography (Fig. 4) with the *E. coli* expressed coat protein lacking 15 amino acid residues at the C-terminus (pCC4C15). This indicates that the C-terminus is essential for dimer formation or perhaps this deletion fails to form the expected coat protein subunit structure.

In vitro assembly of virus particles with *E. coli*-expressed coat protein

Partially purified full-length and deletion mutation forms of the coat protein were tested for their ability to assemble *in vitro* into virus particles. The *in vitro* assembly conditions that produce RNA-containing virus particles with native coat protein and *in vitro*-transcribed RNA 2 (Fox and Young, 1993) were used to assay the *E. coli*-expressed coat protein. Under these assembly conditions, only coat protein expressed from pCC4FL (full

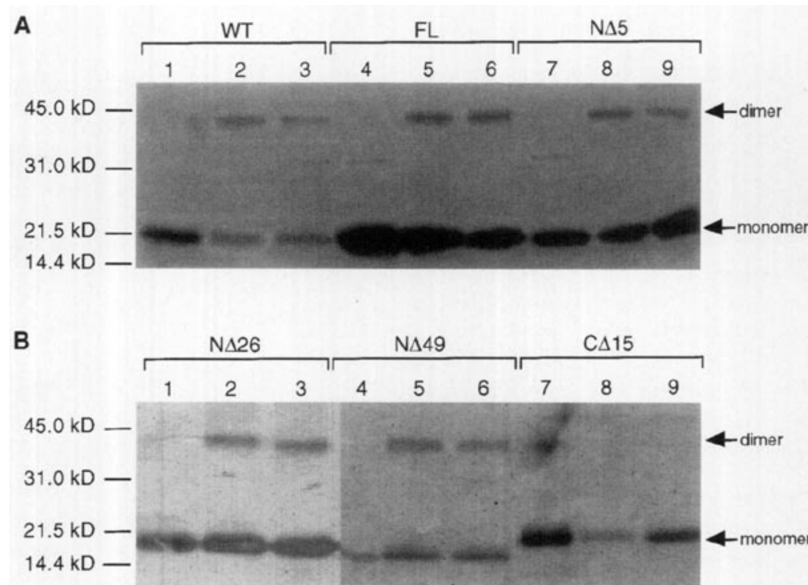

FIG. 3. Western blot analysis of glutaraldehyde cross-linked WT, FL, NΔ5, NΔ26, NΔ49, and CΔ15 coat proteins. (A) Lanes 1, 4, 7 are noncross-linked WT, FL, and NΔ5 coat protein controls, respectively. Lanes 2–3, 5–6, and 8–9 are cross-link products in the presence of 0.032 and 0.016 m*M* glutaraldehyde, respectively. (B) Lanes 1, 4, 7 are noncross-linked NΔ26, NΔ49, and CΔ15 controls, respectively. Lanes 2–3, 5–6, and 8–9 are cross-link products in the presence of 0.032 and 0.016 m*M* glutaraldehyde, respectively. The position of coat protein dimers and monomers (arrows) and the molecular weight standards are indicated.

length) and pCC4N5 (the 5 amino acid N-terminal truncation), but not from pCC4N26, pCC4N49 or pCC4C15, formed virus particles (Fig. 5, Table 1). Virus particles, assembled from coat protein expressed from pCC4FL and pCC4N5, sediment on sucrose gradients at similar positions as the particles assembled from native coat protein (data not shown). The particles isolated from sucrose gradients, negatively stained and examined in the electron microscope, appeared similar to the native particles (Fig. 5). Even though the coat proteins expressed from pCC4N26, pCC4N49, or pCC4C15 failed to assemble into virus particles, they did appear to form aggregates. The nature of these aggregates is under investigation. Analysis with cryoelectron microscopy and image reconstruction techniques demonstrates that at 2.5-nm resolution the particles assembled from the *E. coli*-expressed full-length coat protein are virtually identical to virions purified from infected plants (Fig. 6).

