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Scaffold properties are a key determinant of the size and shape of self-assembled virus-derived particles

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Abstract

Controlling the geometry of self-assembly will enable a greater diversity of nanoparticles than now available. Viral capsid proteins, one starting point for investigating self-assembly, have evolved to form regular particles. The polyomavirus SV40 assembles from pentameric subunits and can encapsidate anionic cargos. On short ssRNA (814 nt), SV40 pentamers form 22-nm-diameter capsids. On RNA too long to fit a $T=1$ particle, pentamers form strings of 22-nm particles and heterogeneous particles of 29 to 40 nm diameter. However, on dsDNA SV40 forms 50 nm particles composed of 72 pentamers. A 7.2-Å resolution cryo-EM image reconstruction of 22-nm particles shows that they are built of twelve pentamers arranged with $T=1$ icosahedral symmetry. At threefold vertices, pentamers each contribute to a three-helix triangle. This geometry of interaction is not seen in crystal structures of $T=7$ viruses and provides a structural basis for the smaller capsids. We propose that the heterogeneous particles are actually mosaics formed by combining different geometries of interaction from $T=1$ capsids and virions. Assembly can be trapped in novel conformations because SV40 interpentamer contacts are relatively strong. The implication is that by virtue of their large catalog of interactions, SV40 pentamers have the ability to self-assemble on and conform to a broad range of shapes.

Keywords

SV40; self-assembly; capsid; scaffold; capsid assembly; virus assembly

Interest in the *in vitro* assembled SV40 particle stems from its use as a model for self-assembly,(1, 2) for production of protein-coated nanoparticles,(3, 4) and its potential as a vector for gene and drug delivery.(5) SV40 has human tropism, transduces many cell types and organs, and infects non-dividing cells. Furthermore, SV40 is non-immunogenic(6, 7) and non-pathogenic for humans.(8-10) Recombinant SV40 capsid proteins spontaneously assemble into virus-like particles (VLPs) that package SV40 or plasmid DNA.(5, 11-13)

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Supporting Information

Experimental method details and supplementary figures as described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession codes

The cryo-EM density map has been deposited to EMDDataBank.org and the accession number is EMD-2375.

Remarkably, the packaging capacity for naked dsDNA was greater than the native virus (up to 17 Kbp(14)).

SV40 is a small non-enveloped primate polyomavirus with a 5.2 kb double-stranded circular DNA genome.(15) The DNA forms a minichromosome with a nucleosome structure similar to cellular chromatin. The minichromosome is enclosed in a 50 nm diameter capsid composed of 72 pentamers of the major viral protein VP1 arranged in a $T=7$ icosahedral lattice. (16)(17) VP1 pentamers are held together by long C-terminal arms.(16). In addition to VP1, a single molecule of a minor coat protein, VP2 or VP3, is tightly anchored to each pentamer.(18, 19) Both VP1 and VP2/3 have nucleic acid binding domains with non-specific binding activities, but only Vp1 is required for assembly.(20)

Current models of virus capsid assembly (see recent reviews(21, 22)) suggest that assembly is based on weak interactions of multivalent subunits. Weak association energy prevents kinetic traps and allows dissociation of misincorporated subunits. In the absence of cellular factors, recombinant SV40 VP1 pentamers assemble aggressively under non-physiological conditions (high salt, low pH or the addition of CaCl_2).(20) Consistent with theory, these conditions trap many complexes other than 50 nm icosahedra, including 22 nm icosahedra, 32-35 nm spherical particles, and tubes.(20, 23) Under milder conditions chaperones were required for assembly of 50 nm particles,(24) which we suggest indicates a kinetic rather than thermodynamic barrier to assembly under more physiological ionic conditions.

