

## SV40 assembly *in vivo* and *in vitro*

Ariella Oppenheim\*, O. Ben-nun-Shaul, S. Mukherjee<sup>1</sup> and M. Abd-El-Latif

Hebrew University-Hadassah Medical School, Jerusalem, Israel

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The Simian virus 40 (SV40) capsid is a  $T = 7d$  icosahedral lattice  $\sim 45$  nm in diameter surrounding the  $\sim 5$  kb circular minichromosome. The outer shell is composed of 360 monomers of the major capsid protein VP1, tightly bound in 72 pentamers. VP1 is a jellyroll  $\beta$ -barrel, with extending N- and C-terminal arms. The N-terminal arms bind DNA and face the interior of the capsid. The flexible C-arms tie together the 72 pentamers in three distinct kinds of interactions, thus facilitating the formation of a  $T = 7$  icosahedron from identical pentameric building blocks. Assembly *in vivo* was shown to occur by addition of capsomers around the DNA. We apply a combination of biochemical and genetic approaches to study SV40 assembly. Our *in vivo* and *in vitro* studies suggest the following model: one or two capsomers bind at a high affinity to *ses*, the viral DNA encapsidation signal, forming the nucleation centre for assembly. Next, multiple capsomers attach concomitantly, at lower affinity, around the minichromosome. This increases their local concentration facilitating rapid, cooperative assembly reaction. Formation of the icosahedron proceeds either by gradual addition of single pentamers to the growing shell or by concerted assembly of pentamer clusters.

**Keywords:** virus assembly; simian virus 40; cooperative reaction; recombinant capsids

### 1. The virus

Simian virus 40 (SV40) is a small, non-enveloped DNA virus, belonging to the polyomavirus family. It was originally isolated from cultures of these cells that were used to produce poliomyelitis vaccine. SV40 undergoes lytic infection in permissive monkey cells, to a lesser extent also in human cells (semi-permissive), and immortalizes non permissive murine cells. The virus is usually propagated in African green monkey kidney cell lines such as CV-1 [1].

### 2. Capsid structure

The SV40 capsid is a  $T = 7d$  icosahedral lattice  $\sim 45$  nm in diameter surrounding the  $\sim 5$  kb circular minichromosome (Figure 1(a)). The capsid has a diameter of 45 nm and is composed of the three virally encoded proteins, VP1, VP2 and VP3. The outer shell consists of 360 monomers of the major capsid protein VP1, tightly bound in 72 pentamers [2]. VP1 peptides appear to fold into pentamers, soon after translation, through transient disulfide interactions [36]. Each VP1 monomer forms extensive contacts with its neighbours, via interdigitating  $\beta$ -strands and loops. VP2 and VP3 bridge between the outer shell and the viral genome. VP1 is a jellyroll  $\beta$ -barrel, with extending N- and C-terminal arms [34,55]. The N-terminal arm binds DNA and faces the interior of the capsid. The flexible C-arms tie together the 72 pentamers in three distinct kinds

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\*Corresponding author. Email: [ariella@cc.huji.ac.il](mailto:ariella@cc.huji.ac.il)

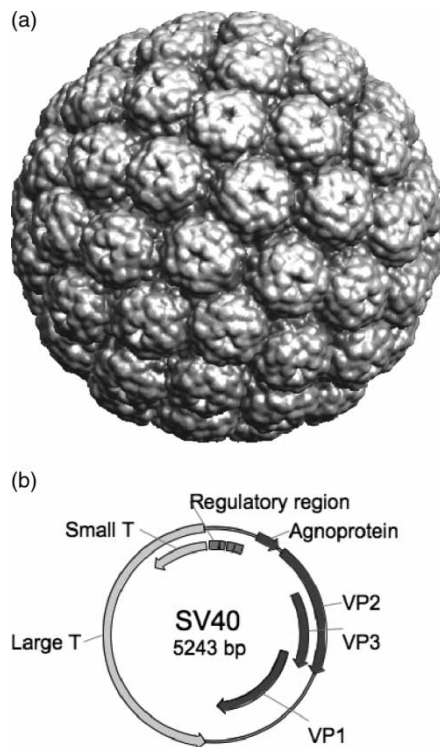


Figure 1. (a) The capsid structure from X-ray crystallography (from VIPER site [http://viperdb.scripps.edu/info\\_page.php?VDB=1sva](http://viperdb.scripps.edu/info_page.php?VDB=1sva)). (b) physical map of the SV40 genome.

of interactions, thus facilitating the formation of a  $T = 7$  icosahedron from identical pentameric building blocks [34,55].

