Role of Electrostatics in the Assembly Pathway of a Single-Stranded RNA Virus

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ABSTRACT

We have recently discovered (R. D. Cadena-Nava et al., J. Virol. 86:3318–3326, 2012, doi:10.1128/JVI.06566-11) that the in vitro packaging of RNA by the capsid protein (CP) of cowpea chlorotic mottle virus is optimal when there is a significant excess of CP, specifically that complete packaging of all of the RNA in solution requires sufficient CP to provide charge matching of the N-terminal positively charged arginine-rich motifs (ARMS) of the CPs with the negatively charged phosphate backbone of the RNA. We show here that packaging results from the initial formation of a charge-matched protocapsid consisting of RNA decorated by a disordered arrangement of CPs. This protocapsid reorganizes into the final, icosahedrally symmetric nucleocapsid by displacing the excess CPs from the RNA to the exterior surface of the emerging capsid through electrostatic attraction between the ARMs of the excess CP and the negative charge density of the capsid exterior. As a test of this scenario, we prepare CP mutants with extra and missing (relative to the wild type) cationic residues and show that a correspondingly smaller and larger excess, respectively, of CP is needed for complete packaging of RNA.

IMPORTANCE

Cowpea chlorotic mottle virus (CCMV) has long been studied as a model system for the assembly of single-stranded RNA viruses. While much is known about the electrostatic interactions within the CCMV virion, relatively little is known about these interactions during assembly, i.e., within intermediate states preceding the final nucleocapsid structure. Theoretical models and coarse-grained molecular dynamics simulations suggest that viruses like CCMV assemble by the bulk adsorption of CPs onto the RNA driven by electrostatic attraction, followed by structural reorganization into the final capsid. Such a mechanism facilitates assembly by condensing the RNA for packaging while simultaneously concentrating the local density of CP for capsid nucleation. We provide experimental evidence of such a mechanism by demonstrating that efficient assembly is initiated by the formation of a disordered protocapsid complex whose stoichiometry is governed by electrostatics (charge matching of the anionic RNA and the cationic N termini of the CP).

Unlike double-stranded DNA viruses, which generally assemble by first forming an empty capsid into which their stiff, extended genome subsequently is packaged by strong molecular motors powered by ATP hydrolysis, viruses with positive-sense single-stranded RNA (ssRNA) genomes spontaneously assemble by coupling the packaging of their flexible, compact genome to the formation of their capsid through concerted capsid protein (CP)-RNA and CP-CP attractive interactions (1).

Strong electrostatic attraction between the CP and the ssRNA genome provides the initial driving force for assembly of a number of such viruses. Consistent with this fact, their CPs typically contain extended, positively charged, arginine-rich motifs (ARMS) that bind strongly to the negatively charged phosphate backbone of RNA (2). Removing these ARMS, either by mutation or proteolytic cleavage, destroys the ability of the CP to package the RNA (3–5). Furthermore, CP alone, i.e., in the absence of the viral genome or an appropriate anionic scaffold, often is unable to assemble into capsid-like structures under physiological conditions (6–8).

Equilibrium thermodynamics and statistical mechanics have been used to understand how electrostatics influences the stability of fully formed RNA viruses and the optimum lengths of their packaged genomes. Specifically, the observation that the interiors of most RNA viruses are (negatively) overcharged (2), meaning that the negative charge brought by the nucleic acid is greater than the positive charge brought by the basic residues lining the interior of the capsid, has been examined in detail (9–12). While recent coarse-grained molecular dynamics simulations (11, 13–17) have begun to elucidate the role of electrostatics during assembly, experimental data remain scarce due to the difficulty of resolving the inherently short-lived assembly intermediates.

In a previous study on the in vitro self-assembly of cowpea chlorotic mottle virus (CCMV) CP and RNAs of various lengths (18), we reported that optimal packaging conditions required that the assembly mixture contain a relative concentration of CP/RNA...
in excess of what is found in the final capsid structure. More precisely, complete packaging of all of the RNA in solution, irrespective of its length (from 140 nucleotides [nt] to 12,000 nt), called for the total positive charge brought by the ARMs of the CP to be approximately equal to the total negative charge brought by the RNA. For lengths like those of CCMV RNA1 and RNA2, each of which is packaged separately in vivo and involves about 3,000 nt, as many as 300 CPs (150 CP dimers) are required for complete packaging (6), consistent with the fact that the ARM of CCMV CP carries a net charge of +10. The role of this specific amount of excess CP within the pathway of assembly remained unclear; e.g., does it interact directly with the RNA or with other CPs within the intermediate states, or is it simply required by the law of mass action for driving one capsid-worth of CP onto the RNA? The inability to probe any of the assembly intermediates left us without answers to these questions.

