The simian virus 40 minor structural protein Vp3, but not Vp2, is essential for infectious virion formation

Editte Gharakhanian, Luz Muñoz† and Luz Mayorca

Department of Biological Sciences, California State University at Long Beach, 1250 Bellflower Blvd, Long Beach, CA 90840-3702, USA

The SV40 capsid is composed of pentameric capsomeres of the major structural protein Vp1. The two minor structural proteins, Vp2 and Vp3, interact with the capsid. Here, the roles of Vp2 and Vp3 were explored during the course of SV40 infection. Start codons of Vp2, Vp3, or both Vp2 and Vp3, were destroyed by site-directed mutagenesis, and mutant genomes were transfected into CV-1 cells. SV40ΔVp2 produced plaques and infectious virion particles with titres indistinguishable from wild-type. SV40ΔVp3 and SV40 ΔVp2/Vp3 were defective in plaque formation and rendered no infectious particles. All three mutants showed normal nuclear localization of T-Ag and Vp1; they also showed packaging of SV40 DNA by nuclease digestion assays. Thus, Vp3 is essential for formation of infectious SV40 particles, whereas Vp2 is not. One critical role of full-length Vp3 appears to be in virus–cell interactions at post-packaging steps of a permissive infection.

Simian virus 40 (SV40) belongs to the polyomavirus family of DNA tumour viruses. In simian cells, SV40 initiates a permissive infection leading to virion progeny. SV40 entry into cells involves caveolar endocytosis leading to virus accumulation and uncoating in the endoplasmic reticulum (Anderson et al., 1996; Pelkmans et al., 2001; Norkin et al., 2002). Late in permissive infection, the viral structural proteins Vp1, Vp2 and Vp3 are synthesized in the cytoplasm and are transported to the nucleus for assembly of the icosahedral capsid (Tooze, 1980). The capsomeric unit is composed of pentamers of Vp1. In polyomavirus, each Vp1 pentamer is tightly associated with one Vp2 or Vp3 molecule through hydrophobic interactions (Barouch & Harrison, 1994; Chen et al., 1998). Vp2 and Vp3 bridge Vp1 capsomeres to the SV40 genome which is complexed into nucleosomes with cellular histones (Rayment et al., 1982; Baker et al., 1988; Liddington et al., 1991; Stehle et al., 1996). The entire 234 amino acid sequence of Vp3 is repeated in the C terminus of a myristylated Vp2, leaving 118 unique residues at the Vp2 N terminus. Several functions have been mapped to the C-terminal 40 amino acids common to both Vp2 and Vp3 (Vp2/3). They include a DNA-binding domain and a nuclear localization signal (Gharakhanian et al., 1987, 1988; Gharakhanian & Kasamatsu, 1990; Clever et al., 1991, 1993; Dean et al., 1995). A Vp1-binding domain has been mapped to an internal domain in the Vp2/3 common region (Gordon-Shaag et al., 2002). Yet, not much is known about the significance of the Vp2-specific 118 residues. Recently, Mannova et al. (2002) have shown that in the closely related mouse polyomavirus, Vp2− and Vp3− viruses form viral particles but show reduced infectivity; a mutant encoding non-myristylated Vp2 shows a smaller drop in infectivity. Here, we have explored the significance of full-length Vp2 and Vp3 in formation of SV40 infectious virions. We report that SV40 harbouring either a mutagenized Vp2 initiation codon or mutagenized Vp2 and Vp3 initiation codons is defective in plaque formation in CV-1 cells; SV40 harbouring only a mutagenized Vp2 initiation codon forms plaques and yields titres indistinguishable from wild-type. All three mutants expressed early and late SV40 gene products, correctly localized them in the nucleus and packaged SV40 DNA, suggesting that a critical function for full-length Vp3 may lie at post-packaging steps of the productive infection.