The mechanism of *in vitro* assembly of nucleic acid-filled VLPs is the subject of continued investigation.(25) With SV40, short dsDNA of 600 bp yields virus-like ~50 nm particles even in the absence of chaperones.(26) Short RNA molecules, up to 814 nt long, led exclusively to assembly of 22 nm particles composed of twelve pentamers.(27) These data suggest that nucleic acid serves as a scaffold and also a nucleating factor.(26) The emerging molecular mechanism, which appears to be general to viruses that assemble around nucleic acids, is based on trade-offs between the stability of protein-protein interaction, the work required to package the nucleic acid, and electrostatic interactions between incoming subunits and the nucleic acid/growing capsid.(25, 27-30)

In the current study we investigate how a scaffold can redirect SV40 assembly using different nucleic acids as scaffolding. We used different lengths of ssRNA and the physically similar ssDNA, and also dsDNA including supercoiled plasmids (which are more compact than relaxed or linear DNA). While dsDNA uniformly yields $T=7$ particles, long ssRNA and ssDNA can yield chains of $T=1$ particles and irregular particles averaging about 30 nm in diameter. Cryo-EM image reconstruction shows that $T=1$ SV40 is based on an interpentamer contacts not found in $T=7$ particles.

RESULTS AND DISCUSSION

Assembly on RNA substrate produced the predominant small particles

Stiff dsDNA substrates (0.6 to 5.2 kbp) led to ~50 nm particles(2, 11) whereas a short ssRNA (814 nt or 0.8 knt) substrate led to 22 nm particles.(27) To further elaborate on how nucleic acid influences capsid structure we tested the hypothesis that a larger scaffold, represented by longer RNA molecules (>0.8 knt), would be sufficient to induce formation of larger capsids.

Assembly of SV40 VP1 with 1.9 or 3.2 knt RNA was tested under mild conditions of 125 mM NaCl, 50 mM MOPS pH 7.2 at room temperature. After 15 minutes samples were examined by negative stain transmission electron microscopy (TEM). We observed a heterogeneous mixture of particles 22-40 nm in diameter (Figure 1A). For both RNAs, the

size distributions of these two assembly reactions were similar. The majority (about 58%) of the particles had a uniform 22 nm diameter (Supplementary Figure S1A, B). The remaining particles were more heterogeneous with a peak diameter of 34 nm.

We speculated that the RNA used was not long enough to create the SV40 virionsized, 50 nm $T=7$ particles. Thus, we then examined 8 and 11 knt RNA substrates, which bracket the mass of the 5.2 kb dsDNA of SV40 genome. The products of the assembly reaction were, however, very similar to those with smaller RNA (Figure 1B). We did not observe 50 nm particles. Unexpectedly, assembly with a longer RNA substrate (either 8 or 11 knt) yielded a slightly higher proportion of the 22-nm particles (about 70%, Supplementary Figure S1C, D).

Combining histograms for the four RNA substrates (1.9, 3.2, 8, and 11 knt), we observed about 62% of 22-nm-diameter particles, while the remaining particles were a mixture ranging from 29 to 40 nm diameter (Figure 1C). The most frequent large diameter, 32-37 nm, was clearly smaller than a 50 nm native virion.

Characterization of assembly reactions assembled on 1900 nt RNA

To determine the pentamer:RNA stoichiometry of particles assembled with 1.9 knt RNA we separated mixtures by size exclusion chromatography (SEC) using either a UV-detector or a multi-angle laser light scattering detector (MALLS) coupled with a refractive index detector. By SEC, we did not observe the anticipated major peak eluting at 17 minutes in our standard Superose 6 column, which corresponds to 22-nm particles assembled on the 0.8 knt RNA substrate.⁽²⁷⁾ Instead, the major peak appeared substantially earlier at 15.7 min (Figure 2A), indicating a larger Stokes' radius. UV absorbance of the fractions eluted from 15 to 16 minutes, including the 15.7 minute peak, showed an A_{260}/A_{280} ratio of 1.52 ± 0.002 , suggesting a composition of 24 VP1 pentamers per 1.9 knt RNA molecule.⁽³¹⁾

In control experiments, SEC-MALLS analysis of VP1 assembled on an 0.8 knt RNA showed a single peak of particle populations with an average mass of 2.6 ± 0.03 MDa and an average size of 22.4 ± 0.4 nm, in agreement with the calculated mass (2.67 MDa) and diameter (22 nm) expected for $T=1$ particles.⁽²⁷⁾ In contrast, SEC-MALLS analysis of the particles assembled on 1.9 knt RNA showed overlapping populations of particles (Figure 2B) in agreement with the TEM results (Figure 1A, C). In the leading edge of the peak (12-14 minute), which includes the void volume, the average mass falls steeply with elution volume, indicating the presence of large aggregates (Fig 2B, red data points). More uniform particles were observed on the trailing side of the peak at 15.7 minutes, with a molecular mass of 5.4 to 6.0 MDa. This range is consistent with the 5.5 MDa mass of a complex of 24 VP1 pentamers and 1 RNA suggested by absorbance (Figure 2A).