Recent progress in our ability to control the attractive forces that drive assembly has allowed us to trap stable (i.e., long-lived) assembly intermediates and characterize their coarse-grained structure by cryoelectron microscopy (cryo-EM) (19). These intermediates, formed under conditions of weak CP-CP attraction found at neutral pH and low ionic strength, appear as disordered complexes consisting of an undefined number of CP molecules bound to single RNA molecules through strong electrostatic attraction. The complexes, which we refer to as protocapsids, were irreversibly converted to well-formed nucleocapsids upon increasing CP-CP attraction by acetylation the assembly buffer, a two-step assembly protocol. Interestingly, we observed that one-step protocols that failed to generate these particular intermediates also failed to produce well-formed nucleocapsids and instead gave rise to kinetically trapped aggregated structures.

In the work presented here, we consider CPs carrying different amounts of cationic charge and systematically quantify the average number of CPs that must bind the RNA within each protocapsid in order to drive nucleocapsid formation. From this, we verify that the intermediate electrostatic conditions required for packaging involve approximate charge matching of the ARMs of the bound CP and the phosphate backbone of the RNA, distinct from the overcharged state found for the final assembly products. More explicitly, we show that the formation of a charge-neutral complex is the key requirement for efficient assembly under the in vitro conditions described. Lastly, we show that the structural reorganization that leads to the final nucleocapsid involves the translocation of the excess CP, from the RNA to the exterior surface of the forming capsid, in a process that is facilitated by the electrostatic attraction between the ARMs of the displaced CP and the negative surface charge density of the capsid.

**MATERIALS AND METHODS**

**CCMV purification and labeling.** CCMV was purified from infected California cowpea plants (*Vigna unguiculata* cv. Black Eye) as previously described by Bancroft (20). Alexa Fluor-647 N-hydroxysuccinimide ester (AF647-NHS; Molecular Probes) was covalently linked to solvent-exposed lysines on the exterior surface of CCMV by direct mixing of AF647-NHS, determined by UV-visible (UV-Vis) spectroscopy according to the manufacturer.

**Purification of CP.** CCMV CP was isolated from fluoroscintently labeled CCMV with a Dol of 0.05, as well as from unlabeled CCMV, as previously described by Annamalai and Rao (21). The CP concentration and the degree of RNA contamination were measured by UV-Vis spectrophotometry; only CP solutions with 280-nm/260-nm absorbance ratios of greater than 1.5 (corresponding to <0.5% RNA contamination) were used in assembly reactions. SDS-PAGE and matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry were used to make certain that the purified protein was not cleaved.

**Synthesis of RNA.** BMV RNA1 was generated by in vitro transcription of p17B1 plasmid (22) with T7 RNA polymerase, rATP, rGTP, rCTP, and rUTP. RNA was purified in Tris-EDTA (TE) buffer by digesting the template DNA with DNase I, followed by repeated washing of the digestion products using a 100-kDa Amicon filter device.

A 500-nt RNA consisting of the 5′-end of BMV RNA1 was prepared by PCR amplification of the first 300 bp of the p17B1 plasmid, followed by in vitro transcription and purification according to the protocol mentioned above.