SV40 ΔVp2, SV40ΔVp3 and SV40ΔVp2/Vp3 genomes were generated by site-directed mutagenesis of the initiation codons of Vp2, Vp3, or both Vp2 and Vp3, respectively, in the parent plasmid pSV40 (Transformer Kit, Clontech). The mammalian expression vector pSV40 contains the wild-type SV40 genome and has been described before (Clever & Kasamatsu, 1993). To minimize revertants, all three bases of each initiation codon were changed (ATG→GCC); mutations were confirmed by sequencing (DNA Sequencing Facility, California State Univ., Northridge). Mutant and wild-type SV40 genomes were excised from pSV40 and were used in transient lipid-mediated DNA transfections (Lipofectamine, Gibco-BRL) of simian CV-1 cells (ATCC). Mock transfections included all reagents and
manipulations minus input DNA. Western blots of transfected cell lysates using anti-Vp3 antibodies confirmed lack of Vp2 or Vp3, or both Vp2 and Vp3 expression in cells transfected with SV40ΔVp2, SV40ΔVp3 or SV40ΔVp2/Vp3, respectively (Fig. 1a). Transfected cells were tracked for cytopathic effect (CPE) and plaque formation by light microscopy and plaque assays (Fig. 1). CPE was detected by day 7–11 post-transfection (p.t.) in cells transfected with wild-type SV40 or SV40ΔVp2 (Fig. 1b, c); in repeated experiments, onset of CPE for wild-type- and SV40ΔVp2-transfected cells was identical and showed a range relative to passage age of cells. Microscopic monitoring of SV40ΔVp3-transfected cells uncovered distinct differences in onset and progression of CPE relative to wild-type (Fig. 1b, c). A delayed, limited and transient CPE was detected in cells transfected with SV40ΔVp3. CPE onset was consistently delayed by 2 days relative to wild-type, cell vesicularization was limited, and CPE was no longer detectable after 4 days post-onset. Cells transfected with SV40ΔVp2/Vp3 or mock-transfected showed no CPE when monitored up to 20 days p.t. (Fig. 1b, c). Repeated plaque assays consistently yielded plaques only in SV40ΔVp2- and SV40-transfected plates. Plaques generated following transfections with SV40ΔVp2 DNA were picked at 14 days p.t. and were subjected to three rounds of plaque purifications; titres were determined following infections. SV40ΔVp2 consistently yielded titres and plaque sizes indistinguishable from wild-type SV40 (Fig. 1c, d). SV40ΔVp2 virions were isolated by a novel small-scale virus mini-preparation that we have described elsewhere (Orlando et al. 2000). DNA from plaque-purified mutant SV40 was subjected to PCR amplification of the mutated AUG codon region and the amplified fragment was sequenced. Sequencing confirmed ATG→GCC mutation at the Vp2 initiation codon of SV40ΔVp2 infectious virions. SV40ΔVp2/Vp3- and SV40ΔVp3-transfected plates were also ‘picked’ at 14 and 20 days p.t. but did not yield productive infections (Fig. 1c) nor amplified SV40 DNA (see Fig. 3b).

In order to further map the block in infectious virion formation seen with SV40ΔVp3 and SV40ΔVp2/Vp3, earlier events of the permissive infection were studied. For immunofluorescent microscopy studies, CV-1 cells were seeded onto glass coverslips and transfected with wild-type or mutant SV40 genomes as described above. Transfected cells were harvested 48 h p.t. and were subjected to indirect double-labelled immunofluorescent microscopy studies using MAbs against large T-Ag (Calbiochem) and polyclonal antibodies against GST–Vp1 (kind gift of Ariella Oppenheim, Hebrew University-Hadassah Medical School, Jerusalem, Israel). Each mutant transfection showed wild-type patterns of expression and nuclear localization of T-Ag and Vp1 (Fig. 2). Anti-T-Ag staining was used to assess possible variations in transfection efficiencies and frequencies of nuclear localization of Vp1 among different mutants. Both wild-type and mutant plasmids led to transfection efficiencies of 11–15 %. In cells transfected with wild-type SV40, SV40ΔVp2 or SV40ΔVp3, between 30 and 55 % of positively transfected cells showed nuclear Vp1; cells transfected with SV40ΔVp2/Vp3 consistently exhibited a slightly lower percentage of nuclear Vp1 at 22 %.