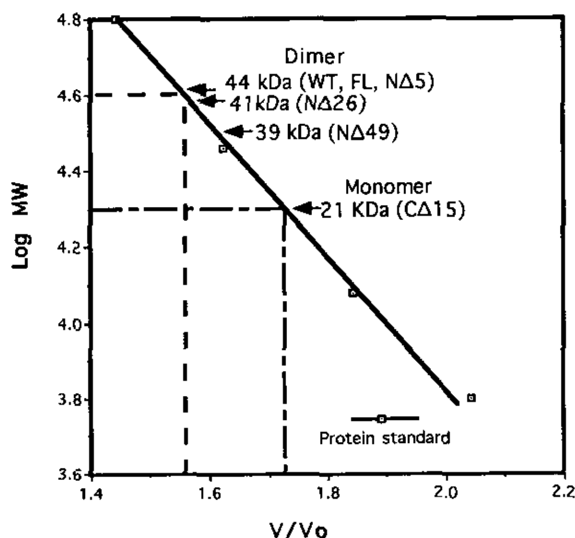

FIG. 4. FPLC size exclusion chromatography profile of full-length and deletion forms of the CCMV coat protein. A plot of the elution volume divided by the exclusion volume (V/V_0) and the Log molecular weight (Log MW) for the WT, FL, NΔ5, NΔ26, NΔ49, and CΔ15 are indicated by arrows. The expected region of coat protein monomer and dimer positions are indicated as well as molecular weight standards.

The various forms of the *E. coli*-expressed coat protein were also assayed for their ability to form empty particles. *In vitro* assembly conditions previously determined to favor the formation of empty particles with purified native coat protein (Fox and Young, 1993) were used in the assay. Upon examination in the electron microscope it was observed that coat protein expressed from pCC4FL, pCC4N5, and pCC4N26, but not pCC4N49 or pCC4C15, produced empty virus particles (Table 1). The empty particles formed were indistinguishable from empty particles assembled from the native coat protein controls (Fig. 5B).

DISCUSSION

Our goal is to determine how protein–protein and protein–RNA interactions control CCMV virus assembly. Here we show that CCMV coat protein, expressed in *E. coli* and partially purified, assembles into particles that are indistinguishable from native virus particles. Since virus particles can be assembled from expressed protein and *in vitro*-transcribed RNA(s), mutagenesis experi-