**Synthesis and purification of mutant CP.** Two mutant variants, R10P and R13P/R14G, were genetically engineered by introducing specific mutations into the wild-type PET15b–CCMV plasmid template using standard QuikChange site-directed mutagenesis techniques (Stratagene). Here, R13P/R14G refers to the double mutant variant in which two arginine residues of the N-terminal ARM were replaced with single proline and glycine residues, resulting in an overall +8 charged ARM. In the case of R10P, in addition to the replacement of a single arginine of the N-terminal ARM into a proline, a 10-amino-acid residue extension also was inserted into the ARM, resulting in an overall +12 charged ARM. In both cases, the mutations were confirmed by DNA sequencing (MWG Eurofins, Germany), and the plasmid DNA was transformed into *E. coli* BL21 (DE3) pLysS (bearing an N-terminal hexahistidine tag; Novagen) for protein expression. Starting cultures were grown overnight at 37°C from glycerol stock cells in 7 ml of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Overnight cultures were used to inoculate 0.5 liters of LB medium containing ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) and grown to an optical density at 600 nm (OD600) of 0.6. Protein expression was induced following addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM at 30°C for 5 h. The cells were harvested by centrifugation (10,000 g for 15 min) and lysed using BugBuster according to standard procedures (Novagen). R10P and R13P/R14G were purified using nickel affinity chromatography with a modified version of the supplier’s protocol (Novagen). Weakly bound and other unwanted proteins were washed with 0.1 M phosphate buffer (pH 8.0), 0.3 M NaCl, and 12.5 mM imidazole before washing with 0.1 M phosphate buffer (pH 8.0), 1.5 M NaCl, 12.5 mM imidazole to remove bound RNA. Purified protein was eluted with 0.1 M phosphate buffer (pH 8.0), 1.5 M NaCl, 0.25 M imidazole before dialyzing overnight in 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 10 mm MgCl2, 1 mM EDTA to remove excess imidazole. The purified protein was dialyzed to 50 mM sodium acetate buffer (pH 5.0), 1 M NaCl, and 1 mM Na2SO4 to induce empty capsid formation and frozen at –20°C.

Prior to removal of the His tag, the samples were thawed and dialyzed against 1 M NaCl, 20 mM Tris, pH 7.2, buffer for 6 h at 4°C. After dilution with 10× cleavage buffer (Novagen), 1 U of biotinylated thrombin (Novagen) was added per mg of CP, and the reaction was carried out at 4°C for 18 h. After digestion, agarose/streptavidin beads (Novagen) were added to the sample, incubated for 30 min at room temperature, and then removed by filtering through a 1-mm spin filter. The buffer was exchanged with 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, and 10 mM imidazole using a 10,000-molecular-weight-cutoff (MWCO) Amicon spin filter (Millipore). The sample was purified from the cleaved His tag as well as the uncleaved CP by using a 10-m1 His tag affinity column (GE Healthcare). Finally, it was concentrated and buffer exchanged to buffer B (plus 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM dithio-
thioglycolic acid (DTT)) with a 10-kDa Amicon filter. The integrity of the samples was double-checked by 15% SDS-PAGE and MALDI-TOF.

**Two-step in vitro assembly reactions.** Assembly reactions were performed by following the previously optimized two-step protocol (19). Specifically, 30 nM BMV RNA1 and various concentrations of CP were mixed under conditions that prevent their interaction (neutral pH and high ionic strength; 1 M NaCl, 20 mM Tris [pH 7.2], 1 mM EDTA, 1 mM DTT, 1 mM PMSF). This noninteracting mixture of RNA and CP was subjected to two subsequent dialysis and equilibration steps, each carried out for at least 6 h at 4°C. Here, we have assumed that 6 h is sufficient for the reaction mixtures to reach equilibrium. The first step turns on CP-RNA attraction while maintaining weak CP-CP attraction (neutral pH and low ionic strength; 50 mM NaCl, 50 mM Tris-HCl [pH 7.2 or 7.0], 10 mM KCl, 5 mM MgCl2) and results in the formation of disordered CP-RNA complexes that serve as the intermediate templates (protoparticles) to proper assembly. The second step turns on CP-CP attraction (low pH and low ionic strength; 50 mM sodium acetate [pH 4.5], 8 mM magnesium acetate) and triggers the irreversible formation of the final nucleocapsid structure.

For assembly reactions involving 500-nt RNA, 500 nM RNA was mixed with 12.5 μM CP and subjected to the two-step assembly protocol described above.

**Cryo-EM.** Three μl of assembly reaction was deposited on a Quantifoil holey carbon grid (200 mesh; R2/1) that had been previously glow discharged. The grids then were blotted and flash-frozen by rapid plunging into liquid ethane cooled to liquid nitrogen temperature. Micrographs were acquired using an FEI Tecnai G2 TF20 microscope operated at an accelerating voltage of 200 kV. Images were recorded at 3- to 4-μm underfocus with a TIETZ F415MP 4,000 (4k)-by-4k pixel charge-coupled device (CCD) camera. Total beam exposure was maintained between 20 and 40 e/Å2 for cryo-EM samples.

**Negative-stain EM.** Six μl of assembly reaction was deposited on glow-discharged copper grids (400-mesh) that previously had been coated with Parlodion and carbon. After 1 min, the grids were blotted and stained with 6 μl of 2% uranyl acetate for 1 min, followed by complete stain removal and storage in a desiccator overnight.