To define the heterogeneous mixture, reactions were separated on a continuous 10-40% sucrose gradient, where we observed a broad band of light scattering solute. TEM of the four fractions (Figure 3) showed presence of 22-nm particles in the top 2 fractions (fractions #1, #2); fraction #3 was dominated by doublets of 22-nm particles and fraction #4 contained a mixture of particles with diameters of ~35 nm and 22 nm, some of which appeared to be associated together.

Assembly on DNA substrate

The inability of RNA, in contrast to dsDNA,^(2, 11) to serve as a scaffold for a $T=7$ capsid, could be due either to the chemical difference between ribose and deoxyribose or to the physical properties of greater flexibility in single stranded nucleic acid. To distinguish between these possibilities we used a 3.2 knt ssDNA from M13 as an assembly substrate.

The ssDNA was the same length as the 3.2 knt RNA used in the earlier assembly studies (Figure 1A, right). Similar to results obtained with 3.2 knt RNA, the assembly products on the ssDNA were smaller than 50 nm and heterogeneous in size (Figure 4 and Supplementary Figure S1E). These findings suggest that flexibility and/or compactability of the nucleic acid is a key factor in determining capsid size and geometry.

We consequently anticipated that the less flexible dsDNA would dictate the formation of larger capsids. Building on previous studies,(2) we examined the effect of double-stranded circular supercoiled DNA (scDNA) molecules, both 2.4 and 5.2 kbp, on assembly of VP1 pentamers. By TEM, both DNAs yielded particles with diameters of approximately 50 nm, similar to virions (Figure 4). Notably, the 2.4 kbp dsDNA is less than half the size of the SV40 genome. Taking together, our results show that VP1 assembled on single stranded RNA (1.9, 3.2, 8, and 11 knt) or DNA (3.2 knt) forms the majority of $T=1$ particles and some intermediate sized particles; assembly with double-stranded circular supercoiled DNA (2.4, 5.2 kbp) forms large $T=7$ particles. These results support the hypothesis that flexibility of the nucleic acid (single stranded vs double stranded) rather than the number of nucleotides correlates with switching between $T=1$ and $T=7$ geometry.

Cooperativity of assembly on different substrates

Cooperativity of capsid assembly, attributable to strong protein-protein interactions, was evaluated by EMSA (electron mobility shift assay) of a nucleic acid scaffold titrated with capsid protein.(25, 31) High cooperativity is seen as a bimodal distribution of bands for free nucleic acid and assembled capsid, while low cooperativity leads to a gradually shifted electrophoretic rate.(25, 32)

Titration of ‘short’ RNA (0.8 knt) with VP1 gave a bimodal distribution,(27) indicating high cooperativity. In contrast, binding of VP1 to longer RNA, 3.2 knt, led to gradual shift of migration in the agarose gel (Figure 5A, left). Ethidium-stained material moved as a single band whose migration was progressively retarded as more VP1 was added. A similar gradual shift was seen with longer RNA molecules, 8 and 11 knt RNA substrates (Supplementary Figure S2). Likewise, assembly on the 3200 nt ssDNA showed a similar pattern (Figure 5A, right).

Unlike ssRNA and ssDNA, scDNA is compact but decidedly double stranded. On the scDNA substrates, a substantial concentration of pentamers was required before any shift in DNA migration was observed, then the migration was gradually retarded (Fig 5B). These results are essentially identical to those observed for linear dsDNA.(2)

Cryo-EM analysis of assembly products of VP1 on 1.9 knt RNA

Simple binding studies provide an unclear picture of the role of a flexible nucleic acid on assembly. The different morphologies of pentamer-RNA and pentamer-DNA complexes – 22 nm particles, strings of 22 nm particles, heterogeneous particles, and 50 nm virus-like particles – require a structural explanation.