Next, packaging of viral DNA was assessed following transfections with wild-type or mutant SV40, or mock-transfections. Cells were harvested at either 72 h p.t. (Fig. 3a) or at 20 day p.t. (Fig. 3b). Packaged DNA was isolated as described in Orlando et al. (2000). Briefly, cellular debris was removed by centrifugation and supernatants were treated with excess DNase to remove all non-packaged DNA, followed by proteinase K, DTT and organic extraction to remove capsid proteins. Viral DNA was amplified by PCR using SV40-derived primers that render a 250 bp expected band. Under these conditions, up to 1 µg of naked pSV40 DNA does not render the expected PCR product (Fig. 3b, lane 5), whereas 100 ng of pSV40 DNA added to mock-transfection harvests following DNase and protease treatments does render a 250 bp PCR product (Fig. 3a, lane 3). At 72 h p.t., all three mutants and wild-type SV40 showed the presence of SV40-derived PCR product, suggesting packaging of DNA early after transfection in the absence of full-length Vp2, Vp3, or both Vp2 and Vp3 (Fig. 3a). At 20 days p.t., however, PCR products were visible only in wild-type SV40 and SV40ΔVp2 transfections, suggesting that persistent infectious virions form only in the presence of full-length Vp3 (Fig. 3b).

The individual roles of full-length SV40 Vp2 and Vp3 in the course of a permissive infection have not been adequately explored to date. A recent study on mouse polyoma virus has shown that in the absence of either polyoma Vp2 or Vp3, virus particles formed but showed reduced infectivity (Mannova et al., 2002). In this study, we have generated SV40 mutants with defective initiation codons for Vp2, Vp3, or both, and have studied them for expression and nuclear localization of early and late SV40 gene products, SV40 DNA packaging, onset of CPE and production of...
(a) Mock, SV40, pSV40/Vp3, SV40ΔVp2, and SV40ΔVp3 were assayed by immunoblotting. Vp2 and Vp3 proteins are indicated.

(b) Day 11 and Day 14 images of mock, SV40, SV40ΔVp2, SV40ΔVp3, and SV40ΔVp2/Vp3.

(c) Summary of infection phenotypes of mutants:

<table>
<thead>
<tr>
<th>Input DNA</th>
<th>Onset of CPE (days p.t.)</th>
<th>Mean plaque diameter (mm)</th>
<th>Titer (p.f.u./ml)</th>
</tr>
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<tbody>
<tr>
<td>Mock</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pSV40</td>
<td>7-11</td>
<td>2.91±0.042</td>
<td>5.3 x 10^{-5}</td>
</tr>
<tr>
<td>SV40ΔVp2</td>
<td>7-11</td>
<td>3.00±0.018</td>
<td>6.1 x 10^{-5}</td>
</tr>
<tr>
<td>SV40ΔVp3</td>
<td>9-14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SV40ΔVp2/Vp3</td>
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(d) 14 days p.t. and 15 days p.i. images of SV40, SV40ΔVp2, SV40ΔVp3, SV40ΔVp2/Vp3, and Mock.
infectious virions in permissive CV-1 cells. Our results show that full-length Vp3 is essential for formation of infectious virions, whereas full-length Vp2 is not. All three mutants continued to express and correctly localize to the nucleus both the early gene product, T-Antigen, and the late gene product, major structural protein Vp1. All three continued to package SV40 DNA into DNase-resistant structures. These results suggest that one major role of full-length Vp3 may be at post-packaging stages of the permissive infection. These may include interaction of assembled virion particles with the infected cell for progression of infection, entry into cells for secondary waves of infection, or uncoating in the ER following entry. In our study, SV40ΔVp3 led to transient, delayed CPE in

