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# $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ of simian virus 40 Vp1 are essential for inter-pentamer disulfide-linkage and stabilization in cell-free lysates

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**Previous studies have implicated disulfide bonds between Vp1 molecules in the stabilization of the simian virus 40 (SV40) capsid. To identify the cysteine residues involved in intermolecular disulfide interactions, systematic oligo-directed mutagenesis of cysteine codons to serine codons was initiated. Wild-type and mutant Vp1 proteins were produced in rabbit reticulocyte lysates and were allowed to interact post-translationally. Disulfide-linked Vp1 complexes were assessed via non-reducing SDS–PAGE and via sucrose-gradient sedimentation. Wild-type Vp1 forms 7S pentamers followed by 12S disulfide-linked multi-pentameric complexes in cell-free lysates. Mutagenesis of all seven cysteine codons abolished Vp1 12S complexes, but did not affect pentamer formation. A quadruple Vp1 mutant at $Cys^{49}$, $Cys^{87}$, $Cys^{254}$ and $Cys^{267}$ continued to form 12S complexes, whereas the major products of the $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ triple mutant Vp1 were 7S pentamers. Single and double mutant Vp1 proteins at the three cysteines affected continued to form 12S complexes, but to a lesser extent. Thus, inter-pentamer disulfide bonds at $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ are essential and sufficient for stabilization of Vp1 complexes in cell-free lysates. These results are in agreement with previous structural studies of SV40 that implicated the same three residues in disulfide linkage in the capsid. Possible parameters for the involvement of the three cysteines are discussed.**

## Introduction

Simian virus 40 (SV40) is a DNA tumour virus belonging to the genus *Polyomavirus*. The capsomeric unit of the virus is the Vp1 pentamer (Baker *et al.*, 1988; Rayment *et al.*, 1982). Late in permissive infection, Vp1 molecules, along with the minor structural proteins Vp2 and Vp3, are synthesized in the cytoplasm of the host cell and travel to the nucleus for assembly of the icosahedral capsid (Lin *et al.*, 1984). Several studies have implicated disulfide bonds in the stabilization of the viral capsid. Treatment of SV40 and polyoma virions with the chelating agent EGTA and with the reducing agent DTT leads to virion dissociation (Brady *et al.*, 1977, 1978, 1980). Ng & Bina (1981) have shown that treatment of SV40 virions with DTT exposes viral DNA rendering it sensitive to digestion with micrococcal nuclease. Recently, SV40 Vp1 molecules expressed in *Spodoptera frugiperda* (Sf9) insect cells were shown to form virus-like particles which were sensitive to DTT and to calcium chelation (Kosukegawa *et al.*, 1996). Since Vp2 and Vp3 contain no cysteine residues and interact non-covalently with the capsid, disulfide bonds occurring in the capsid involve Vp1 molecules (Walter & Deppert, 1974). Liddington *et al.* (1991) visualized the structure of SV40 by X-ray crystallography at a resolution of $3{\cdot}8 \times 10^{-10}$ m, and showed that three of the seven Vp1 cysteine residues in the SV40 capsid were unavailable during mercury labelling, suggesting that they may be involved in covalent disulfide bonds. These are cysteine residues at positions 9, 104 and 207 ($Cys^{9}$, $Cys^{104}$ and $Cys^{207}$, respectively). However, no disulfide bonds were detected at the monomer or pentamer levels. In more recent structural studies, the SV40 structure was further refined to $3{\cdot}1 \times 10^{-10}$ m, and disulfide bonds were detected between $Cys^{104}$ residues of B and B′ SV40 Vp1 molecules at inter-pentameric level (Stehle *et al.*, 1996).