Low-dose cryo-micrographs of SV40 VP1 assembled on 1.9 knt RNA showed the expected small 22-nm particles (Figure 6A, black arrows) and larger assemblies. Image analysis of the 29-40 nm particles, even when segregated based on size, did not yield stable reconstructions, suggesting that these particles are structurally heterogeneous.

Reconstruction of small particles proved to be straightforward. A total of 4386 individual small particles were manually selected from 254 micrographs. When translationally aligned, an average image showed two concentric shells consistent with a capsid layer and an RNA layer (Figure 6A, inset). The density between these two layers is stronger than background,

suggesting the presence of structural elements connecting the capsid and RNA (Supplementary Figure S3). Interestingly, the density at the center of the capsid was relatively weak.

The final 3-D reconstruction was calculated from 3511 particles to a resolution of 7.2 Å based on a Fourier shell correlation of 0.5 (Figure 6B, D). The isosurface rendering of the 22-nm particle clearly showed 12 pentamers located at each fivefold vertex, a characteristic of $T=1$ icosahedral architecture (Figure 6B). The diameter of the pentamer in the $T=1$ particle is about 89 Å, which matches well with the diameter (90 Å) of a pentamer in the $T=7$ capsid.(33) Figure 6C presents docking analysis of an atomic model of a pentamer from a $T=7$ capsid (colored ribbons, PDB entry 1SVA) into the cryo-EM density map of $T=1$ particle (wire mesh). The core of VP1 was well fit by the EM density and had room for the invading arm of adjacent pentamers. However, the C-terminal arms (C-arms) of the monomers, which connect adjacent pentamers, protruded from density (Figure 6C). These results indicate that the quaternary structural configuration of the pentamers is preserved in the $T=1$ particle while the interpentameric contacts are not.

Using the pentamer density as a restraint, we modeled interpentamer interactions. Compared to the $T=7$ capsid, the pentamers in the $T=1$ particles are packed more closely and at a steeper angle, creating a smaller radius of curvature and more extensive interpentamer interactions at each icosahedral twofold and threefold (Figure 8). Interpentamer contacts in the SV40 capsid involve the long, flexible C-arms of VP1 subunits invading into neighboring pentamers.(16, 33)

In the $T=7$ capsid pentameric pentamers and hexameric pentamers form trivalent connections (α - α' - α'') at the quasi-threefold axes.(16, 33) In the $T=1$ cryo-EM density we observed a distinctly different trivalent interaction; this contact is at an icosahedral threefold axis, not a quasi-threefold axis. When rendered at high contour, three short segments of density are present around the threefold axis (Figure 6A, yellow color). The short C-arm α -helix from the $T=7$ model (residues 301-312) sticks out from the $T=1$ cryo-EM density map (Figure 6C, 8A). Because the pentapeptide K²⁹⁶NPYP serves as a hinge orienting the C-arm, (34) we broke the model between residues P³⁰⁰-I³⁰¹ and fit the C-terminal region to cryo-EM density. Tilting the C-arm helix $\sim 69^\circ$ away from its $T=7$ position allows it to fit well to the cryo-EM density (Figure 8 and Supplementary Figure S4), resulting in a new structural model.

In the $T=1$ particle the short C-arm α -helix (residues 301-312) is roughly tangent to the capsid. A complex of three helices forms a triangular frame that surrounds the threefold axis (Figure 8A). In the $T=7$ particle these helices form a parallel three-helix bundle (Supplementary Figure S4). The hydrophobic contacts of this complex, along with protein-RNA interaction, provides energy needed to stabilize a $T=1$ particle (Figure 8B). Analogous hydrophobic interactions have been observed in $T=1$ VLPs in BK polyomavirus(35) and $T=1$ human papillomavirus.(36) Residues beyond the C-arm helix are not well ordered.