Fig. 2. SV40ΔVp2, SV40ΔVp3 and SV40ΔVp2/ΔVp3 lead to expression and nuclear localization of T-Ag and Vp1. CV-1 cells were grown on cover slips and were transfected with linear wild-type or mutant SV40 containing the indicated point mutations in Vp2 or Vp3 start codons. Cells were harvested 48 h p.t. by methanol fixation and were subjected to double labelled indirect immunofluorescent microscopy using mouse anti-T-Ag (αT-Ag) and rabbit anti-GST–Vp1 (αGST-Vp1) followed by TITC-conjugated rabbit anti-mouse and FITC-conjugated goat anti-rabbit secondary antibodies (Sigma). Cells were mounted on slides and observed with an Olympus microscope using a 100× objective.
CV-1 cells which best supports the first alternative. Interestingly, Gordon-Shaag et al. (2003) have most recently shown that poly(ADP-ribose) polymerase (PARP) is involved in the magnitude of CPE in SV40-infected CV-1 cells, and that Vp3 stimulates PARP.

Since our mutants contain only mutagenized initiating AUG codons, translation initiation from internal AUGs could theoretically continue. There are no internal AUG codons in the Vp2-specific 118 amino acid coding region; however, there are two internal AUGs in the Vp2/Vp3 common region, which would render polypeptides of length 150 and 50 amino acids, corresponding to the Vp2/Vp3 C terminus. In cells transfected with SV40 Vp2/Vp3 DNA, immunofluorescent microscopy studies using anti-Vp3 polyclonal antibodies show a weak but positive nuclear staining, and Western blots show a weak Vp3-related 150 amino acid band (data not shown). Thus, an N-terminally truncated Vp3 may be expressed with continued nuclear localization and DNA-binding functions, attributing the observed post-packaging role for Vp3 to the N-terminal domains of the protein.

Our results indicate that infectious SV40 virions can form in the absence of full-length Vp2. SV40ΔVp2 infection is indistinguishable from wild-type SV40 infection as assessed by onset of CPE, plaque size and viral titres. SV40ΔVp3 elicits a limited CPE in transfected cells, whereas SV40ΔVp2/Vp3 does not, suggesting that Vp2 may have a secondary role in pre-CPE stages of the productive infection. A less likely alternative may be that the Met-118 of Vp2, which is changed to an alanine codon in SV40ΔVp3, is essential for infectious virion formation. The only study pertaining to a specific role of SV40 Vp2 in infection dates back to 1977, prior to full knowledge of the SV40 restriction or genomic map (Cole et al., 1977). In that study, SV40 deletion mutants generated by restriction and S1 nuclease digests were assessed for plaque formation; mutations presumed to map to the N-terminal unique region of Vp2 continued to form plaques and infectious particles but plaque onset was delayed and plaque size was smaller relative to wild-type infections. Our study indicates that a full-length Vp2 is not essential in a productive SV40 infection; it also suggests that the Vp2-unique coding region may be dispensable as long as the Vp3 reading frame and initiating AUG codon are maintained. Dispensability of viral coding regions in formation of infectious virions, is a current area of study in vector development for gene therapy.

SV40 has been identified as an attractive vector for high-efficiency gene transfer into various human cells (Sandalon et al., 1997; Sandalon & Oppenheim, 1997; Rund et al., 1998;
Dalyot-Herman et al., 1999; Goldstein et al., 2002). Chang & Wilson (1986) have suggested the packaging limit for SV40 to be between 284 and 460 extra base pairs. Removing the Vp2-unique coding sequences along with the 5’-untranslated region would significantly increase the packaging limit of SV40 in gene replacement studies.

Future biochemical and electron microscopic analyses of these mutants can further shed light on the state and structure of the packaged complexes of SV40ΔVp3 and SV40ΔVp2/Vp3, as well as the SV40ΔVp2 infectious virions.

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REFERENCES


