FOR THE RECORD

A structural rationale for SV40 Vp1 temperature-sensitive mutants and their complementation

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Abstract

Two groups of temperature-sensitive (*ts*) mutants, termed *ts* B and *ts* C, have mutations in the major capsid protein of SV40, Vp1. These mutants have virion assembly defects at the nonpermissive temperature, but can complement one another when two mutants, one from each group, coinfect a cell. A third group of mutants, termed *ts* BC, have related phenotypes, but do not complement other mutants. We found that the mutations fall into two structural and functional classes. All *ts* C and one *ts* BC mutations map to the region close to the Ca²⁺ binding sites, and are predicted to disrupt the insertion of the distal part of the C-terminal invading arm (C-arm) into the receiving clamp. They share a severe defect in assembly at the nonpermissive temperature, with few capsid proteins attached to the viral minichromosome. By contrast, all *ts* B and most *ts* BC mutations map to a contiguous region including acceptor sites for the proximal part of the C-arm and intrapentamer contacts. These mutants form assembly intermediates that carry substantial capsid proteins on the minichromosome. Thus, accurate virion assembly is prevented by mutations that disrupt interactions between the receiving pentamer and both the proximal and distal parts of the C-arms, with the latter having a greater effect. The distinct spatial localization and assembly defects of the two classes of mutants provide a rationale for their intracistronic complementation and suggest models of capsid assembly.

Keywords: viral assembly; complementation; temperature-sensitive mutants; SV40; capsid

SV40 is a small DNA virus belonging to the polyomaviridae family. Virus particles are ~450 Å in diameter with icosahedral symmetry. Viral capsids contain 360 copies of a major structural protein, Vp1, arranged as 72 pentameric building blocks on the viral surface (Rayment et al. 1982). Mature virus particles also contain ~72 copies of

the minor structural proteins Vp2 and Vp3; four host histones (H2A, H2B, H3, and H4); and the viral genome, a double-stranded, circular DNA of about 5200 base pairs, together forming a “minichromosome.” The capsid structure is known at atomic resolution (Liddington et al. 1991; Stehle et al. 1996). The all-pentamer organization requires that the interactions between pentamers holding the capsid together cannot conform to the Caspar-Klug rules of quasisymmetry (Caspar and Klug 1962). Instead, the capsid is tied together by long C-terminal arms (C-arms) that emanate from each Vp1 and invade a

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neighboring pentamer. The arms adopt alternate conformations between pentamers, but at their distal ends they adopt identical conformations, wrapping around the body of a neighboring pentamer and inserting into a clamp that ends at a dual Ca^{2+} binding site.

Five Ca^{2+} -coordinating acidic residues are critical for the formation of an infectious particle (Li et al. 2003). Assembly of the closely related polyomavirus Vp1 pentamer in vitro also requires Ca^{2+} ions (Salunke et al. 1986). The crystal structure of a Vp1 pentamer in complex with a fragment of the common C-terminal segment of Vp2 and Vp3 (hereafter referred to as Vp3) has been determined for polyomavirus (Chen et al. 1998). The Vp3 fragment inserts into the axial cavity of the pentamer, and both the N- and C-terminal ends extend toward the interior of the virus. The Vp3 fragment is nearly identical in sequence to that of SV40, as is the interaction site on Vp1, which is therefore likely to bind Vp3 in an analogous fashion. One Vp1 pentamer bound to one Vp3 is presumed to initiate condensation of the minichromosome for virion assembly in the nucleus. Assembly occurs sequentially, with capsid proteins arranging themselves on the viral minichromosome (Garber et al. 1978, 1980; Baumgartner et al. 1979; Coca-Prados and Hsu 1979; Fernandez-Munoz et al. 1979; Fanning and Baumgartner 1980; Jakobovits and Aloni 1980; La Bella and Vesco 1980). Correct icosahedral virion assembly requires selecting specific intrapentamer and interpentamer bonding, calcium binding, and possibly Vp3 interaction.

