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COMMUNICATION

Fungal Virus Capsids, Cytoplasmic Compartments for the Replication of Double-stranded RNA, Formed as Icosahedral Shells of Asymmetric Gag Dimers

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The primary functions of most virus capsids are to protect the viral genome in the extra-cellular milieu and deliver it to the host. In contrast, the capsids of fungal viruses, like the cores of all other known double stranded RNA viruses, are not involved in host recognition but do shield their genomes, and they also carry out transcription and replication. Nascent (+) strands are extruded from transcribing virions. The capsids of the yeast virus L-A are composed of Gag (capsid protein; 76 kDa), with a few molecules of Gag-Pol (170 kDa). Analysis of these 420 Å diameter shells and those of the fungal P4 virus by cryo-electron microscopy and image reconstruction shows that they share the same novel icosahedral structure. Both capsids consist of 60 equivalent Gag dimers, whose two subunits occupy non-equivalent bonding environments. Stoichiometry data on other double-stranded RNA viruses indicate that the 120-subunit structure is widespread, implying that this molecular architecture has features that are particularly favorable to the design of a capsid that is also a biosynthetic compartment.

Keywords: cryo-electron microscopy; virus capsid protein; three-dimensional image reconstruction; quasi-equivalence; double-stranded RNA

Like all known fungal viruses, L-A virus of *Saccharomyces cerevisiae* and P4 virus of *Ustilago maydis* are spread horizontally by cell mating and heterokaryon formation (Bevan & Mankower, 1963; Wood & Bozarth, 1973). During replication of the dsRNA[†] genomes of L-A and P4, both (+) and (–) strand synthesis occurs within the viral particle (Wickner, 1992). Upon release, the (+) strand serves

both as mRNA and as the species that becomes encapsidated. In this mode of propagation, in which the capsid participates actively in nucleic acid synthesis and never leaves the host cell, its properties may well differ from those of capsids that serve simply to protect a metabolically inactive genome while outside the cell. In this context, we have investigated its structure.

Previously, an estimate of about 120 Gag subunits per capsid was obtained from calculations based on sedimentation coefficients, the RNA:protein ratio, and the molecular weights (Bozarth *et al.*, 1981; Esteban & Wickner, 1986; Fujimura *et al.*, 1992). This figure does not match the values expected for the two simplest icosahedral lattices, which correspond to triangulation numbers of $T = 1$ (60 copies) and $T = 3$ (180 copies; Caspar & Klug,

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[†] Abbreviations used: ds, double-stranded; STEM, scanning transmission electron microscopy.