The pentamers exhibit five fold symmetry with an inward-facing cavity. Each pentamer is associated with a monomer of either VP2 or VP3, anchored in the internal cavity of the pentamer via strong hydrophobic interactions [7]. VP3 translation initiates from an internal initiation codon (AUG) within the VP2 coding sequence, utilizing the same translational frame. Thus both proteins are identical at their carboxy part and are often referred to as VP2/3.

The genome includes the early and late coding regions and a regulatory region in between (Figure 1(b)). The early region encodes for the small and large T-antigens and the late region encodes for the viral capsid proteins, VP1–VP3 and the agnoprotein, the exact function of which is unknown. The regulatory region includes the origin of replication (*ori*), the early and late promoters, the enhancer, and the packaging signal, *ses*. The enhancer is composed of two 72 bp direct repeats.

### 3. The viral life cycle

Soon after infection, the virus produces T-antigens from the early promoter. Large T-antigen has several key functions in the SV40 life cycle acting both in transcription and replication. It is required for viral DNA-replication, mediating the switch from early to late transcription and acts in transactivation of the late genes, leading to biosynthesis of the capsid proteins VP1–VP3. Each of the capsid proteins carries a nuclear localization signal (NLS), facilitating transport to the nucleus for viral encapsidation. Each VP1 pentamer combines with a molecule of either VP2

or VP3 in strong hydrophobic interactions [7,26]. Studies with mutated NLS indicated that VP1 and VP2/3 are transported to the nucleus as a single unit [30], suggesting that VP1<sub>5</sub>VP2/3 is the building block for assembly.

#### 4. *In vivo* assembly

Early studies on SV40, performed by a number of groups, have revealed that assembly occurs inside the nuclei, by addition and organization of the capsomers around the viral minichromosome, rather than by incorporation of DNA into pre-formed capsids (for representative references see Ref. [10]). Recently, participation of chaperones in SV40 assembly has been demonstrated [8].

##### 4.1 *The assembly pathway*

Pulse-chase and velocity gradient sedimentation experiments indicated that viral morphogenesis proceeded via a series of intermediates: 100S replicating chromatin → 75S intermediate → 200 S pre-virions → 240S mature virions [3,10,20]. The 75S nucleoprotein complex, containing a small amount of capsid proteins was found to rapidly convert to 200S pre-virions, with a full complement of capsid proteins. Assembly intermediates with partial capsids were only seen in studies with virions mutated in VP1 [5,42]. Notable assembly intermediates also accumulated in virions with mutations at the amino-terminus of the T-antigen gene [52], in a region demonstrated to encode for the co-chaperone J-domain [54], supporting the requirement for chaperones.

Icosahedral capsid assembly must be an efficient, high fidelity process. The final product is a topologically closed, stable sphere-like particle. Assembly is most likely initiated by a nucleation centre, in a slow, rate limiting step [58,59]. This ensures an extremely robust assembly reaction regardless of protein concentration, avoiding 'kinetic traps' [17]. In SV40, the 75S species which is the only intermediate seen in the *in vivo* assembly studies and which contains chromatin with a small amount of VP1 and VP2/3 [4,21,53], is most likely the nucleation centre attached to the viral genome.