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We have previously reported the development of a cell-free system to assay protein–protein interactions among SV40 structural proteins (Gharakhanian *et al*., 1988, 1995). The cell-free lysate was used to map the Vp1-interactive domain of Vp3 and to show the formation of Vp1 pentamers and multi-pentamer complexes *in vitro*. Here, we have utilized the cell-free system to identify the specific Vp1 cysteines involved in inter-pentamer disulfide linkage. Systematic site-directed mutagenesis of individual Vp1 cysteine codons into serine codons was followed by transcription and translation of wild-type and mutant Vp1 genes in rabbit reticulocyte lysates. Translation products were assayed for multimerization by non-reducing SDS–PAGE and by sucrose-gradient sedimentation. Using this methodology, we have previously reported formation of non-covalently linked 7S Vp1 pentamers followed by formation of disulfide-linked 12S, multi-pentameric complexes of Vp1 (Gharakhanian *et al*., 1995). In this study, destruction of all seven cysteine codons resulted in no 12S complexes, directly implicating disulfide bonds in stabilization of inter-pentamer Vp1 interactions. Vp1 proteins that contained serine residues instead of cysteines at $Cys^{49}$, $Cys^{87}$, $Cys^{254}$ and $Cys^{267}$ continued to form disulfide-linked 12S complexes similar to wild-type Vp1, whereas $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ triple mutant Vp1 proteins did not. Single and double mutants at the three cysteines affected continued to form 12S complexes, but to lesser degrees. Thus, our results show that $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ are sufficient and essential for formation of Vp1 inter-pentamer disulfides in cell-free lysates. While single cysteines at positions 9, 104 or 207 are sufficient for formation of inter-pentamer disulfide bonds *in vitro*, all three cysteines participate in the process for maximum complex formation. These results are in agreement with SV40 structural studies which have implicated the same three Vp1 cysteines by mercury labelling (Liddington *et al*., 1991), and which have actually detected $Cys^{104}$-mediated disulfide bonds only at the inter-pentamer level of organization (Stehle *et al*., 1996).

## Methods

■ **Bacterial strains.** *Escherichia coli* BMH 71-18 *mutS* cells (Clontech) were used during the construction of the mutant plasmids since this strain is deficient in DNA mismatch repair. *E. coli* DH5α cells were used for routine bacterial transformations and plasmid amplifications.

■ **Plasmids.** The pSp6Vp1 plasmid (Gharakhanian *et al*., 1995) is a transcription vector that contains the SP6 promoter, an ampicillin resistance gene and the SV40 Vp1 gene. This plasmid was used during oligo-directed mutagenesis to generate the mutant Vp1 genes. Another plasmid, pUC19M (Clontech), was used in the mutagenesis reaction as a control. This plasmid has an amber stop codon in the *lacZ* gene.

■ **Restriction and modifying enzymes.** The restriction enzymes used were from New England Biolabs unless otherwise indicated. The T4 DNA polymerase and T4 DNA ligase were from Clontech.

■ **Construction of mutant pSp6Vp1 plasmids by oligo-directed mutagenesis.** Mutagenesis was performed following the protocols in the Transformer Site-Directed Mutagenesis Kit from Clontech (1993) with some minor modifications. Two primers, the selection and the mutagenic primers, were annealed to the template at the same time. The mutagenic primer encoded a point mutation that changed a specific cysteine codon into a serine codon. The selection primer was used to change a unique restriction site on the vector to another unique restriction site. In this case, the *Afl*III restriction site on the plasmid was changed to a *Bgl*II restriction site.

After mutagenesis, DNA was extracted from mutant colonies and subjected to restriction digest analysis using *Afl*III and *Bgl*II enzymes. Mutant samples that were linearized by *Bgl*II but not by *Afl*III were further analysed for the desired G-to-C base change at a given cysteine codon by dideoxynucleotide sequencing using the Sequenase Quick-Denature Plasmid Sequencing Kit (Amersham). Polyacrylamide (6%)–urea sequencing gels were fixed with a solution of 5% acetic acid–15% ethanol for 3 min. Dried gels were exposed to Kodak BioMax MR1 film overnight at room temperature. The autoradiograms were developed in an automatic film processor (Kodak).