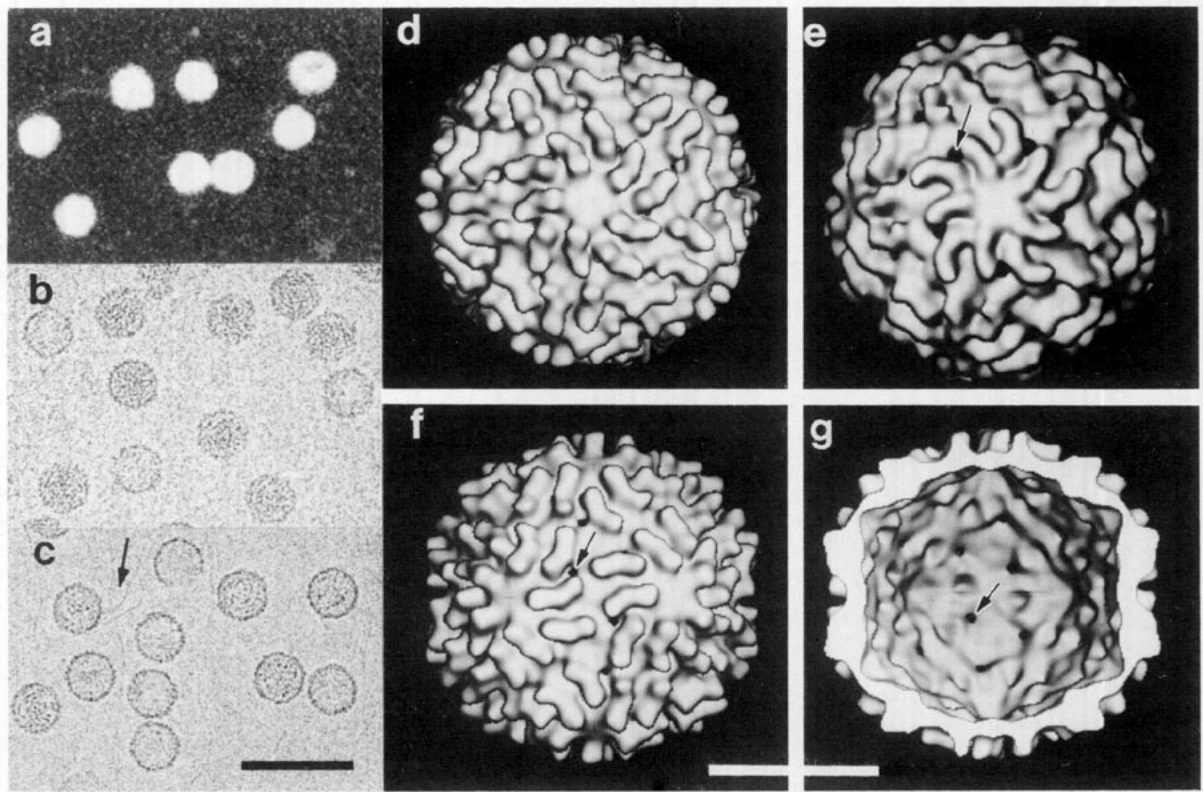


Figure 1. a, STEM micrograph of L-A recorded at the Brookhaven Biotechnology Resource (Wall & Hainfeld, 1986). Mass measurements were calculated from 621 full and 26 empty capsids. b, Cryo-micrograph of L-A. c, Cryomicrograph of P4, arrow denotes filamentous material presumed to be RNA. The magnifications in a, b, and c are the same. The bar represents 1000 Å. d and e, Reconstructions of L-A and P4, as viewed along an axis of 5-fold symmetry. f and g, L-A outer and inner surfaces as viewed along a 2-fold axis of symmetry. The bar represents 200 Å. Arrows in e, f, and g note the holes in the capsids. L-A virions from *Saccharomyces cerevisiae* strain TF229 (Esteban & Wickner, 1986) and UMV virions from *Ustilago maydis* strain P4 (Bozarth *et al.*, 1981) were grown and purified, and cryo-electron microscopy and 3 dimensional reconstructions were carried out essentially as described (Baker *et al.*, 1989; Dryden *et al.*, 1993); in addition, extensive use was made of the "Polar Fourier Transform" (PFT) algorithm, an iterative, model-based procedure for determination of orientations (Cheng *et al.*, 1994). These L-A reconstructions were based on 37 (empty) and 42 (full), respectively, and the P4 reconstruction on 60 particles. In addition, independent reconstructions were calculated of L-A (full) and P4 (empty and partially empty, respectively), yielding entirely consistent results. The resolution of the 3 dimensional maps was 26 Å according to the criterion of Conway *et al.* (1994); moreover, cross-common-lines phase residuals (Crowther, 1971; Fuller, 1987) were found to remain statistically significant to this limit. If icosahedral symmetry were to be imposed on a set of non-symmetric particles, or on capsids with incorrectly determined orientation angles (data not shown), these quantitative measures break down at much lower resolution, i.e. at the point at which it is no longer valid to describe the particles as spherically symmetric. This inferred resolution is close to the limit of information imposed by the first zero of the contrast transfer function, which was at $\sim(24 \text{ \AA})^{-1}$ for the micrograph analyzed, as measured by optical diffraction.

1962). To confirm the stoichiometry, we measured the masses of purified L-A capsids from dark-field scanning transmission electron micrographs (STEM; Wall & Hainfeld, 1986; Figure 1a), obtaining $12.0(\pm 0.86)$ MDa for the full capsid, and $7.9(\pm 0.86)$ MDa for the empty capsid. These data correspond to $116(\pm 11)$ subunits and $104(\pm 11)$ subunits per capsid, respectively, and support the 120-subunit model.