**Gel-shift titration assay.** A 10-μl aliquot of each assembly reaction mixture was mixed with 3 μl of glycerol and loaded into a 1% agarose gel in virus electrophoresis buffer (0.1 M sodium acetate, 1 mM EDTA, pH 4.5 or 5.5). The samples were electrophoresed at 4°C at 50 V and stained with a solution of 5 g/ml ethidium bromide (EtBr). The gels were visualized with an FX Pro plus fluorimeter/phosphorimager (Bio-Rad) by exciting the RNA-intercalated etidium bromide and measuring the emitted fluorescence intensity.

**Velocity sedimentation by sucrose density gradient centrifugation.** Ten to 40% (wt/vol) sucrose gradients were made by subjecting 3.5 ml of 2% uranyl acetate for 1 min, followed by complete ethanol addition. The gradients were loaded with 0.2 ml of sample and centrifuged for 3 h at 30,000 rpm at 4°C in an SW50.1 rotor (Beckman Coulter) and manually fractionated by pipetting 0.2-μl aliquots from top to bottom. The 19 extracted fractions were loaded into a 96-well flat-bottom plate. The fluorescence of the labeled CP (AF647; excitation wavelength, 650 nm; emission wavelength, 668 nm) was measured for each fraction using a Tecan M1000 plate reader.

**RNase A digestion assay.** Assembly reaction mixtures containing 150 and 90 CP2/RNA (mol CP dimer/mol RNA ratio) that were subjected to the first step of the two-step assembly protocol, as well as wild-type (wt) CCMV and naked BMV RNA1, were equilibrated in neutral-pH, low-ionic-strength assembly buffer with various concentrations of RNase A for 90 min on ice. Ten-μl aliquots then were mixed with 3 μl glycerol and loaded into a 1% agarose gel in virus electrophoresis buffer (0.1 M sodium acetate, 1 mM EDTA, pH 5.5), constituting the second step (pH lowering) of assembly.

![FIG 1 CP-RNA assembly titration by gel shift assay. A 1% agarose gel was run in low-pH electrophoresis buffer and stained with EtBr. Shown is the titration of a fixed concentration of BMV RNA1 with various amounts of wt capsid protein dimer (CP2), ranging from 0 in the left-most lane and increasing to the right. Molar CP2/RNA ratios are reported above each lane. The right-most lane contains wt CCMV. The band positions of assembled capsids (†), naked RNA (‡), and incomplete CP-RNA complexes (†) are noted at the left of the gel. We define complete packaging to occur at the point in the titration that corresponds to the disappearance of incomplete CP-RNA complexes (CP2/RNA ratio, 140). Regions I, II, and III (see the text), i.e., lanes 1 to 6, 7 to 14, and 15 to 19, respectively, correspond to smears of increasingly CP-bound RNAs (1), coexisting incomplete complexes and nucleocapsids (II), and nucleocapsids with increasing amounts of CP bound on their exteriors (III).](http://jvi.asm.org/content/jvi/82/22/10474/F1)

**RESULTS AND DISCUSSION**

Assembly of RNA and wt CP. wt CP, purified from CCMV virions harvested from infected California cowpea plants (Vigna ungiculata cv. Black Eye), form stable dimers (CP2) in solution that serve as the fundamental assembling units during packaging (23). The RNA used is a 3,200-nt *in vitro* transcript of genomic RNA1 from brome mosaic virus (BMV RNA1). We choose to work with heterologous BMV RNA1 instead of the equal-length genomic RNA1 from CCMV in order to reduce the role of sequence-specific interactions that may exist between CP and RNA that have co-evolved; specific interactions cannot be completely ruled out, however, due to the high degree of sequence homology between CCMV and BMV RNA.

To quantify the extent of packaging as a function of the relative concentrations of CP2 and RNA, mixtures of 30 nM RNA and various concentrations of CP2 (from 0 to 5.4 μM) were subjected to the previously optimized two-step, pH- and ionic strength-dependent assembly protocol described in Materials and Methods. Gel shift titration assays (Fig. 1) of the final assembly products, stained with ethidium bromide (EtBr), show that the amount of packaged RNA, defined experimentally as the integrated EtBr stain intensity migrating with the same electrophoretic mobility as CCMV virions, depends strongly on the relative amount of CP2 present in the assembly mixture. Three regions of the assembly titration are evident. Region I, spanning CP2/RNA ratios of 0 to 50, shows no packaging; instead, all of the RNA signal is present as broad smears with electrophoretic mobilities consistent with a distribution of incomplete CP-RNA complexes. Region II, spanning CP2/RNA ratios of 50 to 140, shows a coexistence between packaged RNA and incomplete complexes, with the extent of packaging increasing at higher concentrations of CP2. Region III, corresponding to CP2/RNA ratios above 140, exhibits complete packaging, although it should be noted that higher ratios begin to migrate slightly more slowly than those for CCMV for reasons that will be described later. Quantitative measurements of the total packaged RNA signal are plotted in Fig. 2c in red.