Three classes of temperature-sensitive (*ts*) mutants have been defined within the Vp1 gene: *ts* B, *ts* C, and *ts* BC (Tegtmeyer and Ozer 1971; Chou and Martin 1974; Dubbs et al. 1974; Lai and Nathans 1974; Robb et al. 1974; Tevethia et al. 1974; Tevethia and Ripper 1977; Noonan and Butel 1978; Cosman and Tevethia 1981; Mertz 1984). *ts* B mutants complement *ts* C mutants, while *ts* BC mutants do not complement other mutants (Chou and Martin 1974, 1975; Lai and Nathans 1975, 1976). Lai and Nathan have proposed that complementation occurs at the protein level, but the molecular basis has not been explained. The block in assembly of *ts* mutants leads to the accumulation of two classes of assembly intermediates: 75S particles comprising SV40 minichromosomes with little or no capsid proteins, and 100–160S intermediates with extensive association of capsid proteins (Garber et al. 1978, 1980; Bina et al. 1983a,b; Blasquez et al. 1983; Ng and Bina 1984). In contrast, mature virions have a sedimentation coefficient of 240S. Based on the observation of the intermediates, nuclear virion assembly has been proposed to proceed in an orderly and stepwise fashion involving the addition of structural proteins to an SV40 minichromosome (Garber et al. 1978, 1980; Bina et al. 1983a; Blasquez et al. 1983; Ng and Bina 1984). Mapping the *ts* mutants onto the capsid structure has allowed us to correlate them with

their phenotypes, provide a rationale for intracistronic (that is, within one gene) complementation, and to provide further clues into the assembly process.

Results and Discussion

We mapped Vp1 *ts* mutations onto the SV40 capsid structure and onto a model of the Vp1–Vp3 complex (see Materials and Methods). Twenty Vp1 *ts* mutants have been previously sequenced (Behm et al. 1988), and seven further mutants were sequenced in this study (Table 1); of these, 21 are distinct Vp1 mutants. All mutations map to structural elements that form inter- and intrapentamer interactions within the viral capsid (Table 1). Table 1 also lists the reported assembly intermediates that accumulate at the nonpermissive temperature (Garber et al. 1978, 1980; Bina et al. 1983a,b; Blasquez et al. 1983; Ng and Bina 1984). The mutation sites fall into two major regions, I and II (shown in Figs. 1, 2, colored according to their *ts* group), each with a distinctive phenotype at the nonpermissive temperature. It is noteworthy that none of the mutants are found in residues within the C-terminal arms themselves. Presumably, such mutants are too disruptive to be tolerated at any temperature.

Interaction with the proximal part of the invading arm is required for accurate capsid assembly

Region I encompasses a contiguous region that includes a small “jelly-roll” domain inserted between strands E and F (which packs against the side of the CHEF β -sheet, creating the acceptor site for the proximal part of the invading C-arm), as well as surrounding loops on the middle and upper surface of the pentamer. All 10 *ts* B mutations and four out of five *ts* BC mutations map to this region (Fig. 1). Of these, three *ts* B mutations (at Gly 163, Ala 166, and Ala 195) and three *ts* BC mutations (at Thr 170, His 193, and Asp 200) map to the jelly-roll domain itself; four *ts* B mutations (at Gln 54, Pro 58, Glu 83, and Ala 71) and one *ts* BC mutation (at Pro 283) map to loops and strands that make direct contact with the jelly roll domain; three *ts* B mutations (at His 136, Gly 139, and Ser 147) are more distant, and map to loops that interlock neighboring monomers at the top of the pentamer. Region I mutants share a common phenotype at the nonpermissive temperature: a defect in assembly leading to the accumulation of intermediates of heterogeneous size (100–160S) carrying substantial amounts of Vp1 (Table 1) (Bina et al. 1983a,b; Blasquez et al. 1983).

Given the conserved phenotype and spatial clustering of the Region I mutations, the structural basis for the assembly defect is likely to have the same origin: compromised pentamer–pentamer contacts that are mediated by the proximal part of the invading arm. In 11 out

Table 1. Location of *ts* mutations on the Vp1 secondary and tertiary structure and their reported assembly intermediates