To investigate their capsid geometry, P4 and L-A were examined by cryo-electron microscopy (Adrian *et al.*, 1984) and three-dimensional image reconstruction (Crowther, 1971; Fuller, 1987; Baker *et al.*, 1989). Both viruses are similar in size (~ 420 Å) and appearance (Figure 1b and c). P4 proved to be less stable than L-A upon storage, as evidenced by large numbers of empty capsids and the presence of free RNA. Reconstructions were calculated for both

viruses, treating empty and full particles separately. The resulting renditions of the capsids were highly reproducible (e.g. Figure 1d to g; Figure 2). Although icosahedral symmetry was imposed in the reconstruction procedure, analysis of the images by quantitative criteria attested that the capsids do indeed observe this symmetry (Figure 1, legend). Good agreement between the original images and corresponding reprojections from the reconstructions further corroborated their validity.

The capsids of these two fungal viruses exhibit very similar features (Figure 1d and e), with 120 morphological units packed on a $T = 1$ lattice. The capsid is composed of 12 pentons, each consisting of ten curved, elongated subunits arranged in two sets of five. An inner ring of five subunits is surrounded by an outer ring, whose subunits are offset like fish scales relative to those of the inner ring. On the outer

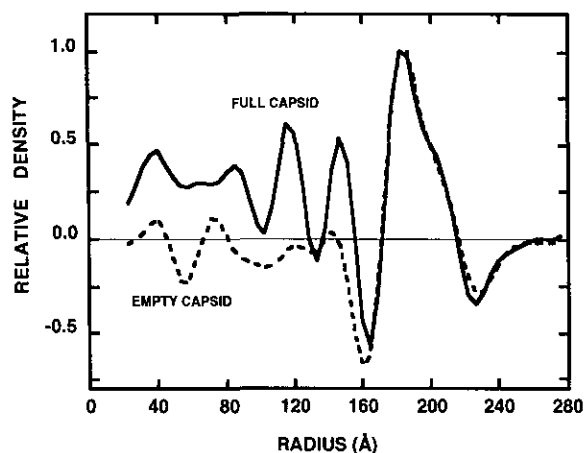


Figure 2. Radial density profiles calculated from the 3 dimensional density maps of full (—) and empty (---) L-A capsids. All particles analyzed were from a single micrograph, hence this comparison is not biased by differences in ice thickness, defocus, or radiation damage. The protein shells ($r = 172$ to 217 Å) are essentially superimposable. As expected, the full capsids show additional density inside the capsid ($r < 160$ Å), but very little of it is immediately adjacent to the inner surface, and we infer that there is at most a small amount of icosahedrally ordered RNA. The inferred absence of extensive interactions between the RNA and the Gag subunits is also consistent with the relative ease with which fungal viruses lose their RNA. The radial profile shows several peaks of RNA density, spaced at approximately equal intervals of 50 Å. It is unlikely that the RNA is organized in a succession of concentric spherical shells because such an appearance is not suggested by the micrographs themselves. The 50 Å spacing is probably accentuated in the reconstruction because this is the predominant spacing between duplexes (or multiple duplexes) of packaged RNA.

surface, the subunits are ~ 65 Å long and 25 to 30 Å wide, and the thickness of the capsid wall varies from about 20 to 50 Å. Although the inner and outer ring subunits occupy non-equivalent bonding environments, the morphological resemblance between them, coupled with the known capsid stoichiometry, suggests that both are monomers of Gag.

An icosahedral capsid structure consisting of 120 subunits is noteworthy in that it invokes non-equivalent packing of subunits. In principle this stoichiometry would also be consistent with octahedral (432) symmetry (Salunke *et al.*, 1989) but the observation of projections that exhibit 5 -fold symmetry (Figure 3) verifies the icosahedral (532) symmetry. We have also considered the possibility that the non-equivalence of the 120 -subunit structure would be resolved if Gag were to consist of two similar domains, like the large subunit of plant comoviruses (Lomonosoff & Johnson, 1991). Thus, the 240 domains could be packed in a quasi-equivalent manner according to orthodox $T = 4$ symmetry. However, there is no local 6 -fold symmetry at the 2 -fold axes at the level of the protruding density features (Figure 1) nor is there evidence for a repeated structure or internal gene