Consistent with previous work (18, 24, 25), we have established that only 50% of the RNA in solution is packaged upon the equil-
completion, and it leaves ambiguous the role of the excess CP2 reactions often require an excess of one or more reagents to reach complete packaging is equal to one. The lines show best-fit sigmoid curves plot has been normalized so that the band intensity corresponding to the point most lane) and increasing to the right; molar mCP2/RNA ratios are reported with EtBr. As described for Fig. 1, all gels show the titration of BMV RNA1 with Shown are 1% agarose gels run in low-pH electrophoresis buffer and stained using mutant CP2 carrying a N-terminal charge of \( \pm \) per dimer. (b) A titration using mutant CP2* carrying a N-terminal charge of +16. Note that the relative position of CP-RNA complexes in the right gel of panel a is different from that in the left gel due to pH changes in the running buffer that occur during electrophoresis. (c) Densitometry profiles generated by plotting the integrated EtBr signal from the capsid band of each lane as a function of mCP2*/RNA. The plot has been normalized so that the band intensity corresponding to the point of complete packaging is equal to one. The lines show best-fit sigmoid curves that are meant to aid the eye in following the extent of packaging as a function of CP2/RNA.

FIG 2 CP-RNA assembly titrations and densitometry of gel shift assays. Shown are 1% agarose gels run in low-pH electrophoresis buffer and stained with EtBr. As described for Fig. 1, all gels show the titration of BMV RNA1 with various amounts of mutant capsid protein dimer (mCP2*), beginning at 0 (left-most lane) and increasing to the right; molar mCP2*/RNA ratios are reported above each lane. The right-most lane contains wt CCMV. The band positions of assembled capsids (+), naked RNA (†), and incomplete CP-RNA complexes (‡) are noted to the left of the gels. (a) An assembly titration involving mutant CP2*, which carries a total N-terminal charge of +24 per dimer. (b) A titration using mutant CP2* carrying a N-terminal charge of +16. Note that the relative position of CP-RNA complexes in the right gel of panel a is different from that in the left gel due to pH changes in the running buffer that occur during electrophoresis. (c) Densitometry profiles generated by plotting the integrated EtBr signal from the capsid band of each lane as a function of mCP2*/RNA. The plot has been normalized so that the band intensity corresponding to the point of complete packaging is equal to one. The lines show best-fit sigmoid curves that are meant to aid the eye in following the extent of packaging as a function of CP2/RNA.

fraction of RNA packaged

FIG 3 Sucrose density gradient velocity sedimentation. Different mixtures containing fluorescently labeled CP dimers (CP2*) were analyzed by sucrose density gradient centrifugation before (a) and after (c) acidification. After centrifugation, the 10 to 40% gradients were fractionated from top (fraction 1) to bottom (fraction 19), and the fluorescence intensity was measured. The sedimentation profile for an assembly reaction consisting of 150 CP2* per RNA is shown in green; a stoichiometrically equivalent mixture of 60 CP2* added per CCMV capsid is shown in red, and CCMV virions that were fluorescently labeled (CCMV*) are shown in gray. The peak intensity for CCMV* has been renormalized to match the peak intensity for the assembly reaction. Inlays b and d show representative cryo-EM images of the assembly complexes before and after acidification, respectively. Note that none of the CP2* present in the assembly mixtures sediments as free dimer (which is found in the top fractions of the gradient), i.e., all of the CP2* is bound in CP-RNA complexes.

Electrostatics of RNA Virus Assembly

The green curve in Fig. 3a is the sedimentation profile for the assembly intermediates generated by equilibrating 150 fluorescently labeled capsid protein dimers (CP2*) per BMV RNA1 at neutral pH and low ionic strength. The position and width of the sedimentation peak compared to that of fluorescently labeled CCMV virions (CCMV*) (Fig. 3a, gray curve) are consistent with the distribution of disordered, capsid-sized CP-RNA complexes observed by cryo-EM (19). Figure 3b shows one such protocapsid. Additionally, we see that essentially all of the CP2* present in the assembly mixture sediments between fractions 6 and 12, signifying that it is bound to RNA, in contrast to free (i.e., unbound) CP2* that has been shown to sediment between fractions 1 and 2 (19, 23). This observation demonstrates that the excess CP2 required for complete RNA packaging is tightly bound within the nucelocapsid complex; thus, it is actively involved in the reorganization process that leads to the final virion.