<i>ts</i> Mutant	Residue/substitution ^a	Structural element ^b	Structural region	Assembly intermediate (S) ^c
C260 ^d	Gly40 Glu ¹	AB	II	75
C1641	Val47 Met ²	B	II	ND
B228	Gln54 Lys ¹	BC1	I	100/160
B218	Pro58 Arg ¹	BC1	I	100/160
B204, 211,265	Ala71 Val ¹	BC2	I	100/160
B8 ^e	Ala71 Thr ¹	BC2	I	ND
B8 ^e	Glu83 Asp ¹	BC2	I	ND
B1640	His136 Tyr ²	DE	I	ND
B1606	Gly139 Asp ²	DE	I	ND
B1603	Ser 147 Leu ²	DE	I	ND
B221	Gly163 Ala ¹	E'	I	ND
B201	Ala166 Thr ¹	E'E''	I	100/160
BC1602,1607	Thr170 Ile ²	E'E''	I	ND
BC223	His193 Tyr ¹	E''	I	100/160
B4	Ala195 Val ¹	E''	I	ND
BC1605	Asp200 Asn ²	E''E'''	I	ND
C219	Pro212 Ser ¹	E'''F	II	75
C240	Leu244 Ile ¹	G2	II	75
C260 ^d	Pro252 Leu ¹	G2	II	75
BC208, 214,216,217,248,274	Pro283 Ser ¹	I	I	100/160
BC11	Lys287 Thr ¹	I	II	75

The nomenclature used is the same as that in Liddington et al. (1991) and Stehle and Harrison (1997).

^a Amino acid substitutions determined by Behm et al. (1988),¹ or in this study.²

^b Secondary structural elements of a monomer are labeled: (β -strands) B, E', E'', G2, and I; (loops) AB, BC1, BC2, DE, E'E'', E''E''', and E'''F.

^c Types of assembly intermediates found at nonpermissive temperature, S, Svedberg value. (From Garber et al. 1978, 1980; Bina et al. 1983a,b; Blasquez et al. 1983; Ng and Bina 1984.)

^d *ts* C260 has two amino acid substitutions.

^e *ts* B8 has two amino acid substitutions.

ND, Not known.

of 14 cases, this appears to be fairly direct, while in three other cases we surmise that the effect is mediated through a more global effect on pentamer integrity. The presence of substantial amounts of capsid proteins in the assembly intermediates suggests that the initial interaction of pentamers with Vp3 and the minichromosome, as well as cross-linking of the distal part of the invading arms with the clamp and Ca²⁺ binding sites, can proceed with some efficiency. However, we suggest that subsequent steps in assembly, which require correct choices among twofold and threefold subassemblies, are compromised.

The Ca²⁺ binding sites and distal C-arm interactions are required for the initiation of minichromosome packaging

Region II mutations cluster at the base of the pentamer: the region includes the clamp for the distal part of the C-arm, the two Ca²⁺ binding sites that cross-link arms from three pentamers, and residues that lie between the Ca²⁺ sites and the Vp3 binding surface (Figs. 1, 2). Region II mutants comprise all four *ts* C mutants and one of the *ts* BC mutants (at Lys 287) (Table 1). Assembly intermediates of three out of the four *ts* C mutants and the *ts* BC mutant have been reported: they all have a severe assembly defect with few viral proteins attached to the mini-

chromosome (Table 1; Garber et al. 1978, 1980; Bina et al. 1983a,b; Ng and Bina 1984). Closer inspection of the mutants shows that some are clearly predicted to disturb salt bridges that maintain the integrity of the Ca²⁺ binding sites (Fig. 2). This is consistent with our previous finding that mutations of Ca²⁺-coordinating residues can profoundly affect particle assembly (Li et al. 2003).

For other region II mutations, notably at Leu 244 and Pro 252, it is possible that the assembly defects arise from disturbing the interaction with Vp3 (Fig. 2). Leu 244 and Pro 252 are on the G strand, and orient a small seven-amino acid loop, colored green in Figure 2; two such loops, from neighboring Vp1 monomers within the pentamer, make contact with the two ends of the Vp3 helix. The mutations may dislocate these loops, affecting the Vp1–Vp3 interaction. However, Leu 244 also packs against Pro 252, which in turn, packs against the loop containing the Site II Ca²⁺ coordinating residue, Glu 157, whose integrity influences virion assembly (Li et al. 2003). We therefore cannot distinguish between these two sources of the assembly defect by simple inspection of the structure. However, we have studied the role of Vp3 in virion formation by mutating the Vp1–Vp3 contact sites predicted by our three-dimensional model (see Materials and Methods), and shown that virion