Capsid-RNA interaction was not well ordered. When rendered at a very low contour level ($\sigma=0.13$) some density was observed under the twofold axis, close to the last visible residue in the X-ray structure at the N-terminus (Figure 7), where the DNA binding domain of VP1 is located.(37) The lack of coherent signal connecting the RNA to the atomic model strongly suggests that this peptide is not well ordered, in agreement with the X-ray data of the $T=7$ capsid (16, 33).

The internal sphere of density in the reconstruction was attributed to the encapsidated 1.9 knt RNA (Figure 6B, 7). The RNA forms a hollow sphere of density, measuring 26 Å in

thickness on average. The low density center is evident in CTF-corrected averages (Figure 6A inset), indicating that it is not a reconstruction artifact. Of note, the volume attributed to RNA could only accommodate <1.7 knt of the 1.9 knt substrate, suggesting that at least some of the RNA was extruded from the capsid.

In this study we have made three critical observations:

- i. Pentamer-pentamer association in a $T=1$ particle is based on different set of contacts than found in a $T=7$ structure, indicating that SV40 pentamers actually encode a constellation of stable interactions. These are likely to be accidental consequences of the $T=7$ complex found in infections. These accidents in an evolved structure should be a caution for those interested in designing self-assembling molecules.
- ii. The RNA substrate always yields a large fraction of $T=1$ particles even though the RNA may be too big to fit in a $T=1$ cage. The fundamental lessons here is that SV40 $T=1$ assembly, and presumably also $T=7$ assembly, does not conform to the global features of the substrate, rather it is trapped by the early stages of assembly, consistent with the very strong association energy estimated from curve fitting $T=1$ assembly kinetics (27) and the kinetic traps observed in pH-induced assembly of empty particles (20). This hypothesis emphasizes our view that substrate flexibility is a critical determinant of SV40 local quaternary structure.
- iii. Though theory has consistently shown the advantages to driving assembly of empty and nucleic acid-filled capsids by weak interactions between multivalent subunits (21, 38), SV40 assembly is driven (and trapped by) strong interactions. This observation begs the question: why don't pentamers assemble spontaneously in the absence of RNA? We suggest that assembly is prevented by a high kinetic barrier which can be overcome only with the aid of a catalyst such as nucleic acid (as shown in this paper and our previous studies) or a chaperone (24).

These results show that regular capsids can be based on very strong local interactions if nucleation of assembly and subsequent elongation is strictly regulated. One likely explanation for such regulation is that nucleic acid chaperones the transition to an assembly active state. Another alternative is that assembly proceeds by an induced fit mechanism (39).

Controlling the geometry of self-assembly will allow formation of novel designed structures. Viruses are the quintessential example of uniform self-assembly *in vivo* and yet *in vitro* can be led off-path. Here we examine how the capsid protein of SV40, a $T=7$ dsDNA virus, can form $T=1$ and irregular particles using a single-stranded nucleic acid substrate. We have solved the structure of $T=1$ SV40 capsid to subnanometer resolution by cryo-EM image reconstruction and observed that the C-terminal arms of VP1 adopted a conformation not seen in the $T=7$ structure. Thus, the morphological switch for SV40 capsid assembly and geometry is achieved through the C-terminal arms, which are capable of adopting multiple conformations.

We propose that this switch is directed by substrates. In this study that role is played by nucleic acid. Stiff dsDNA substrates, whether a linear 600-mer (2) or a compact scDNA plasmid (11, 14) (see also Figure 4), led to 50 nm virus-like particles (presumably $T=7$). Conversely, ssRNA of 0.8 knt led exclusively to 22 nm particles.(1)

Between the two extremes of 50 nm $T=7$ particles and 22 nm $T=1$ particles are those based on ssRNA and ssDNA that is too long to fit into a $T=1$ capsid. In the range of samples tested in this paper, a mixture of predominantly $T=1$ capsid (~60%) was formed along with a heterogeneous population with a range of diameters centered around 34 nm diameter (Figure