##### 4.2 *The SV40 packaging signal ses*

All three capsid proteins were found to bind DNA nonspecifically at a high affinity [9,35]. This raised the question how they recognize the viral minichromosome within the nucleus, in the presence of a large excess of cellular chromatin. We have identified a specific encapsidation signal in the SV40 genome, *ses*. *ses* is present within the viral regulatory region [43], encompassing the GC-boxes, that include six GGGCGG elements present within three 21 bp repeats and that bind transcription factor Sp1 [22]. Genetic investigations indicated that *ses* is composed of multiple, redundant elements that are partly interchangeable [13]. Biochemical studies showed dramatic cooperation of VP1<sub>5</sub>VP2/3 with Sp1 in binding at *ses* [25], suggesting that Sp1 and *ses* function together in recruitment of the capsomers to the SV40 minichromosome. Confirmation of the role of *ses* in capsomer recognition was obtained from experiments which demonstrated that excess of cellular DNA did not compete with *ses* for capsomer binding [26]. We have also observed that *ses* cannot be separated from the origin of replication [43] and that binding of VP2/3 repress both the early and late promoters [23], suggesting that *ses* also has a regulatory role. We have proposed that as the infection cycle progresses and the level of capsid proteins in the nucleus becomes sufficiently high, they are recruited to the packaging signal *ses* by Sp1, turning off the early and late promoters and forming the nucleation centre for assembly [25]. Thus, promoter turn off and the initiation of assembly

are tightly coupled. We have further proposed that *ses* regulates the transition from replication and late transcription to assembly and that the Sp1-VP1<sub>5</sub>VP2/3-*ses* recruitment complex serves as a nucleation centre for capsid assembly [26].

#### 4.3 Nucleosomal rearrangement and chromatin condensation

The 5.2 kb circular DNA molecule comprising the viral genome is complexed in 20–22 nucleosome and referred to as a minichromosome. The structure of the spherical, condensed circular chromatin molecule within the virion is not known. Mathematical computations [38] indicated that the internal space within an SV40 allows inclusion of 21 nucleosomes. Minichromosomes isolated from infected cells appeared mostly as globular structures when observed by cryo-electron microscopy (EM) [16].

The early and late viral promoters are localized near the *ori* in a small region, which is nuclease sensitive, as is typical of transcriptionally active genes [57]. Detailed studies by electron microscopy have revealed that some (15–25%) of the intracellular viral minichromosomes are free of nucleosomes in the nuclease-sensitive region [32,51]. In the mature virion, the unique DNase I-sensitive region disappears [28] and the average nucleosome spacing is increased, suggesting that during virion development the nucleosomes are redistributed around the viral minichromosome [11]. Nucleosomal reorganization is triggered by the capsid proteins [6,44]. It occurs by displacement of transcription and replication factors from the regulatory region [44], and is possibly mediated by PARP-1 [27]. Consistent with this scenario, we have recently found that Sp1, which binds at five of the six GC-boxes during both early and late transcription [23] is not present in the mature virion [47]. Minichromosome condensation is likely to be an energy-dependent process, which may be driven partly by interactions and binding of the capsomers in the formation of the icosahedral shell.

### 5. *In vitro* assembly

We have developed an experimental system for assembly studies, based on production of recombinant capsid proteins expressed in insect Sf9 cells and supercoiled plasmid DNA produced in *Escherichia coli*. To facilitate functional, biological assay of the assembly process we use a reporter plasmid carrying the *luc* gene. We use a quantitative assay measuring luciferase enzymatic activity in infected cells, which evaluates not only particle formation, but also their infectivity.

#### 5.1 The experimental system

When expressed in insect cells the capsid proteins assemble spontaneously in the nuclei of infected cells to form virus-like particles, VLPs [49]. Our source for capsid proteins is nuclear extract of infected Sf9 cells. We have found that purified proteins are not active in assembly [40,50], indicating that some nuclear factors are required. We also found that VP2 and VP3 do not enhance assembly (or infectivity) of the nanoparticles. Both proteins were not included in the experiments described below.

The experimental system is based on disassembly of VLPs produced in Sf9 cells and their reassembly in the presence of supercoiled plasmid DNA. SV40 was previously shown to disassemble into VP1 pentamers in the presence of reducing and chelating agents [12,31,49]. We have found that the VLPs are significantly less stable than the virus. In contrast to wild type SV40, which required both DTT and EGTA for disassociation, VLPs consisting of VP1–VP3 completely dissociated by incubation with either Dithiothreitol (DTT) or ethylene glycol tetraacetic acid (EGTA) alone [49]. For the present study we have chosen to dissociate the

particles by DTT and to avoid the use of chelating agents, as they might interfere in subsequent steps of DNA binding and assembly. As observed by EM, the VLPs start dissociating at 5 mM DTT and at 15 mM they are no longer visible (not shown).