Figure 1. Mapping of Vp1 *ts* mutants onto an SV40 pentamer. In the center, a Vp1 pentamer is shown as a space-filling model with Vp1 monomers in different colors. A single invading arm from a neighboring pentamer is in marine blue. The C-arms emanating from the central pentamer are omitted for clarity. The *upper* zoom is a cutaway view of the upper part of the pentamer, showing one Vp1 monomer as β -strands and loops (in gray). The small “jelly-roll” domain is shown in a darker gray. All *tsB* (red) and three *tsBC* (blue) mutation sites are shown. Mutations are predicted to disrupt interactions with the proximal part of the invading arm (shown as a red dotted line), either directly via destabilization of the acceptor groove created by the packing of the small jelly-roll domain against the CHEF sheet of Vp1, or indirectly, via a more global destabilization of the pentamer. There are no *tsC* mutation sites in this region. The *lower* zoom shows a similar cutaway of the lower part of the pentamer, with the invading C-arm in marine blue, *tsC* mutation sites in green, and one *tsBC* mutation site (at K287) in blue. Primed numbers indicate residues from a neighboring Vp1 within the pentamer. There are no *tsB* mutations in this region. These mutations are predicted to disrupt interactions between either the Ca^{2+} binding sites (white arrow) and the C-arm or between the interior of the pentamer and Vp3 (see Fig. 2).

Figure 2. Close-up of the paired Ca^{2+} binding sites, I and II, at the interface between two Vp1 monomers within a pentamer (in gray and orange), an invading arm (marine blue), and Vp3 (in red). *tsC* mutants are in green, and the *tsBC* mutant, Lys 287, is in blue. Primed numbers indicate residues from a neighboring Vp1 within a pentamer. Two pairs of *tsC* mutations, at L244 and P252, from neighboring Vp1 monomers, contact either end of the VP3 helix. Ca^{2+} coordinating residues are in dark gray. Underlined numbers indicate Ca^{2+} coordinating residues from the invading arm. The two Ca^{2+} ions are shown as blue spheres. Vp3 residues are circled.

assembly can occur in the presence of small or negligible amounts of Vp3 (Nakanishi et al. 2006). While this preliminary result favors the notion that disruption of the Ca^{2+} coordination sites has a greater effect on capsid assembly than disruption of Vp1–Vp3 interactions, it does not formally rule out the possibility that a dysfunctional (rather than absent) Vp1–Vp3 interaction could be the cause of aberrant capsid assembly in the case of Leu 244 and Pro 252 mutations.

Given the consistent and severe assembly defects of the Region II mutants, we speculate that pentamer–pentamer cross-linking mediated by the distal parts of the invading C-arms and the Ca^{2+} binding sites is a critical early step in the addition of pentamers to the minichromosome. Previous work from others has identified the importance of calcium in stabilizing the structure of preassembled particles (Brady et al. 1977; Christiansen et al. 1977; Stehle et al. 1996). Furthermore, we have previously described a Ca^{2+} -coordinating mutant, E330K, which forms particles but is unable to bind cells or penetrate into the cytoplasm (Li et al. 2003), and proposed that the substituted lysine makes salt bridges with the side chains of the Ca^{2+} -coordinating Glu 48 and Glu 216, thus displacing the Ca^{2+} ion from site 1. We hypothesize that the failure of this mutant to infect cells arises from its inability to break this salt bridge. Therefore, our work provides further support for the role of Ca^{2+} ions, both

their binding and release, in multiple steps of the viral life cycle.