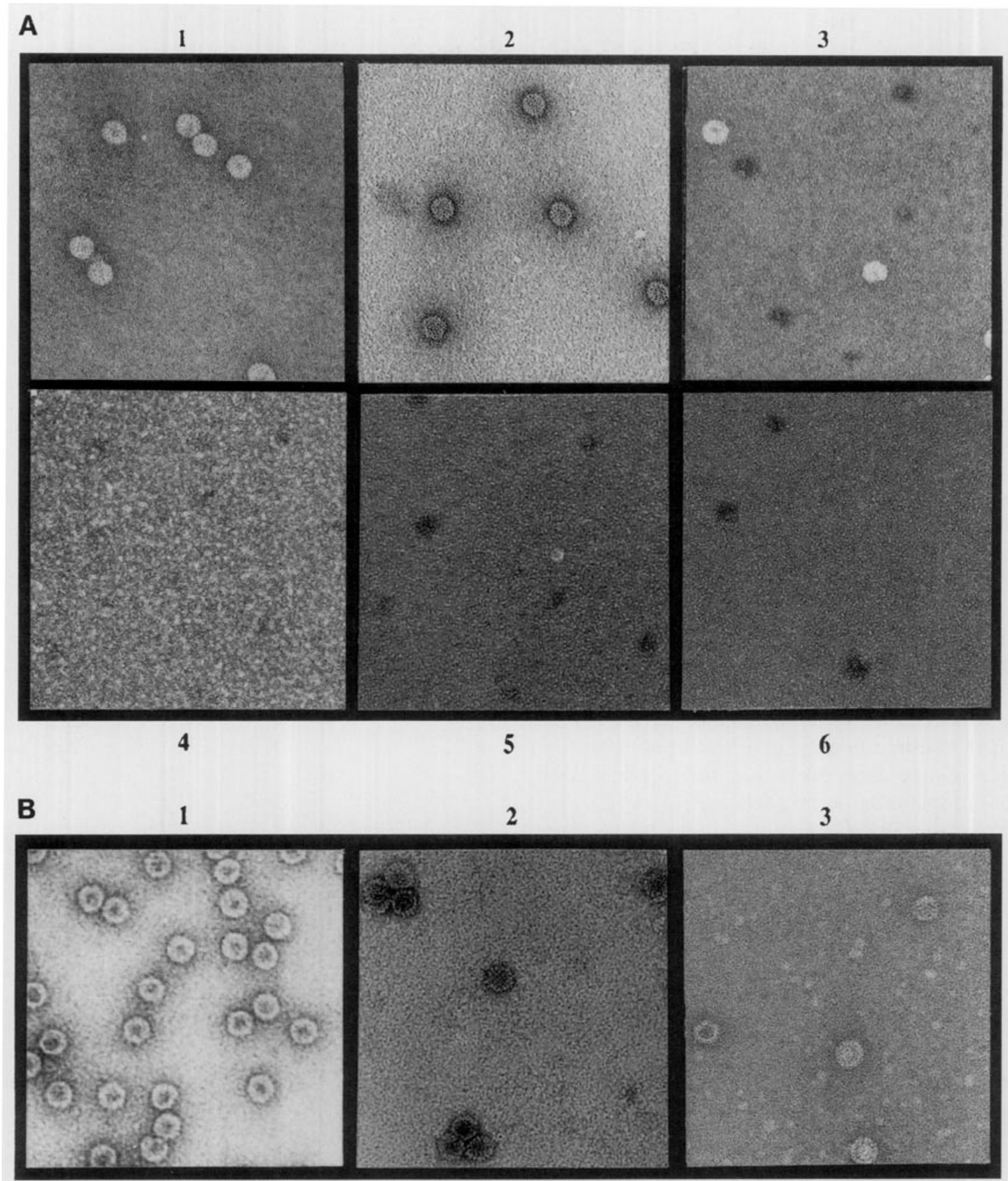

FIG. 5. Negative-stain preparations of *in vitro*-assembled particles from partially purified coat protein expressed in *E. coli.* (A) Full particle assemblies with CCMV RNA 2 and coat protein: (1) purified from virions isolated from infected plants; (2) FL; (3) NΔ5; (4) NΔ26; (5) NΔ49; and (6) CΔ15. (B) Empty particle assembly reactions with coat protein from (1) purified virions isolated from infected plants; (2) FL; and (3) NΔ26.

ments provide a powerful approach to examine the interactions which dictate viral assembly.

The potential role of the coat protein N- and C-termini in virion assembly is suggested by the structure of CCMV determined to 3.2Å resolution by X-ray crystallography (Speir *et al.*, 1994). The virion is made up of 180 copies of the coat protein subunit arranged with a $T = 3$ quasi-symmetry and organized in 20 hexameric and 12 pentameric capsomers. A striking feature of the coat protein subunit structure is the presence of N- and C-terminal "arms" that extend away from the central, eight-stranded, antiparallel β-barrel core (Fig. 7). Each coat protein subunit consists of a canonical β-barrel fold (formed by amino acids 52–176) from which long N-terminal (residues 1–51; 1–27 are not ordered in the crystal structure) and C-terminal arms (residues 176–190) extend in opposite directions. The N- and C-terminal arms may provide an intricate network of "ropes" which "tie" subunits to-

TABLE 1

In Vitro Assembly of Full and Empty CCMV Particles with Full-Length and Deletion Forms of Coat Protein Partially Purified from *E. coli*

Protein	Full particles	Empty particles
WT	+	+
FL	+	+
NΔ5	+	+
NΔ26	−	+
NΔ49	−	−
CΔ15	−	−

gether. The extended C-terminal arms facilitate the intercapsomer contacts. This arm in each subunit points away from its capsomer of origin and interacts with the twofold-related subunit in an adjacent subunit. The N-terminal arms provide additional stability for the interpenetrating C-terminal arms by "clamping" the arms between the β-barrel module and the extended N-terminal arm. The N-terminal extension also appears to stabilize hexameric capsomers. The six N-terminal arms of the hexameric capsomer intertwine at the icosahedral threefold axis to form a unique hexameric tubular structure (termed the "β-hexamer"). This structure is made up of six short parallel β-strands that are hydrogen-bonded together (residues 27−35). The five N-terminal arms of the pentamer capsomer also approach the icosahedral fivefold axis but do not form an ordered "β-pentamer".