Using this method, four types of Cys-to-Ser mutants in pSp6Vp1 were generated. Three single Cys-to-Ser codon mutants at positions 9, 104 and 207 (pSp6Vp1.C9S, pSp6Vp1.C104S and pSp6Vp1.C207S, respectively) were generated. Additionally, one double Cys-to-Ser codon mutant at positions 9 and 207 (pSp6Vp1.C9S.C207S), one triple Cys-to-Ser codon mutant at positions 9, 104 and 207 (pSp6Vp1.C9S.C104S.C207S) and one quadruple Cys-to-Ser codon mutant at positions 49, 87, 254 and 267 (pSp6Vp1–4X) were produced. Please see Table 1.

To generate the plasmid that encodes serine at all seven cysteine codon positions (pSp6Vp1–7X), the mutant plasmid pSp6Vp1.C9S.C104S.C207S was used in the site-directed mutagenesis reaction. The unique *Bgl*II site was changed back to *Afl*III. After mutagenesis, primary screening was done by restriction digest analysis. Confirmation of the presence of the desired mutations was done by dideoxynucleotide sequencing.

■ **Construction of mutant plasmids by directional subcloning.** The double Cys-to-Ser Vp1 mutant plasmids at positions 9 and 104, and at positions 104 and 207, (pSp6Vp1.C9S.C104S and pSp6Vp1.C104S.C207S, respectively) were generated by directional subcloning. The restriction endonucleases *Afl*III and *Apa*I (Boehringer Mannheim) cut at 1699 and 2258 bp, respectively, to produce a band of 559 bp. This 559 bp fragment contains the cysteine codons at positions 87, 104, and 207. The remaining 3·7 kb contains the cysteine codons at positions 9, 254 and 267. To generate pSp6Vp1.C9S.C104S, the 3·7 kb region from pSp6Vp1.C9S was used as the vector and the 559 bp region from pSp6Vp1.C104S was used as the insert. To generate pSp6Vp1.C104S.C207S, the 3·7 kb region of the wild-type pSp6Vp1 was used as the vector and the 559 bp region of pSp6Vp1.C9S.C104S.C207S was used as the insert.

Plasmid DNA was digested first with *Afl*III for 1 h at 37 °C then with *Apa*I at 30 °C for 2 h. After digestion, the samples were electrophoresed in 1% agarose. The appropriate bands were purified from the gel using the QiaExII DNA Extraction Kit (Qiagen). After DNA extraction, 1:1 molar ratios of insert to vector were prepared. Before ligation, the insert–vector solution was placed in a beaker with boiling water until the water came to room temperature. T4 DNA ligase (Boehringer Mannheim) was added and the solution was kept at 12 °C overnight to allow the reaction to proceed. Plasmid DNA was then transformed into *E. coli* DH5α cells. To confirm successful subcloning, plasmid DNA was extracted from the resulting colonies and mutagenized region(s) were analysed by dideoxynucleotide sequencing. After the mutant plasmids

**Table 1.** Summary of Cys-to-Ser mutant Vp1 plasmids and proteins used

| Protein name | Plasmid name | Cysteine residue(s) changed to Serine | Method of generation |
|---|---|---|---|
| Vp1–7X | pSp6Vp1–7X | $Cys^{9}$, $Cys^{49}$, $Cys^{87}$, $Cys^{104}$, $Cys^{207}$, $Cys^{254}$ and $Cys^{267}$ | Site-directed mutagenesis |
| Vp1–4X | pSp6Vp1–4X | $Cys^{49}$, $Cys^{87}$, $Cys^{254}$ and $Cys^{267}$ | Site-directed mutagenesis |
| Vp1.C9S. C.104S.C207S | pSp6Vp1.C9S.C104S.C207S | $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ | Site-directed mutagenesis |
| Vp1.C9S | pSp6Vp1.C9S | $Cys^{9}$ | Site-directed mutagenesis |
| Vp1.C104S | pSp6Vp1.C104S | $Cys^{104}$ | Site-directed mutagenesis |
| Vp1.C207S | pSp6Vp1.C207S | $Cys^{207}$ | Site-directed mutagenesis |
| Vp1.C9S.C104S | pSp6Vp1.C9S.C104S | $Cys^{9}$ and $Cys^{104}$ | Directional subcloning |
| Vp1.C9S.C207S | pSp6Vp1.C9S.C207S | $Cys^{9}$ and $Cys^{207}$ | Site-directed mutagenesis |
| Vp1.C104S.C207S | pSp6Vp1.C104S.C207S | $Cys^{104}$ and $Cys^{207}$ | Directional subcloning |