As the DNA-binding domain of VP1 is at the internal face of the capsid, we have anticipated that the pentamers would assemble around the DNA. Based on this rationale, the disassembly-assembly reaction was planned in three steps: step A, dissociation of the VLPs in DTT; step B, the addition of DNA with concomitant dilution of the DTT to allow VP1-DNA binding and assembly and step C, stabilization of the re-assembled capsids at pH 5.2. The complete procedure has been described in detail [40].

### 5.2 The disassembly-assembly reaction

As expected, the reaction absolutely depends on presence of VP1 and DNA [40]. The yield, measured as *luc*-transducing units (TU), was highly reproducible with a small standard error of repeated experiments. As a control we used a mutant VP1, VP1 $\Delta$ C, with a deletion of the entire carboxy terminal arm that links between pentamers. As the DNA-binding domain is at the amino terminus of VP1, this mutant retains DNA-binding activity but cannot assemble into capsids [46]. We found that replacement of VP1 by VP1 $\Delta$ C completely abolished packaging. Purified VP1 was not active in the reaction, indicating a requirement for nuclear factors. We therefore, routinely use in the reaction nuclear extracts of Sf9 cells containing VP1. We also found the VP2 and VP3 do not enhance the assembly reaction or infectivity of the assembled products.

The optimal temperature for the reaction is 37°C and optimal salt concentration was 160 mM monovalent salts suggesting an enzymatic activity, provided by the nuclear extracts. The optimal salt concentration may also represent a balance between the need, on one hand, to stabilize the particles (VLPs are stabilized at 1 M NaCl), and on the other hand to allow protein-DNA interaction, which are destabilized at a high ionic strength. The reaction requires ATP and Mg<sup>++</sup>, consistent with participation of enzymatic activity [40].

Analysis of the assembled nanoparticles by equilibrium sedimentation in a CsCl density gradient showed that the peak of TU coincided with the peak of DNA at a density of 1.35 g/cm<sup>3</sup>, like wild type SV40. The nanoparticles contain a single protein (VP1) and no histone octamers. The packaged DNA released from the purified particles and analysed by electrophoresis appeared as a single distinct DNA band, suggesting that complete molecules were packaged [40,50]. Under electron microscopy the purified particles appear well dispersed and of uniform shape and size (~45 nm; Figure 2(b)), like wild type SV40 (Figure 2(c)). The purified particles appeared to be more permeable to the stain than wild type SV40, most likely because of the absence of histone proteins, which were shown to occupy much of the space within the particles [38]. It may be noted that prior to packaging many of the VLPs were smaller (Figure 2(a)), presumably  $T = 1$  and 3 structures. Thus, it appears that the present reaction conditions direct the formation of  $T = 7$  particles.

We tested the assembled particles for DNA protection by extensive DNase I digestion, after raising the pH from 5.2 to ~ 8. The results consistently demonstrated that the packaged DNA was DNase resistant.

### 5.3 Stoichiometry of the reaction and cooperativity of VP1

The reaction yield increases linearly with increasing DNA input, reaching an optimum at 1  $\mu$ g DNA per reaction (Figure 3(a)). Higher DNA concentrations were inhibitory, may be because they sequestered VP1 pentamers and did not allow the assembly intermediates to go to completion. When VP1 concentration was varied the reaction was sigmoid, indicating cooperativity and reached saturation at 5  $\mu$ g VP1 per reaction (Figure 3(b)). The optimal