1). One mechanism used to contain overlong RNA was formation of oligomers of $T=1$ particles. For a 1.9 knt ssRNA where the majority of particles were $T=1$, the UV 260/280 absorbance (Fig 2A) the average molecular mass obtained by SEC-MALLS (Figure 2B) were consistent with the 5.6 MDa complex of 24 pentamers and one RNA. Our TEM results, in particular after sucrose gradient fractionation, indicated the presence of numerous doublets of $T=1$ particles providing a clear structural basis of this stoichiometry (Figure 1, 3). Similar doublet of capsids were recently described for Cowpea Chlorotic Mottle Virus (CCMV) assembled on long RNA.(40) Indeed, the gradual changes in migration of long RNA in EMSA experiments were likely to be due to forming multiplets of $T=1$ capsids (Figure 5). Therefore we suggest that the high proportion of single $T=1$ particles observed by TEM may correspond to doublets that dissociated during sample handling, or singlets with the excess, unpackaged RNA extruding out.

The 7.2Å cryo-EM structure of $T=1$ SV40 shows how the local geometry of interaction between pentamers (Figure 6, 8 and supplementary Figure S4) leads to a much smaller radius of curvature than seen in $T=7$ particles. In a $T=1$ particle we observed a novel threefold interaction modulated by the short C-arm helix (residues 301-312). Hydrophobic contacts between helices stabilized a triangular complex that is tangent to the virus surface (Figure 8); the same helices in a $T=7$ structure form a three-helix bundle that is perpendicular to the capsid surface (Supplementary Figure S4).(16) Modeling the three-helix triangle was consistent with a flexible hinge following Pro300, no other significant changes were necessary to model the $T=1$ particle.(34) Though not clearly resolved in this structure, it is evident based on density of the pentamers that the rest of the C-terminal arm goes on to invade an adjacent pentamer, as it does in the $T=7$ structure.(16) The SV40 $T=1$ particle is very similar to the $T=1$ capsids formed by the BK polyomavirus,(35) except for the twofold interaction in the SV40 $T=1$ particle (Figure 7), which is modulated by RNA. No corresponding twofold density was observed in the BK virus $T=1$ structure, which was not surprising as its assembly was induced by Ca^{++} and not an internal scaffold of nucleic acid.

Packaged RNA in the $T=1$ structure formed a hollow internal sphere (Figure 7). The internal cavity is not an artifact of icosahedral reconstruction or the contrast transfer function (CTF) as weak central density is evident when CTF-corrected images are overlaid to generate a circularly averaged image (Figure 4A inset). The volume of the RNA density, assuming relatively close packing and high occupancy, could accommodate a maximum of 1.7 knt; the actual occupancy is likely to be much less. The RNA only approaches the capsid near icosahedral twofold axes, corresponding to the expected locations of the basic N-terminal segment of VP1. In the crystal structure, the first 14 amino acids of VP1 are disordered and the next seven form part of a clamp that interacts with a C-terminal arm invading from an adjacent pentamer.(16) Thus, the RNA density indicates a similar arrangement is to be found in our $T=1$ particles.

How are different types of assembly products formed on identical RNA molecules? Elrad and Hagan(29) predicted that if the nucleic acid were beyond a certain length, it would not be encapsulated entirely. In such cases, the uncontained segment may act like free nucleic acid, nucleating assembly of a second capsid and resulting in a doublet. However, in addition to polymers of $T=1$ particles and the choice between $T=1$ and $T=7$ particles, we observe intermediate sizes.

The large heterogeneous particles observed on long ssRNA and long ssDNA by TEM had a continuum of sizes (Figure 1, 4, 7). Even for subsets of particles sorted by size, we were unable to generate stable reconstructions whether we imposed icosahedral symmetry or assumed no intrinsic symmetry. While it is possible that the mixture includes $T=3$ and $T=4$ icosahedra and 24 pentamer octahedra, it is more likely that the majority of these particles

are truly irregular. The relatively narrow range of sizes suggests that the number of pentamers recruited by a given piece of nucleic acid must be restrained by electrostatic complementarity and the local geometry of pentamer-pentamer interaction. It is readily conceivable that mixing the $T=1$ threefold interaction ($\alpha-\alpha-\alpha$, Figure 8) with those found in a $T=7$ particle (Supplementary Figure S4) could allow a variety of different irregular particles. A $T=7$ SV40 capsid has a C-arm helix mediated contacts at quasi-threefold ($\alpha-\alpha'-\alpha''$) and quasi-twofold ($\beta-\beta'$) vertices; there is a direct contact between subunits at twofold axes.⁽¹⁶⁾ An irregular complex would have a mosaic of interactions allowing a roughly spherical particle with a variety of disclinations to achieve a local energy minimum that accommodates protein-protein and protein-nucleic acid interactions.^(29, 41, 42)