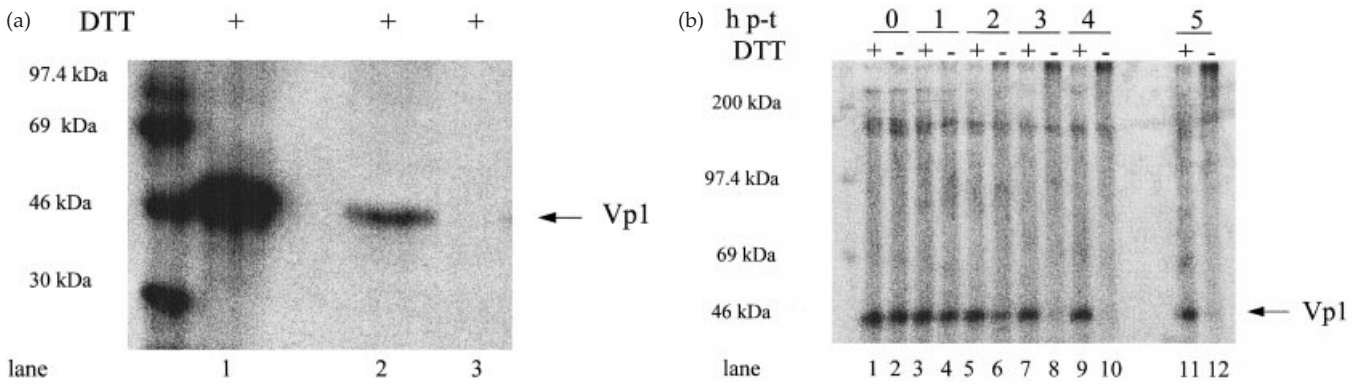


Fig. 1. Vp1 is the major translation product of pSp6Vp1-directed translation and forms disulfide-bonded multimers in rabbit reticulocyte lysates. (*a*) *In vitro* transcription–translation products (5 μl) of wild-type pSp6Vp1 plasmid were subjected to reducing SDS–PAGE directly after translation (lane 1), or subsequent to immunoprecipitation with polyclonal antibodies against a neutralizing Vp1 peptide (lane 2). Immunoprecipitation of 5 μl rabbit reticulocyte lysate after mock translation (pSp6Vp1 plasmid was not added during translation) with anti-Vp1 antibodies is shown in lane 3. (*b*) *In vitro* transcription–translation products of wild-type pSp6Vp1 plasmid were either reduced in 5 mM DTT (+DTT, odd-numbered lanes) or not reduced (−DTT, even-numbered lanes), alkylated with iodoacetamide and then separated by 10% non-reducing SDS–PAGE and visualized by autoradiography. Samples in lanes 1 and 2 were incubated for 0 h; samples in lanes 3 and 4 were incubated for 1 h; samples in lanes 5 and 6 were incubated for 2 h; samples in lanes 7 and 8 were incubated for 3 h; samples in lanes 9 and 10 were incubated for 4 h; samples in lanes 11 and 12 were incubated for 5 h. h p-t, hours post-translation.

were identified, large-scale plasmid isolations were done to prepare plasmid DNA for subsequent reactions using the Qiagen Plasmid Maxi Kit (Qiagen).