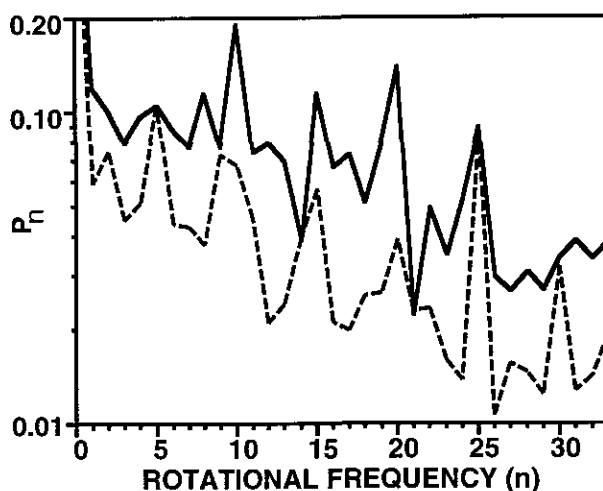


Figure 3. Rotational power spectrum analysis (Crowther & Amos, 1971) of images of an L-A virion (—) and a P4 capsid (---) which were used in the reconstructions (Figure 1) and happened to be oriented within 2° of a 5 -fold axis of symmetry. Both plots demonstrate predominant 5 -fold character that is consistent for structures with icosahedral (532) symmetry. Each semi-log plot is normalized so the power of the zero-order harmonic, P_0 is 1.0 . The higher total azimuthal power in the L-A plot reflects the larger mass of the virion compared to the empty P4 capsid. The order 5 harmonics (P_n , where $n = 5, 10, 15, \dots, 30$) represent 31% of the total azimuthal power

$$\left(\sum_{n=1,30} P_n \right)$$

for this P4 capsid and 29% for this L-A virion. For comparison, the average order- 5 power for randomly oriented virions (P4: $n = 147$; L-A: $n = 92$) was $17(\pm 2)\%$, which is close to the expected value (20%) for a random structure.

duplication in the *gag* sequence (data not shown). It has been reported on the basis of multiple sequence alignments, that a subtle homology pertains between residues 203 and 448 of L-A Gag and the VP3 capsid protein of picornaviruses (Bruenn *et al.*, 1989). This homology suggests that, like VP3, this Gag domain may fold into the canonical eight-stranded antiparallel β -barrel exhibited by many capsid proteins (Rossmann & Johnson, 1989; Stuart, 1993). In principle, the amino (residues 1 to 202) and carboxy (residues 446 to 668) termini of Gag are both long enough to fold into another copy of this module, but there is no indication on the basis of sequence homology that they do so. We thus conclude that L-A and P4 capsids both consist of 120 Gag subunits of which 60 copies are located in each of two non-equivalent bonding environments.

Both virions have 60 holes of ~ 15 Å diameter that extend through the capsid wall. These holes, three per facet and spaced ~ 60 Å apart (Figure 1e,f and g), might be channels through which freshly synthesized (+) strand RNA is extruded into the cytoplasm. As such, they should be large enough to allow ingress of nucleotides and egress of freshly synthesized RNA,

yet small enough to exclude potentially damaging nucleases or proteases.

Like fungal virus capsids, the cores of other dsRNA viruses (reoviruses (Dryden *et al.*, 1993), rotaviruses (Mansell & Patton, 1990)), and the enveloped bacteriophage $\phi 6$ (Mindich & Bamford, 1988) actively participate in the synthesis of viral nucleic acids, whereas their outer capsids play the conventional role of protecting the genome in the extracellular environment. Interestingly, each of these particles is thought to contain 120 copies of at least one protein subunit. Conversely, no other kind of virus has yet been found to have 120 capsid protein subunits. This stoichiometry and the associated molecular architecture would therefore appear to be widely favored for the construction of specialized intracellular compartments for transcription and replication that protect the dsRNA genomes from host cell enzymes, and may also serve to retain them in the cytoplasm.

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