A variety of pentamer-pentamer interactions are possible in an irregular particle. The selection could be related to the kinetics of bond formation, the stability of individual interactions, and the work required to force the nucleic acid into the appropriate conformation.⁽²⁵⁾ Assembly of $T=1$ SV40 particles is very fast and yields a very stable complex.⁽¹⁾ On long RNA, assembly starting near both ends of an extended molecule would result in a doublet of 22 nm capsids.⁽⁴³⁾ Thus, the interactions resulting in an intermediate-sized particle are likely to be determined by local conformation of the nucleic acid substrate. A highly folded RNA that is incommensurate with the volume of a 22 nm capsid would give rise to a ~35 nm particle. Supporting this concept, recent data suggest that viral RNA favors a loose prolate structure.^(44, 45)

In contrast to 22 nm and 35 nm capsids, only ~50 nm $T=7$ icosahedra were formed on double-stranded DNA, regardless of its size. It appears that the stiffness of dsDNA (~50 nm persistence length) prevents pentamer-pentamer interaction with a smaller radius of curvature. Again, this can be put into the context of the amount of work required to deform the DNA compared to the strength of protein-protein interactions.^(25, 46) EMSA data show that cooperative interaction is required for capsid formation on DNA (Figure 4).

We conclude that the product of SV40 pentamer assembly is modulated by the template used. Pentamers are capable of several different protein-protein interactions that result in a range of products. The polymorphism of capsid conformations seen with long ssRNA is likely the result of properties of the nucleic acid and the site of assembly initiation. The C-terminal arms of VP1 allow flexible interaction between neighboring pentamers. A critical implication is that SV40 pentamer assembly will be far more accommodating of substrate geometry and thus far more useful for coating nanostructures. This study provides a broader perspective of supramolecular assembly.

METHODS

Protein and nucleic acids for assembly reaction

SV40 VP1 VLPs production, disassembly and reassembly reactions were performed as described (11). For assembly reactions the nucleic acid templates for ssRNA were 1900 nt (Xenopus elongation factor), 3200 nt (CCMV RNA1), 8000 nt and 11000 nt (pBR322 and pBR322-GFP plasmids, respectively); for ssDNA was 3200 nt (bacteriophage M13 truncation mutant); for supercoiled circular dsDNA were minicircle (2.4 kbp) and pGL3-control plasmid (5.2 kbp). Details are in Supplementary Information.

Sucrose gradients

Samples of assembled SV40 (260 μ l) were loaded on a ~13ml 10-60% linear sucrose gradient in assembly buffer and centrifuged for 2 hours at 40,000 rpm in a Beckman SW40 swinging bucket rotor. Bands were extracted by side puncture with a syringe.

Analysis of nucleoprotein complexes

For TEM, specimens were negatively stained with 2% UA. SEC and SEC-MALLS were set up as described in (11). See Supporting Information for more details.

Cryo-electron microscopy and data processing

The sample was applied on a Quantifoil® holey-carbon grid (R2/2) for 25s at 4 °C under 100% humidity using FEI Vitrobot™. The sample was blotted and immediately frozen in a liquid ethane bath. The specimen, kept at liquid nitrogen temperature (< -176 °C), was imaged in a JEOL 3200FS electron microscopy operated at 300 kV with an in-column energy filter using a slit width of 20 eV. Images were recorded on a Gatan UltraScan 4000 CCD camera at a pixel size of 0.18 nm under low-dose condition ($\sim 20 \text{ e}^- \text{Å}^{-2}$).