## ■ Multimerization assays of the wild-type and mutant proteins

*In vitro* transcription–translation. Production of wild-type and mutant Vp1 proteins was accomplished using the TNT Coupled Reticulocyte Lysate System from Promega. As directed by the manufacturer, transcription and translation were performed at 30 °C for 2 h in one reaction using 1 μg plasmid DNA and 60 μCi L-[$^{35}$S]methionine (> 1000 Ci/mmol; Amersham). To confirm that Vp1 protein is the major translation product of the Vp1 gene in the system, the translation product was immunoprecipitated as described by Gharakhanian *et al.* (1995) using polyclonal antibodies that were raised against a neutralizing Vp1 peptide (Fasching, 1998; C. Fasching and others, un-

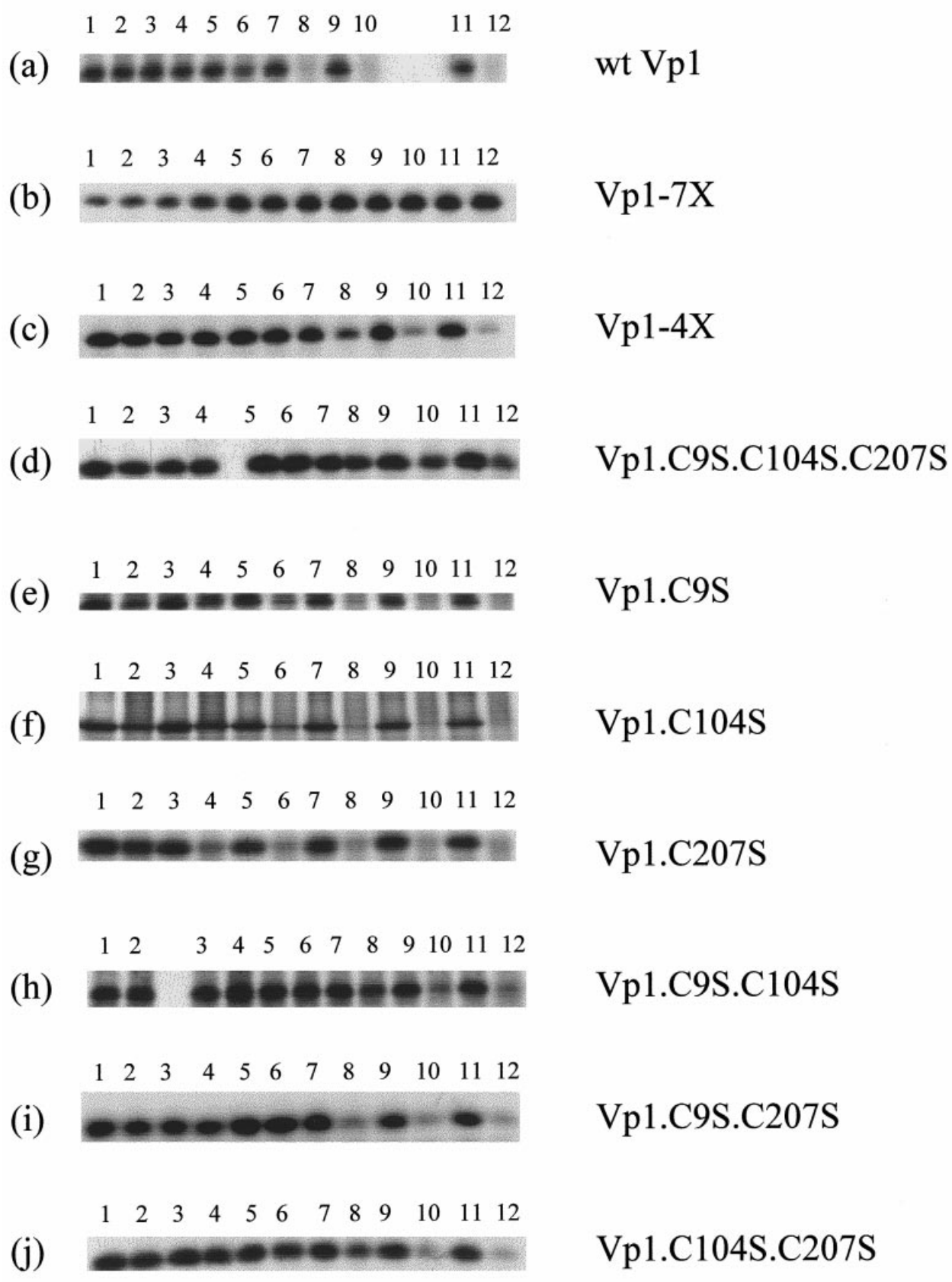


Fig. 2. For legend see facing page.