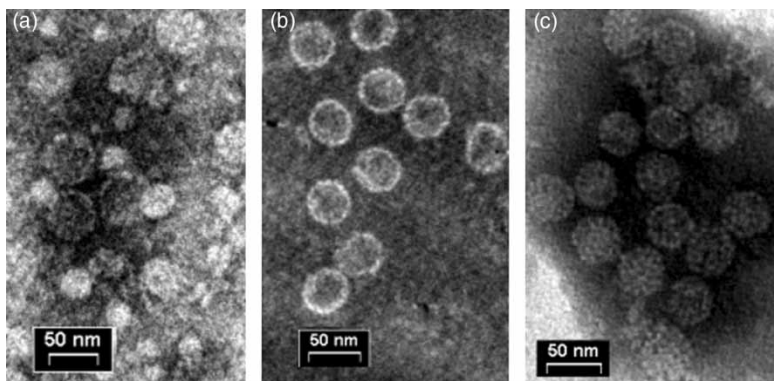


Figure 2. Structure of the nanoparticles. Transmission electron microscopy pictures of (a) VLPs, (b) *in vitro* packaged nanoparticles and (c) wild type SV40. Samples were adsorbed onto formvar-carbon-coated copper grids and stained with 1% sodium phosphotungstate, pH 7.0. The samples were viewed in a Philips CM-12 electron microscope, using a voltage of 100 kV and photographed at a magnification of 53,000 $\times$  (from Ref. [40]).

VP1:DNA ratio on a weight basis, 5:1, corresponded to a 1:1 capsid to DNA molar ratio (MW of a VP1 capsid is  $\sim 15$  MDa, and of a 5 kbp DNA molecule is  $\sim 3$  MDa). At high VP1 concentrations the reaction levels off. It is possible that excess VP1 molecules assemble into empty particles.

Different nuclear extract preparations contained variable levels of VP1. This is not surprising, since VP1 level depends on a number of biological factors that are not fully controlled. Taking advantage of this variability we analysed 14 different nuclear extract preparations with different VP1 contents. The results (Figure 3(c)) showed dramatic sigmoid correlation between reaction yield and VP1 content. Extracts that contained less than  $1 \mu\text{g}/\mu\text{l}$  VP1 had very low activity. These data were analysed according to the Hill equation, which provides a way to quantify cooperativity of a reaction [29]. The resulting Hill coefficient was  $5.8 \pm 0.6$  (Figure 3(d)), suggesting that under the present conditions (in presence of nuclear factors) cooperativity was between at least 6 and 7 partners. For comparison, the Hill coefficient of the cooperative binding of 4  $\text{O}_2$  molecules to the haemoglobin tetramer is 2.8 [19,29].

#### 5.4 The reaction process

How does assembly *in vitro* proceed? Studies on binding of VP1 to complete SV40 molecules have demonstrated that it binds at multiple sites along the entire molecule (Figure 4; [47]). In those experiments, we have used the VP1- $\Delta\text{C}$  mutant, which retains DNA binding but cannot assemble into capsids. We propose that the *in vitro* assembly reaction begins by binding of the disassembled VP1 pentamers along the supercoiled DNA molecules (Figure 5). This increases the local concentration of VP1, facilitating concerted assembly, as seen by the high VP1 cooperativity and Hill coefficient (Figure 3). Supporting this scenario we found that DNA-binding is a rapid reaction that is completed within a few minutes, while complete assembly of the nanoparticles is the rate-limiting step, requiring hours to go to completion (our unpublished results). The DNA appears to function as a scaffold for capsid formation, as described for CCMV [39]. However, for recombinant VP1 of members of the polyomaviridae family the DNA scaffold is not obligatory as we and others have observed that it can assemble into empty capsids in the absence of DNA [18,24,33,45,48,49,56]. For *in vitro* assembly *ses* is not required, since