Implicit in this observation is the fact that each RNA molecule is capable of binding an amount of CP2 in significant excess of the 90 CP2* found in the final capsid. This is not surprising, provided that CP-RNA binding is nonspecific and predominantly electrostatic in nature. Consider that BMV RNA1 carries a charge of −3,200, and that extending from each CP2 is a pair of flexible 26-residue ARMs carrying a combined charge of +20. A purely electrostatic binding model suggests that an upper limit of 160 CP2 (corresponding to a total charge of +3,200) should be able to
bind a single RNA molecule before saturation; our observation that an average of 150 CP2 bind each RNA molecule falls close to this limit.

Cryo-EM (Fig. 3d) confirms that acidifying the assembly reaction mix reorganizes the disordered protocapsids containing an excess of bound CP2 (on average, 150 CP2 per RNA) into well-formed nucleocapsids (19). This process logically requires the unbinding of 60 CP2 from each RNA molecule to allow for the remaining 90 CP2 to assume their place within the capsid lattice. The expected free energy cost of disrupting this number of strong electrostatic interactions is quite large, roughly 1,200 kT. This value can be derived from physical arguments based on an estimate of the entropy gain associated with mobile counterion release upon polycation/nucleic acid binding, which gives a free energy benefit of roughly kT per charge. Since there are +20 charges on the CP2 N termini, we expect binding energies of the order of 20 kT per dimer, a value in accord with equilibrium binding assays (26). It is a remarkable fact that, despite this penalty, nucleocapsid reorganization proceeds spontaneously. The predominant driving force for the rearrangement comes from the formation of a large number of acidic pH-stabilized contacts between the 90 CP2 that make up the capsid (27, 28). In addition to these contacts, velocity sedimentation measurements of the reaction mixture after acidification suggest that extra interactions involving the 60 displaced CP2 also play a role.

The sedimentation profile after acidification (Fig. 3c, green curve) shows no CP2* in fractions 1 and 2, demonstrating that excess CP2* has not been released from the virion as free dimers into solution. Instead, we observe an increase in the fast-sedimenting shoulder that is suggestive of nucleocapsids with excess CP2* bound to their exterior. This interpretation is based on the observed interaction of a stoichiometrically equivalent mixture of CP2* and unlabeled CCMV virions (60 CP2*/CCMV). At neutral pH and low ionic strength, we find that 60% of the added CP2* cosediments with CCMV (Fig. 3a, red curve) and shifts the sedimentation peak from fraction 8 (naked CCMV*) to fraction 9 (60 CP2*/CCMV). This shift, in combination with analytical ultracentrifugation measurements (data not shown), confirms previous observations that excess CP2* binds the exterior surface of CCMV (29), as expected from the electrostatic attraction between the ARMs of the CP2* and the negative surface charge density of CCMV (30). An identical binding assay carried out under acidic conditions (Fig. 3c, red curve) shows that >95% of the CP2* binds the exterior surface of CCMV and generates a broader sedimentation peak with a faster-sedimenting shoulder. The increase in total binding at low pH (the reduction in free CP* signal) reflects the strengthening of lateral CP2–CP2 interactions caused by the decrease in electrostatic repulsions between proximal dimers. Thus, we conclude that the pH-triggered reorganization of the protocapsid involves the displacement of excess CP2 from the RNA to the newly forming capsid exterior. Electrostatic and lateral interactions involving the displaced CP2 in addition to the formation of lateral contacts between the 90 CP2 that constitute the capsid, help to overcome the energetic cost of unbinding the excess CP2 from RNA.

While the binding of excess CP2 to the exterior surface of the assembled nucleocapsids is consistent with the retarded electrophoretic mobilities observed at high CP2/RNA ratios (Fig. 1), resolving the excess CP2 by cryo-EM has proven difficult. Similar assembly reactions carried out using 500-nt truncations of BMV RNA1 that package into “pseudo-T=2” capsids produce particles with additional shells (multishells) of CP2 that can be resolved by cryo-EM (Fig. 4). The assembly products of full-length BMV RNA1, however, do not show multishell formation, and neither do equivalent mixtures of wt CCMV and excess CP2. In the latter cases, the excess surface-bound CP2 seems to be more disordered and harder to visualize. This may be due to the fact that the smaller T=2 core particles support the growth of multishells with curvatures closer to that of the preferred T=3 capsid, whereas the larger T=3 cores are less likely to favor the generation of ordered multishells due to the smaller curvatures they would have to adopt (30).