published). Samples were then incubated at 37 °C for up to 5 h for post-translational Vp1 interactions. Total incubation time at 30 and 37 °C was 7 h. Previous experiments (E. Gharakhanian, unpublished) showed that going beyond 7 h resulted in degraded products and non-specific aggregations.

During the 37 °C incubation, 10 µl samples were removed every hour. Half of the sample from each time-point was treated with 5 µl 20 mM DTT for 30 min at 37 °C as controls; the other half received 5 µl double-distilled water. Five µl 50 mM iodoacetamide was added to each sample to alkylate the free –SH groups; the reaction was carried out in the dark at room temperature for 30 min. The reactions were stopped by addition of 15 µl 2 × SDS–PAGE sample buffer. The samples were either stored at −70 °C or used in SDS–PAGE.

The samples were subjected to 10% non-reducing SDS–PAGE. Thirty min after the tracking dye ran off, the gels were stopped. This was done to see 40 to 200 kDa bands on the same gel. The gels were fixed with 5% acetic acid–5% isopropyl alcohol for 15 min and were subjected to fluorography using Autofluor (National Diagnostics). $^{14}$C-labelled molecular mass markers were also included on the gel.

Densitometric analyses were performed on selected autoradiograms using the Optimas Profile 1-D Gel Densitometry program. Using this program, relative amounts of the Vp1 monomeric bands in reduced and non-reduced samples at 5 h post-translation were tracked. Zero was given as the absorbance value whenever the program could not detect a peak. Vp1 monomer values were calculated by dividing the absorption total value of the non-reduced Vp1 monomeric band by the absorption total value of the reduced Vp1 monomeric band for each of the Vp1 samples. Values are expressed in percentage Vp1 monomers.

**Separation of translation products by sucrose-gradient sedimentation.** Wild-type and mutant Vp1 proteins were produced by *in vitro* transcription–translation as described above and were subjected to post-translational manipulations as described (Gharakhanian *et al.*, 1995) with the following modifications. After translation, one-third of each sample was frozen at −70 °C, one-third was further incubated at 37 °C for 1 h and one-third was incubated for 3 h, to allow for Vp1–Vp1 interactions. At the end of each incubation time, samples were stored at −70 °C. Thirty µl of each thawed sample was loaded onto a 5–20% sucrose gradient with a 50% sucrose cushion. The samples were centrifuged at 35000 r.p.m. at 4 °C for 23 h in an SW41 rotor. Fifteen 0·75 ml fractions were collected from the bottom of each tube and 100 µl of each fraction was precipitated with trichloroacetic acid (TCA). An equal volume of cold 50% TCA was added to each sample in wells lined with filter paper. This was incubated for 15 min at 4 °C. The supernatant was aspirated and the filter paper was gently rinsed with more cold 50% TCA, air dried, and precipitable radioactivity was determined by liquid-scintillation counting. In repeated fractionations, bovine serum albumin (4·5S) peaked at fraction 12, native immunoglobulin G (7S) peaked at fraction 10 and catalase B (11·3S) peaked at fraction 7.

To reconfirm the molecular masses of the 7S and 12S species, 500 µl each of the wild-type Vp1 fraction 10 (7S Vp1) and fraction 6 (12S Vp1) were subjected to gel filtration using Sephacryl S-300 (Sigma) in standard TE buffer. Fractions (1 ml) were collected and 100 µl of each fraction was TCA-precipitated and Vp1 radioactivity was determined as described above. Yeast alcohol dehydrogenase (150 kDa), sweet potato β-amylase (200 kDa), horse spleen apoferritin (443 kDa) and bovine thyroglobulin (670 kDa) were used as markers for calibration (Sigma marker kit).

