



MS2 has a capsid formed of 180 identical proteins organized with icosahedral symmetry as 12 pentamers and 20 hexamers. A single genomic RNA molecule, 3,569 nucleotides long, is encapsulated inside the capsid. Like other ssRNA viruses, the genome does not occupy uniformly the entire available space. Instead, it forms 2 concentric layers adjacent to the luminal capsid surface and has relatively low density near center. However, while the genome of other simple  $T = 3$  ssRNA viruses, such as the well-studied cowpea chlorotic mottle virus (CCMV), is smeared nearly isotropically across the luminal surface, genome distribution in MS2 is anisotropic and correlates with capsid geometry (6). This difference in genome organization helped formulate the hypothesis that genome packaging during MS2 assembly may be guided by different constraints and therefore may follow qualitatively different pathways from those proposed for other simple viruses. For instance, 3 possible models exist for the broad class of icosahedral viruses represented by CCMV (Fig. 1 A–C). The coat proteins of this class will readily assemble and form capsids in vitro around heterologous nucleic acids, other polyanions, and even nanoparticles, and thus the first driving interactions taken into consideration in building these models were nonspecific.

The first model was adapted from the kinetic nucleation–growth approach of Oosawa and Asakura (7), successfully applied to empty capsid assembly first (8), then later extended to the study of assembly kinetics of coat proteins around genomic cargo (9). This model is predicated on strong interactions between coat proteins, relative to RNA–coat–protein interactions. Here, the nucleation step is rate-limiting. Growth occurs through rapid addition of subunits at the growing edge once a critical size oligomer has formed on a precompact RNA molecule. Intermediates along the elongation leg of the assembly pathway are very short-lived (Fig. 1A).

The second model (“en masse” or “micelle/crystallization” model) corresponds to the limit where interactions between RNA and coat protein are dominating. Here, after rapid initial formation of a disordered nucleoprotein complex, the high effective protein concentration increases the chances of multiple weak protein interactions acting cooperatively to stabilize the precursors of a symmetric capsid along the growth pathway (10) (Fig. 1B). Interactions among coat proteins are necessarily weak in this case, which leads to a fluid previrion surface, open to structural transitions, and able to find, in the end, the free-energy minimum, that is, the icosahedral cage structure.

Since the magnitude of interactions between coat proteins, and coat protein and RNA, can be modulated by pH and ionic strength, different assembly scenarios are expected to be favored by the same system. Indeed, coarse-grained simulations and in vitro small-angle X-ray scattering experiments with CCMV both support this expectation (11, 12). This suggests that virus assembly is a resilient process, which can adapt to changes in the chemical environment by switching between mechanisms.

The third model (Fig. 1C) reserves yet another role for RNA, whereby the nucleic acid not only locally concentrates the coat protein, but its strand acts like an extended antenna that guides the RNA-associated coat proteins, through one-dimensional diffusion, toward the live edge of a nucleus from which the virus grows (13). The emphasis on the multifaceted, central role of RNA sets this model as a precursor of the current model for the mechanism of MS2 assembly.

The defining feature of the mechanistic model for MS2 assembly is the pivotal role it assigns to specific interactions between RNA and coat protein throughout the virus growth process (14). Thus, increased coat–protein binding affinity sequences called packaging signals (PS) have been identified on

the MS2 RNA, and PS–protein binding events at low (nanomolar) protein concentration were found to lead to RNA condensation. In this process, RNA-bound proteins are believed to be brought together in ways that avoid potential kinetic traps. Orderly binding and folding of the viral RNA thus orchestrates the growth of the capsid. It would be a fascinating experience to be able to see this process at work in real time, from A to Z. This is what Garmann et al. (4) set out to do.

### In PNAS, Garmann et al. provide a compelling demonstration of the potential held by a wide-field optical microscopy method for tightly controlled, real-time single-particle studies of the ssRNA bacteriophage MS2 assembly with near-molecular accuracy and broad temporal dynamic range.

Their single particle assembly kinetics data (at micromolar dimer concentrations) show that there is a broad distribution of start times for the elongation step, which strongly suggests the existence of a critical nucleus; that is, assembly has to overcome a free-energy barrier before rapid, cooperative growth can take place (4). Interestingly, during the rapid growth phase, the time to capsid completion is 4 orders of magnitude longer than the timescale required for 90 dimers to cross the RNA strand by diffusion. Therefore, only a small fraction of protein–RNA collisions are productive at this protein concentration, with the system having the ability of sampling a large number of pathway steps before proceeding to add new subunits. No major disassembly events could be observed.

The size of the nucleus could not be estimated, since it appears to be smaller than the detection limit, that is, about 6 dimers (dimers, not monomers, are the more stable coat–protein state in solution, prior to assembly) (4). The findings are intriguing because the number of putative PS sites should be greater than that (14), yet it appears that the initiation step corresponding to proteins binding at PS sites could not be detected by the interferometric scattering approach. This could be due to a number of reasons: drift in the background intensity, and/or differences in the buffer ionic strength and protein concentration with respect to previous work (15). In those previous studies, protein concentration was in the nanomolar range, and total assembly times exceeded  $10^3$  s, which would be challenging to achieve with interferometric scattering, due to drift. In addition, lower ionic strength in ref. 15 favored stronger RNA–coat–protein interactions. Could it be that, like other viruses, MS2 is able to switch between mechanisms, for example, depending on the stage of infection? Might it be that PS activity depends on the kinetics of the nucleoprotein complex folding with PS sites fading in and out of existence through a slow ramping of protein concentration, as Dykeman et al. (16) have suggested? What would *in singulo* kinetics look like if the RNA of a different virus were used instead of MS2 RNA? What instrumentation improvements could lower the detection limit of interferometric scattering microscopy to the point where the nature of the critical nucleus could be determined? The path ahead is wide open and promises to add to an already fascinating story.

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