Assembly of RNA and mutant CP with various charges on the ARM. Two mutant variants (R13P/R14G and R10P) differing only in their N-terminal ARMs were recombinantly expressed and used in these studies; specifically, their ARMs carry different total charges (Table 1). An identical binding assay carried out under acidic conditions (Fig. 3c, red curve) shows that >95% of the CP2* binds the exterior surface of CCMV and generates a broader sedimentation peak with a faster-sedimenting shoulder. The increase in total binding at low pH (the reduction in free CP* signal) reflects the strengthening of lateral CP2–CP2 interactions caused by the decrease in electrostatic repulsions between proximal dimers. Thus, we conclude that the pH-triggered reorganization of the protocapsid involves the displacement of excess CP2 from the RNA to the newly forming capsid exterior. Electrostatic and lateral interactions involving the displaced CP2 in addition to the formation of lateral contacts between the 90 CP2 that constitute the capsid, help to overcome the energetic cost of unbinding the excess CP2 from RNA.

FIG 4 Products of an assembly reaction mixture containing a 500-nt truncation of BMV RNA1 and enough CP to completely package the RNA (25 CP2/RNA, which corresponds to charge matching between the CP-ARM and the RNA) were imaged by cryo-EM after acidification. A fraction of the T=2 capsids show a partial second shell consisting of the excess of CP required for charge matching. Scale bar, 50 nm.
noted that previous studies on RNA packaging by wt CP2 have reported similar increases in electrophoretic mobility at low CP2/RNA ratios using slightly different assembly protocols (25). Region II shows a two-state coexistence between incomplete complexes and packaged RNA, and it spans mCP2/RNA ratios of 60 to 165 (mCP16) and 33 to 121 (mCP24). This two-state behavior is suggestive of cooperative assembly and is not observed in the reaction mixtures containing wt CP. Region III shows complete packaging above mCP2/RNA ratios of 180 (mCP16) and 132 (mCP24). Quantitative measurements of the total packaged RNA signal, measured by gel shift titration assay, are plotted in Fig. 2c. Negative-stain electron micrographs (Fig. 5) show that the assembly products obtained using mCP and wt CP are indistinguishable.

Despite the qualitative differences in the gel shift titration assays, Fig. 2c demonstrates that the fraction of RNA packaged at suboptimal ratios of CP2/RNA increases with the charge of the ARM. Consistent with this observation, the CP2/RNA ratio required for complete packaging varies inversely with the total charge on the N termini of the CP2. The product of the number of charges on the ARM and the CP2/RNA ratio required for complete packaging gives +2,900 for mCP16, +2,800 for wt CP, and +3,200 for mCP24, i.e., the product is constant to within about 10%, even as the charge of the ARM varies by as much as 40%. Considering the large (order of 10%) experimental error associated with agarose gel densitometry, these measurements establish that approximate charge neutralization of the RNA (which carries a charge of ~3,200) is a general requirement for complete packaging under the in vivo conditions described. These observations should be contrasted with those of experiments by Ni et al. (31) on the in vitro packaging of RNA by mutant BMV CP with various arrangements of basic residues within the ARM. They were able to show that the length and types of RNA encapsidated depended not only on the total charge of the ARM but also on the primary sequence of the N-terminal residues. A complete understanding of packaging in terms of electrostatic arguments could not be reached by Ni et al., and their experiments serve to remind us of the relative complexity of RNA packaging in vivo.

The effect of RNase on packaging, an unexpected result. The RNase A digestion assay has long been used in in vitro assembly experiments to report on packaging; RNA that survives RNase treatment is considered to be packaged. While employing the standard RNase A digestion assay as a complementary technique for quantifying the extent of packaging, we noticed an unexpected result: RNA packaging was enhanced upon the addition of RNase during the first step of assembly to reaction mixtures containing an insufficient amount of CP2.