Deletion analysis of the coat protein supports the roles of the C- and N-terminal arms in the virus structure. Previous studies indicated that coat protein subunits form dimers in solution (Adolph and Butler, 1977; Adolph, 1975). The high resolution X-ray structure suggests that homodimer formation involves the exchange of C-terminal arms between two coat protein subunits. The stability of the noncovalent dimer is controlled by interactions between the invading C-terminal arm and the β-barrel and the N-terminal "clamp" of the adjacent coat protein subunit. The requirement of the C-terminal arm in dimer formation is demonstrated by the deletion of the 15 amino acids at the C-terminus (pCC4C15). The observation that this mutant cannot form either RNA-containing or empty virus particles *in vitro* strongly implicates coat protein dimers as essential building blocks in CCMV assembly. In contrast, the three deletions at the N-terminus did not prevent dimer formation. The fact that NΔ49 protein was still capable of forming dimers in solution suggests that the "clamp" is not strictly essential for dimer formation under the solution conditions tested. Similar C-termini interactions have been observed in SV40 (Liddington *et al.*, 1991) and polyomavirus (Stehle *et al.*, 1994).

The N-terminal arm of the coat protein is predicted to participate in three different types of interactions. The first 25 amino acids are very basic and most likely interact with the viral RNA on the interior of the virion particles. Amino acids 27−35 form the β-hexamer, which stabilizes hexameric capsomers, and amino acids 44−51 form the "clamp" for the invading C-terminal arm. The first eight amino acid residues are not essential for assembly *in vitro*. Coat protein, lacking the first eight amino acids (pCC4N5), still assembles into empty and RNA-containing particles. A similar mutation in BMV forms infectious virus particles *in vivo* (Sacher and Ahlquist, 1989). In contrast, deletion of the first 25 amino acid residues (pCC4N26) eliminates assembly of RNA-containing particles, but not empty particles. A similar result has been previously reported where the first 25 amino acids were removed by trypsin digestion (Vriend *et al.*, 1981). Removal of this region in BMV, *in vivo*, eliminates virus assembly, but not viral RNA replication (Sacher and Ahlquist, 1989). Our results support an essential role for the basic N-terminus in dictating RNA−protein interactions in viral assembly under defined conditions of pH and ionic strength. The versatility of the *in vitro* assembly system is further demonstrated by the ability to use this same mutant to form empty particles under assembly conditions that favor protein−protein interactions. The deletion of the complete N-terminal arm (pCC4N49) prevents assembly of either RNA-containing or empty parti-