Particles were semi-automatically extracted using e2boxer.py. Three-dimensional reconstruction was performed using AUTO3DEM (v4.02). The final resolution of the map was computed to 7.2 Å from 3511 particles. The structure was modeled and visualized in Chimera. Full methods are described in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SEC	size exclusion chromatography
TEM	transmission electron microscope
MALLS	multi angel light scattering

Figure 1. VP1 assembly on RNA

(A) TEM images of particles formed on 1900 nt (left) and 3200 nt (right) RNA. (B) particles formed on 8000 nt (left) and 11000 nt (right) RNA. Particles were stained with uranyl acetate and visualized at magnification x60,000. (C) Size distribution of capsids assembled on RNA. 785 capsids, from 14 TEM micrographs, were measured. The number of particles for each size is shown on a log scale. The peak of the heterogenous population at 34 nm contained ~10% particles in comparison to the fraction of 22 nm particles (54 versus 497 particles).

Figure 2. Analysis of the capsids assembled on 1900 nt RNA

(A) Protein to RNA ratio assayed by SEC-UV. The assembly products were separated on SEC and analyzed by UV-spectrophotometer. *- anticipated time for elution of 22 nm $T=1$ capsids. (B) Average Molar Mass analyzed by SEC-MALLS.

Figure 3. Sucrose gradient of particles assembled on 1900 nt RNA

Four fractions were collected from top of a sucrose gradient and visualized by TEM. Fractions #1 and #2 contain mostly $T=1$ particles. Fraction #3 is dominated by doublets of $T=1$ capsids. Fraction #4 contains larger particles. Magnification of x60,000.

Figure 4. Assembly on DNA

TEM images of particles assembled on DNA substrates, as designated. Assembly of VP1 to ssDNA led to heterogeneous particles, which were dominated by ~70% of small 22 nm particles (Supplementary Figure S1E). Some free pentamer is in the background. Magnification x60,000.

Figure 5. EMSA analysis showing titration of nucleic acid substrates with increasing molar ratios of VP1

The nucleic acid used in each experiment is designated on top. (A) RNA and ssDNA are linear; (B) scDNA is circular and supercoiled. The reaction products were analyzed on 0.6% agarose gels and stained with EtBr.

Figure 6. Cryo-EM 3-D reconstruction of aT=1 VLP assembled on a 1900 nt RNA
 (A) Micrograph of unstained, vitrified T=1 SV40 VP1 VLP. Inset shows the translationally aligned image. Note that the average image displays two concentric rings of density. (B) Radially color-cued, surface-shaded representation of T=1 SV40 VP1 VLP at 7.2-Å resolution is shown in the front (left) and internal (right) views. The internal RNA shell (blue) is essentially disconnected from the capsid shell by a 14-Å gap. The contour was chosen to render the structure at 100% expected mass. Oval, triangle, and pentagon indicate locations of twofold, threefold and fivefold axes, respectively. (C) The atomic model of one pentavalent pentamer derived from the crystal structure of VP1 (PDB entry 1SVA) was fitted into cryo-EM density map of T=1 SV40 VP1 VLP (wire mesh). VP1 subunits are in different colors. (D) The 7.2-Å resolution estimated for the cryo-EM density map was based on a Fourier shell correlation cutoff of 0.5.

Figure 7. Capsid and RNA interaction

(A) Equatorial section of central 9-Å slide rendered at low contour level. Connected density between capsid and RNA was found located under each twofold axis. (B) Close-up view from (A). The last visible residue (Pro14) at the N-terminus in the X-ray structure located closed to the connected density (red arrowhead). Oval, triangle, and pentagon indicate locations of twofold, threefold and fivefold axes, respectively.

Figure 8. Modeling of the C-terminal arm in T=1 SV40 VP1 VLP

(A) Cryo-EM density map of *T*=1 SV40 VP1 VLP was rendered at 100% expected mass (mesh, gray) and higher contour (surface, yellow). Guided by the cryo-EM density, the short helix (residues 301-312) from the C-terminal arm of VP1 was lifted up from the plane and rotated 69° from vertical axis to horizontal axis. (B) Close-up view of the cryo-EM density fitted with modeled C-terminal arm at the threefold axis. Note that the side chains from the hydrophobic residues are faced to the center cavity.