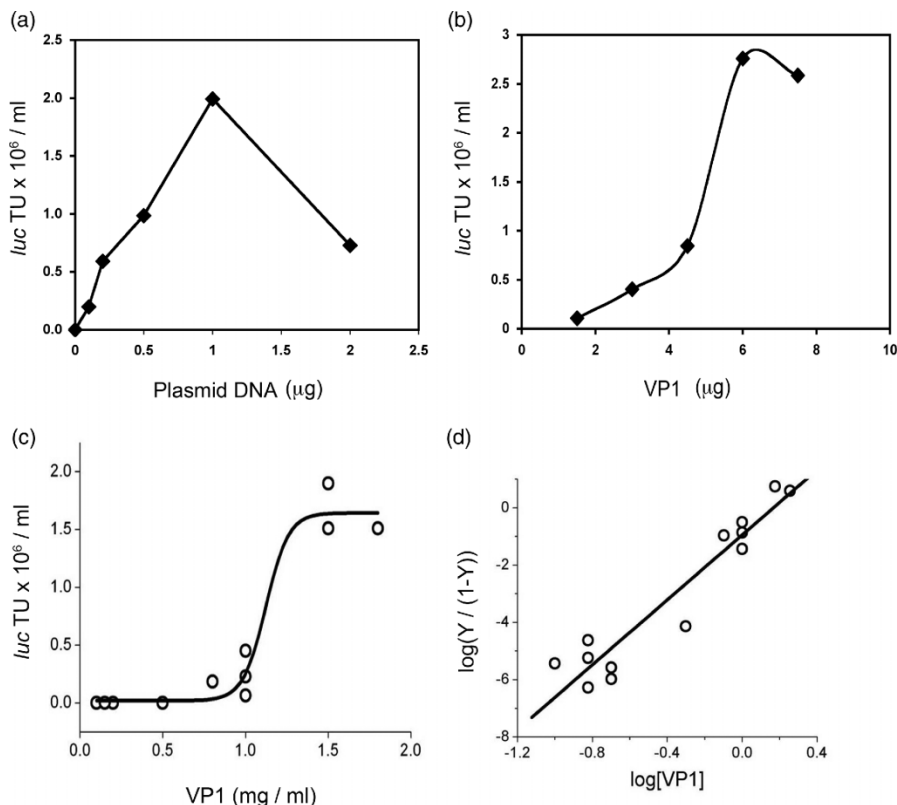


Figure 3. Cooperativity of VP1 in the reaction. Effect of substrate concentration on the reaction yield. (a) DNA; (b) VP1. The amounts used per reaction, as described in Materials and methods are indicated. (c) Fourteen different batches of nuclear extracts were assayed for packaging activity. Only 11 distinct data points are seen because of overlap of some of the data points. (d) Calculation of Hill coefficient from the same set of data as in C.  $Y$  is the fraction of fully assembled VP1, measured as TU (from Ref. [40]).

the plasmid DNA participating in the reaction does not have to compete with cellular DNA for capsomer binding.

## 6. Comparison of the *in vitro* reaction to *in vivo* assembly

The *in vitro* assembly process described above differs from *in vivo* assembly of SV40 primarily by the absence of VP2 and VP3 and the use of naked plasmid DNA rather than a minichromosome. Nevertheless, the findings obtained from this simplified *in vitro* assembly system are in agreement with *in vivo* studies. First, the reaction represents assembly around the SV40 genome, rather than insertion of DNA into preformed shells. Second, our data account for the inability of previous investigators to isolate partially assembled intermediates from cells infected with the wild type virus [3,10,20]. Furthermore, the requirement for host factors, for physiological conditions (temperature, pH and salt concentration) and for ATP and  $Mg^{++}$  is consistent with the participation of chaperones in the *in vitro* system. A number of investigators have recently reported, based on studies with mutant viruses of both polyoma and SV40, that VP2 and VP3 are not required for the assembly process *in vivo*, but rather for infectivity, egress and for the transport of the viral genome to the nucleus [14,15,37,41]. Consistent with these data the particles produced here are significantly less infective than wild type SV40.