Structural basis for intracistronic complementation

The existence of two classes of mutants that are structurally distinct and that influence virion assembly in distinct and consistent ways may provide a rationale for the ability of certain *ts B* and *ts C* mutants to complement each other. Complementation analysis was established to facilitate the genetic analysis of a number of *ts* mutants of SV40, and revealed the presence of more than one complementation group in genes that are expressed late in viral infection (Tegtmeyer and Ozer 1971; Chou and Martin 1974). The two complementation groups, *ts B* and *ts C* arise from mutations in one gene (encoding Vp1), and thus complementation is termed "intracistronic." Such complementation occurs when two mutants are introduced simultaneously into a cell and allowed to replicate through multiple rounds of infection (Tevethia et al. 1974, 1981; Tevethia and Ripper 1977; Cosman and Tevethia 1981), enabling the mixing of mutants Vp1 pentamers synthesized in the cytoplasm prior to transport into the nucleus. Complementation does not occur when viral replication is limited to a single replication cycle at the restrictive temperature (Tevethia et al. 1974, 1981; Tevethia and Ripper 1977; Cosman and Tevethia 1981), since no such mixing of mutant pentamers can occur. To provide a molecular rationale for this complementation, we examined the relationship between pairs of mutants in which intracistronic complementation has been documented (Table 2; Chou and Martin 1974; Tevethia and Ripper 1977; Tevethia et al. 1981). We find that all known complementing pairs carry amino acid substitutions in distinct structural regions (i.e., one in region I, the other in region II).

The simplest model of intracistronic complementation is to postulate the assembly of heterotypic Vp1 pentamers in the cytoplasm of coinfecting cells prior to trafficking to the nucleus; this would allow that a fraction of the pentamer-pentamer contacts forming during capsid assembly contain a wild-type element in both the donor and acceptor elements, which could be sufficient to promote accurate assembly, presumably a highly cooperative process. We do not have direct evidence for or against the formation of such heterotypic pentamers, although we note that complementation occurring between two separate gene products has been previously observed for SV40, that of Vp1 and a mutant Vp3, in which a functional Vp1 nuclear localization signal promotes nuclear entry of the mutant Vp3 via a heterotypic Vp1-Vp3 complex (Ishii et al. 1994). If pentamers are homotypic, then the intracistronic complementation model needs to be more sophisticated, and dependent on the details of the assembly process (which have not been fully elucidated), and thus we can only speculate at this point. One

Table 2. *Vp1 structural region and intracistronic complementation*

<i>ts</i> Mutant (mutation)	Structural region	<i>ts</i> Mutant (mutation)	Structural region
B228 (E54K)	I	C219 (P212S)	II
		C240 (L244I)	II
		C260 (G40E, P252L)	II
B218 (P58R)	I	C219	II
		C240	II
		C260	II
B204, 211, 265 (A71V)	I	C219	II
		C240	II
		C260	II
B8 (A71T, E83D)	I	C1641 (V47M)	II
B1640 (H136Y)	I	C219	II
B1606 (G139D)	I	C219	II
B1603 (S147L)	I	C219	II
B221 (G163A)	I	C219	II
		C240	II
		C260	II
B201 (A166T)	I	C219	II
		C240	II
		C260	II

Complementing pairs are from Chou and Martin (1974), Tevethia and Ripper (1977), and Tevethia et al. (1981). *ts* mutants listed on the left column with amino acid substitution in parentheses complement *ts* mutants listed on the right column.

possibility is that if, as has been proposed (Liddington et al. 1991), assembly is initiated by a series of "five pentamers around one" subassemblies, it is possible to envision five region II pentamers making initial cross-links around a central region I pentamer via its wild-type Ca^{2+} binding sites; lateral interactions between the five region II pentamers (involving wild-type interactions with the proximal parts of the invading arms) might then be sufficient to generate a subassembly with the correct curvature; 12 such subassemblies might then complete the capsid.

Materials and methods

PCR amplification

Most *ts* mutations have been mapped to specific regions of the viral genome by rescuing each mutation with certain restriction endonuclease fragments of the wild-type DNA (Lai and Nathans 1974, 1975). The sequence alterations of 20 mutants have been identified by Bina and colleagues (Behm et al. 1988). Mutations in *ts* B1602, *ts* B1603, *ts* BC1605, *ts* BC1606, *ts* BC1607, *ts* BC1640, and *ts* C1641 were determined in this study. The wild-type F fragment from HindII-HindIII digestion can rescue the *ts* phenotypes of all but *ts* BC1605 and *ts* C1641. *ts* C1641 can be rescued by the wild-type K fragment (Tevethia et al. 1981). For sequence determination PCR amplification was performed in a GeneAmp PCR System 9700 (Perkin-Elmer) using ~30–50 ng of the mutant DNAs (0.014–0.026 $\mu\text{g}/\mu\text{L}$, stored at -20°C) as template DNA. Herculase DNA polymerase