## Results

To study *in vitro* interactions of Vp1 proteins, wild-type and mutant Vp1 genes were separately transcribed and translated in a rabbit reticulocyte lysate system. Post-translational multimerization was assessed by non-reducing SDS–PAGE and by sucrose-gradient centrifugation. The major product of translation in all Vp1-gene-directed translations is the Vp1 protein, based on the monomeric 45 kDa size of the major product and by its immunoprecipitability with anti-Vp1 antibodies (Gharakhanian *et al.*, 1995; Weidman, 1994; present study). In this study, a 45 kDa protein was the major translation product (Fig. 1*a*, lane 1), and it could be immunoprecipitated by polyclonal antibodies against a neutralizing Vp1 peptide (Fig. 1*a*, lane 2); the 45 kDa protein was absent when SV40 DNA was not added to the translation system (Fig. 1*a*, lane 3). As reported previously (Gharakhanian *et al.*, 1995), the 7S species detected in sucrose gradients corresponds to the 225 kDa Vp1 pentamer and the 12S species corresponds to 700 kDa multi-pentamer complexes as assessed by gel-filtration chromatography (data not shown).

### 12S complexes of Vp1 are stabilized by inter-pentamer disulfide bonds *in vitro*

When wild-type Vp1 was subjected to non-reducing SDS–PAGE, only 45 kDa Vp1 monomers were seen immediately after translation and up to 2 h post-translation; after 3 h post-translation, the majority of the Vp1 proteins were migrating as high molecular mass complexes barely entering the resolving gel (Fig. 1*b*, lane 8). By 5 h post-translation, almost all Vp1 molecules migrate as multimers (Fig. 1*b*, lane 12); densitometric analysis of the autoradiograms at that time-point revealed that < 5% of Vp1 molecules were monomeric (data not shown). Multimer complexes were only seen in the unreduced samples and were completely disrupted in the presence of DTT (Fig. 1*b*, lanes 7, 9, 11). Bands with molecular masses of 90, 135 or 210 kDa are seen with some lysate batches and are independent of Vp1-gene-directed translation (Gharakhanian *et al.*, 1988; present study). Wild-type Vp1 gene products were also subjected to sucrose-gradient sedi-

Fig. 2. Disulfide-linked multimerization of wild-type and Cys-to-Ser mutant Vp1 molecules produced in rabbit reticulocyte lysates. *In vitro* transcription–translation products of wild-type and mutant pSp6Vp1 plasmids were incubated for different lengths of time (0–5 h post-translation) and were either reduced in 5 mM DTT (odd-numbered lanes) or not reduced (even-numbered lanes), alkylated with iodoacetamide and then separated by 10% non-reducing SDS–PAGE and visualized by autoradiography. Samples in lanes 1 and 2 were incubated for 0 h; samples in lanes 3 and 4 were incubated for 1 h; samples in lanes 5 and 6 were incubated for 2 h; samples in lanes 7 and 8 were incubated for 3 h; samples in lanes 9 and 10 were incubated for 4 h; samples in lanes 11 and 12 were incubated for 5 h. Only the regions of the gels corresponding to 45 kDa Vp1 are shown.