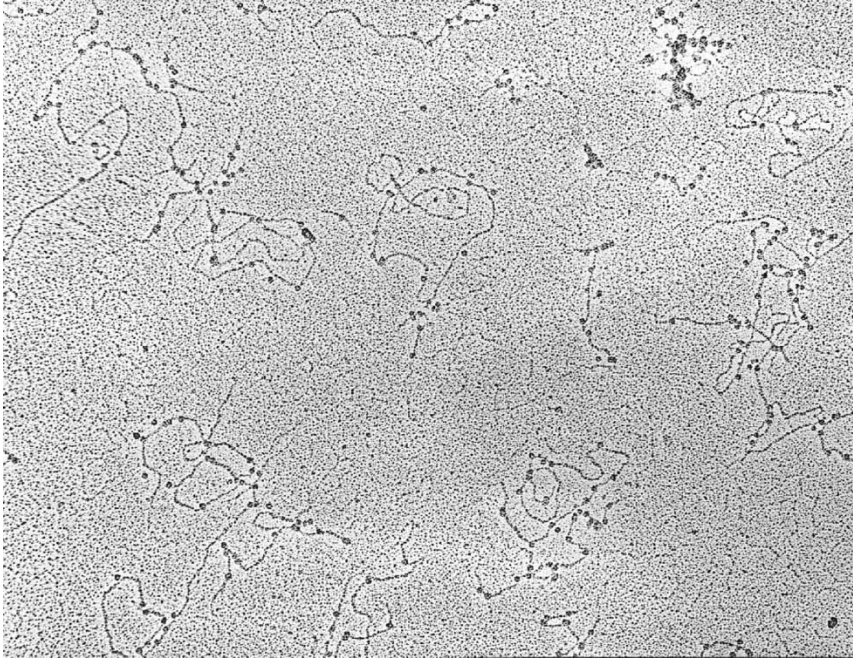


Figure 4. Visualization of protein–DNA interaction. VP1 $\Delta$ C<sub>5</sub>VP3 complexes (1  $\mu$ g, 4.78 pmol) were incubated with circular SV40 DNA (375 ng; 0.11 pmol) and 1.5  $\mu$ g poly[dI-dC] and applied onto grids by BAC spreading (from Ref. [47]).

Additional investigations are needed for full *in vitro* recapitulation of the assembly process, in presence of all three capsid proteins and the viral minichromosome. Nevertheless, the present *in vitro* reaction facilitates an insight into the assembly reaction and will serve for further biochemical and biophysical investigations using both wild type and mutant VP1, and as a basis for identification of participating host factors.

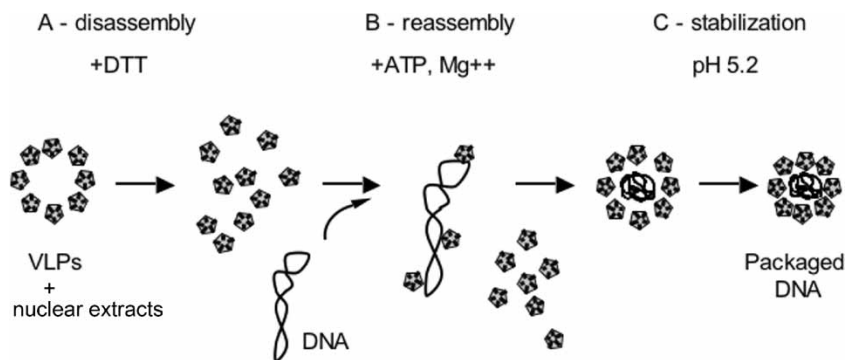


Figure 5. A cartoon describing the *in vitro* assembly reaction. The addition of DTT to nuclear extracts (step A) leads to disassembly of the VLPs. Following the addition of supercoiled DNA (step B) pentamers bind along each DNA molecule. This increases the local concentration of VP1, facilitating concerted assembly. Reassembly may be facilitated by presence of chaperones in the nuclear extracts. Assembly is accompanied by DNA condensation, presumably via the action of topo II. In step C, the capsids are stabilized at pH 5.2 (from Ref. [40]).



## 7. A model for capsid assembly

In polyomaviruses, the icosahedral capsid is assembled from identical 72 VP1 pentamers. Twelve of the 72 pentamers, located at the vertices of the icosahedron (pentavalent pentamers), are surrounded by 5 pentamers each. The other 60 (hexavalent pentamers) are surrounded by 6 pentamers each. Thus identical building blocks are in widely different contacts with their neighbours, depending on their location in the capsid. The virus solved this structural paradox by using flexible C-arms, which can assume different conformations to connect between pentamers [34,55].