(Stratagene) and 2 mM dNTPs were used. The initial denaturation was performed at 92.0°C for 2 min, followed by 30 cycles of the following: 92.0°C for 30 sec, renaturation at 55.0°C for 30 sec, and extension for 1 min at 72°C, with the final extension at 72°C for 10 min. The reaction was done in triplicates for assurance of the nucleotide alteration. For *ts* C1641, a Hirt lysate of cells infected with the mutant was used as the template. For *ts* B1602, *ts* B1603, *ts* BC1606, *ts* BC1607, and *ts* BC1640, the following two primer pairs were used: forward 5' 1623–1641 (CTGGAGTAGACAGCTTCAC) and reverse 5' 2182–2163 (TGTGTAGGTTCCAAAATATCTA), and forward 5' 1567–1581 (AGTGCAAGTGCCAAA) and reverse 5' 2666–2652 (CTTGTTTATTGCAGC). The first primer set was used initially to amplify just the F region, while the second primer set amplified a larger region including the F fragment, yielding equivalent levels of amplification. Sequencing of the PCR products was performed as instructed by Laragen using 5' 1567–1581 (AGTGCAAGTGCCAAA) and 5' 2188–2163 (CCCACCTGTGTAGGTTCCAAA). For *ts* BC1605 and *ts* C1641, the entire Vp1 gene was amplified using forward 5' 1320–1339 (CAGTATTCAGCAAGTAACGTG) and reverse 5' 2666–2652 primers, followed by sequencing using 5' 1320–1339 for *ts* C1641 and 5' 2671–2652 (GTTAACCTGTTTATTGCAGC) for *ts* B1605.

A homology model of the SV40 Vp1 pentamer–Vp3 complex

The crystal structure of the polyomavirus Vp1 pentamer–Vp3 complex is known (Chen et al. 1998), and the structure of SV40 and polyomavirus pentamers are well conserved (Liddington et al. 1991; Stehle et al. 1994, 1996). Based on these structures (PDB codes 1CN3 and 1SVA) we built a homology model in the following way. The polyoma complex was superimposed onto the Vp1 pentamer of SV40 by overlaying the C α positions of the β -sheets of Vp1 (the backbone can be superimposed with a root-mean-square deviation of ~ 0.5 Å). The ordered region of Vp3 observed in the polyoma structure (151–187) is highly conserved within SV40 Vp3 (154–187), and the side chains with library torsion angles were replaced with their SV40 counterparts using TURBO-FRODO. In this model, the first ordered residue of the Vp3 fragment is Phe 157. Phe 157 and Ile 158 nestle into the hydrophobic neck at the top of the conical interior of one Vp1 pentamer. Residues upstream of Phe 157 may extend through the neck and be exposed on the surface of the virion, or fold back toward the base of the Vp1 pentamer, as suggested by Chen et al. (1998). Residues Pro 164, Gly 165, and Gly 166 form a tight turn where they pack into a crevice between the two main β -sheets of Vp1. Residues Trp 175 through Tyr 184 form a hydrophobic α -helix at the base of the pentamer. Vp3 residues Leu 177 and Leu 181 make hydrophobic contacts with Vp1 residues Val 243 and Leu 245, respectively. Based on this model we altered Vp3 residues (Phe 157, Ile 158, Pro 164, Gly 165, Gly 166, Leu 177, and Leu 181) and Vp1 residues (Val 243 and Leu 245), and determined the effect of substitutions on the Vp1–Vp3 interaction and on infectivity of the mutant particles to the new host. The mutations either reduced or eliminated the incorporation of minor capsid proteins into mutant particles. The loss of minor capsid proteins in the particles accompanied the reduction in viability, and the particles with no detectable Vp3 were nearly noninfectious (Nakanishi et al. 2006), indicating that the SV40 homology model constructed here reflects the Vp1–Vp3 interaction occurring in vivo. The Vp1–Vp3 complex was built and inspected using TURBO-FRODO (<http://afmb.cnrs-mrs.fr/rubrique113.html>). Figures were made

using PyMOL Molecular Graphics System (DeLano 2002, <http://www.pymol.org>). The model of the Vp1–Vp3 complex, as well as a detailed description of the environments of all the mutations sites, is available from the corresponding author on request.

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