The products of controlled digestion reactions carried out by equilibrating, at neutral pH, various concentrations of RNase A with naked RNA and wt CCMV were electrophoresed at low pH and stained with EtBr (Fig. 6a and b, respectively). Comparison of the digestion profiles confirms that the capsid of CCMV protects RNA against degradation and RNase has no measurable effect on wt virions. On the other hand, naked RNA is shown to be incompletely digested by low concentrations of RNase A into a distribution of fragments with lengths of a few hundred nucleotides. The decrease in total RNA signal within each lane containing moderate concentrations of RNase suggests that short fragments (less than a few dozen nucleotides) also are generated that cannot be resolved due to their high electrophoretic mobility and lack of double-stranded segments required for EtBr intercalation.

Assembly reaction mixtures containing 150 CP2/RNA and 90 CP2/RNA in the first stage of assembly (neutral pH and low ionic strength) were treated and analyzed identically (Fig. 6c and d, respectively). By gel shift assays run at low pH, we measured no effect of RNase on assembly mixtures containing the required excess of CP2. However, RNase did affect reaction mixtures containing only the stoichiometric amount of CP2 (90 CP2/RNA). The fast-moving smear of stain intensity (corresponding to incomplete complexes) was removed at moderate enzyme concentrations. Additionally (and remarkably), moderate RNase treatment resulted in an increase in the packaged RNA signal as well as the CP2 signal associated with the packaged RNA (which was independently quantified by Coomassie stain densitometry and plotted in Fig. 6c). Taken together, these observations suggest that the addition of RNase A to an assembly reaction mixture containing an insufficient amount of CP2 increases the number of assembled nucleocapsids.

This unexpected increase in assembly products can be understood in terms of our previous observations on the requirements for efficient packaging. Consider that assembly reaction mixtures containing 90 CP2/RNA package only 50% of the RNA in solution upon acidification, and that the remaining RNA is found as incomplete complexes decorated by an insufficient amount of CP2 to trigger capsid formation. Addition of RNase initiates the digestion of a fraction of these incompletely packaged RNAs and allows for the redistribution of their CP2 to other unpackaged RNAs. The redistribution is made possible by the reversible binding equilib...

### TABLE 1 N-terminal sequences of the three CP variants

<table>
<thead>
<tr>
<th>CP</th>
<th>Charge (dimer)</th>
<th>Designation</th>
<th>N-terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>+20</td>
<td>CP</td>
<td>MSTVGTGKLTGQRRAAARKNKRNTTRVVQ</td>
</tr>
<tr>
<td>R13P/R14G</td>
<td>+16</td>
<td>mCP16</td>
<td>MSTVGTGKLTGQPGAAARKNKRNTTRVVQ</td>
</tr>
<tr>
<td>R10P</td>
<td>+24</td>
<td>mCP24</td>
<td>MSTVGTGKLTGQPMQRAGKLTQPPQRRAAARK...</td>
</tr>
</tbody>
</table>

* Cationic residues are labeled in boldface, and the 10-residue extension that has been inserted to generate the mCP24 variant is underlined.
FIG 6 RNase A digestion of RNA, wt CCMV, and CP-RNA assembly reactions monitored by native gel electrophoresis and densitometry. Increasing amounts of RNase A were added to samples preequilibrated in neutral pH assembly buffer and left on ice for 30 min before electrophoresis. Electrophoresis was carried out in 1% native agarose gels cast in low-pH gel electrophoresis buffer. After electrophoresis, gels were stained with both EtBr and Coomassie instant blue protein stain. The RNase/RNA mass ratio of increases from left to right and is indicated above each lane. The left-most lane in each gel contains a dsDNA ladder. (a) RNase digestions of BMV RNA1 stained with EtBr. The dsDNA ladder shows (from top to bottom) 2.0, 1.5, 1.0, and 0.5 kbp. (b to d) RNase digestions of CCMV (b), BMV RNA1 stained with EtBr. The dsDNA ladder shows (from top to bottom) 10.0, 8.0, 6.0, and 5.0 kbp. (e) Densitometry profiles were generated by plotting the integrated EtBr protein stain signal from the capsid band of each lane as a function of the RNase/RNA mass ratio. The plot has been normalized so that the band intensity of bound CP, must be sufficiently high for efficient packaging. This suggests that the rearrangement of the RNA through interaction with the polycationic ARMs of the CP is a key step in assembly. Furthermore, we observe that the structural reorganization that gives rise to the final virion involves the transfer of excess CP from the RNA to the exterior surface of the forming capsid through the electrostatic attraction of the ARMs of the displaced CP and the outward-facing negative-charge density of the remaining RNA-bound CP. This pathway is represented qualitatively in Fig. 7.

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