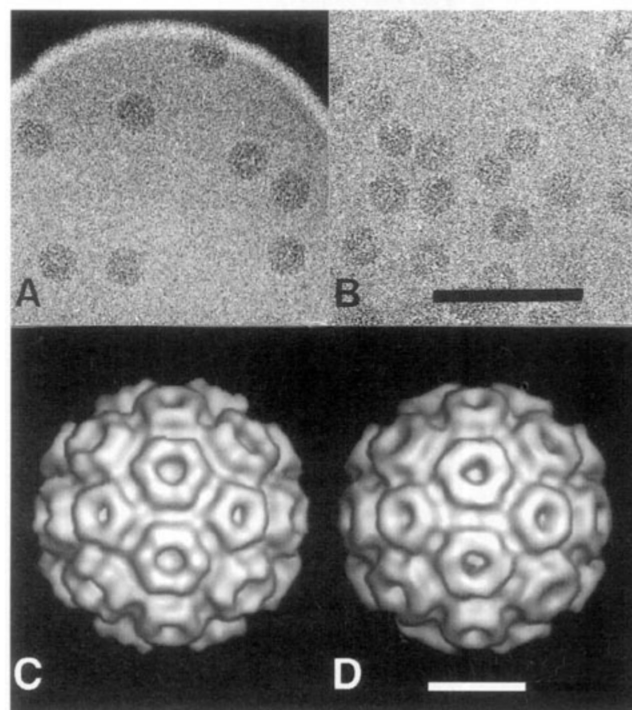

FIG. 6. Cryoelectron microscopy and three-dimensional image reconstructions of native CCMV and CCMV assembled *in vitro*. (A) Electron micrographs of frozen-hydrated native CCMV particles or (B) CCMV particles assembled *in vitro* from coat protein partially purified from *E. coli* and viral RNA 2 transcribed *in vitro*. Shaded, surface representations of reconstructions of native CCMV particles purified from infected plants (C), or CCMV particles assembled *in vitro* from *in vitro*-transcribed RNA 2 and coat protein partially purified from *E. coli* (D). Bar, 100 nm (A, B), 10 nm (C, D).

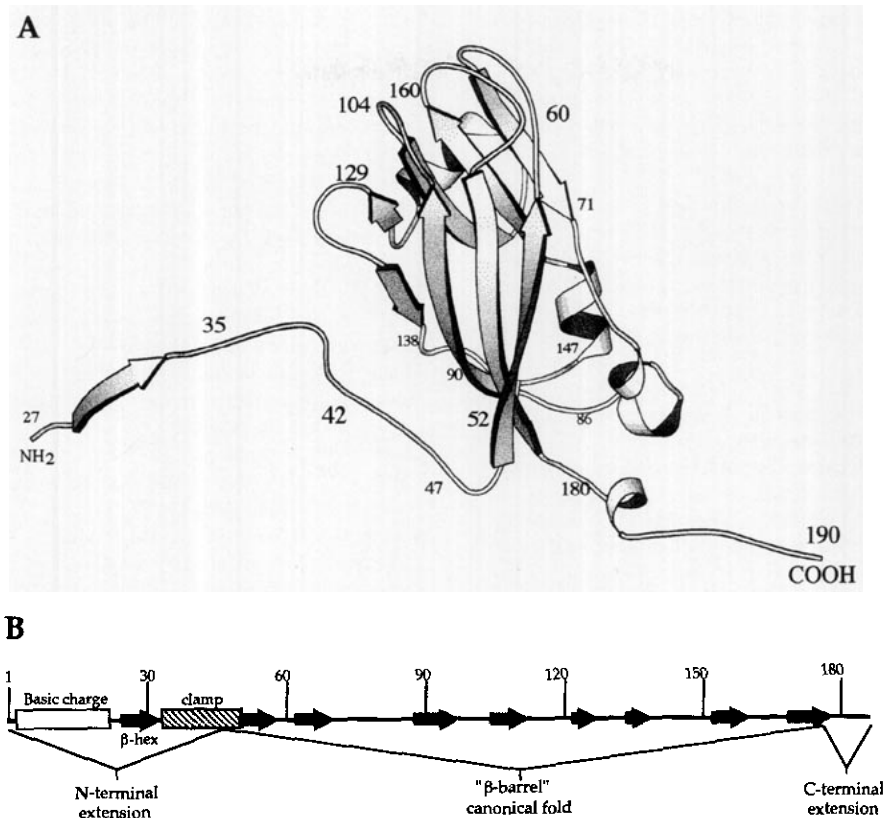

FIG. 7. CCMV coat protein subunit (Speir *et al.*, 1994). (A) Ribbon diagram of the coat protein subunit. The N-terminal arm is formed by amino acid residues 1–51 (residues 1–27 are not shown). The central, eight-stranded, antiparallel, β-barrel core is formed by amino acid residues 51–179. The C-terminal arm is formed by amino acid residues 180–190. (B) Linear representation of the coat protein subunit. White box indicates the basic charged N-terminal residues not viewed in the X-ray structure. Black arrow represents the "β-hexamer". Striped box represents the "clamp" region. Grey arrows represent the eight antiparallel β-sheets that comprise the "β-barrel" core. The three principle domains of the coat protein subunit are indicated below. The numbers indicate coat protein amino acid positions.

cles. Thus it can be concluded that the β-hexamer formation is essential to CCMV assembly.

The ability to express and purify large quantities of mutant forms of the CCMV coat protein, which are able to selfassemble into particles, greatly facilitates future structural and biophysical studies on the details of protein–RNA and protein–protein interactions which dictate CCMV assembly.

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