The need for a number of different contacts and C-arm conformations during assembly adds considerable complexity to the assembly process. Stehle *et al.* [55] proposed that assembly begins and proceeds by stepwise addition of pentamers to one another in the growing shell, in a highly error-prone process, often leading to formation of incorrect structures or ‘dead ends’. They suggested that these ‘dead end’ structures are disassembled by chaperones, thus facilitating formation of the  $T = 7$  icosahedron. An early intermediate on the correct assembly pathway was proposed to be a pentavalent pentamer surrounded by five additional ones. It has been further pointed out that this five fold symmetrical structure already has a relatively defined curvature, which may favour the correct continuation of shell growth [55].

Here, we present an alternative assembly model for SV40 and other polyomaviruses, that avoids the ambiguity at each assembly step, and which takes into account the high cooperativity of VP1 observed in the *in vivo* reaction as described above. Polyomaviruses decrease the initial complexity of assembly from 360 VP1 monomers by folding the monomeric peptides into pentamers soon after translation [36], reducing the number of building blocks to 72. Following the same rationale, we propose that the capsid assembles not from individual pentamers, but rather from larger units that contain several pentamers joined together (pentamer clusters). Structural considerations (our unpublished data) suggest that this larger unit is a cluster composed of a pentavalent pentamer surrounded by five other pentamers (1+5 cluster). 12 such clusters are required to form the complete shell. Specifically, our model proposes that the capsid is formed by cooperative interactions of 12 preformed 1+5 pentamer clusters, bound to a viral minichromosome, which serves as a scaffold for assembly. This model takes into account the Hill coefficient of  $\sim 6$  found for the *in vitro* assembly reaction.

Another, less likely possibility is that the assembly unit is a hexavalent pentamers surrounded by 6 pentamers each (1+6 cluster). In this case, assembly of a complete capsid requires 10 such units and two single pentamers, to be placed at the remaining icosahedral vertices. These two pentamers would complete the icosahedral shell, locking the other 70 pentamers in their position. However, this scenario is less probable based on considerations of symmetry, as well as structural examinations and the types of interpentameric bonds that participate in the 1+5 vs. the 1+6 clusters (our unpublished data).

Our previous studies have suggested that *in vivo*, as the infection cycle progresses and the level of capsid proteins in the nucleus becomes sufficiently high, they are recruited to the packaging signal *ses* by Sp1, turning off the early and late promoters and forming the nucleation centre for assembly [25]. Thus, promoter turn off and assembly are highly coupled.

According to the present model, the nucleation centre is composed of a 1+5 pentamer cluster attached to *ses*. Its formation is followed by rapid binding of multiple 1+5 capsomer clusters around the minichromosome at a lower affinity. The pentamer clusters bind at quasi-random locations, since only part of the DNA is accessible, depending on nucleosomal configuration. Our model further suggests that the minichromosome functions as a scaffold by bringing together the pentamer clusters, increasing their local concentration, facilitating concerted assembly reaction. The central pentamers of the 1+5 clusters form the vertices of the

assembling icosahedron, forcing their surrounding pentamers to combine in the formation of the capsid. The cooperative interactions of the twelve 1+5 pentamer clusters and the formation of low free energy bonds provide the energy for condensing the minichromosome into a constraint topological state. Stability of the icosahedral shell with its low free energy state drives the reaction to completion and ensures irreversibility of the reaction. This model which relies on the viral genome as a scaffold and on larger and fewer building blocks than previously realized, provides for a robust assembly pathway.

## 8. Conclusion

The structure of the SV40 capsid was solved at 3.1 Å resolution over ten years ago, however the process leading to its formation is not well understood. The emerging picture from both *in vivo* and *in vitro* studies suggest that following initial rapid binding of VP1 to DNA and formation of nucleation centre, assembly is achieved by a cooperative interaction between capsomers around the condensing DNA as a scaffold.

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## Note

1. Present address: Department of Biochemistry and Molecular Biology, Oklahoma University Health Sciences Center, Oklahoma City, OK, USA.

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