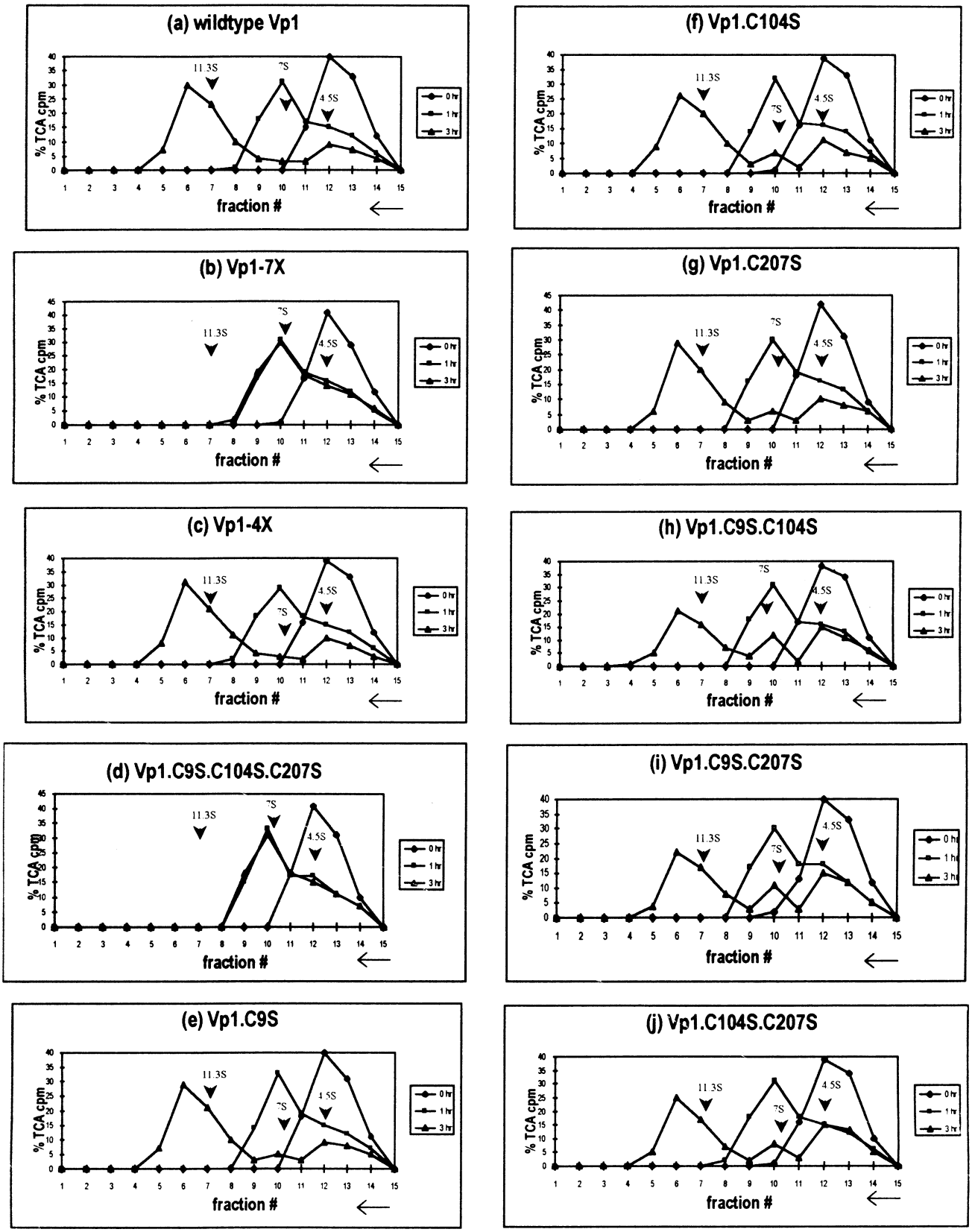


Fig. 3. Sucrose-gradient sedimentation profiles of wild-type and Cys-to-Ser mutant Vp1 molecules produced in rabbit reticulocyte lysates. Wild-type and Cys-to-Ser mutant Vp1 genes were transcribed–translated *in vitro* and were subjected to sucrose-gradient sedimentation and fractionation 0, 1 and 3 h post-translation. Samples were then TCA-precipitated and

mentation and were fractionated 0, 1 and 3 h post-translation. Immediately after translation, Vp1 products sedimented as a 4–5S species, corresponding to monomeric 45 kDa Vp1 molecules. By 1 h post-translation, the majority of Vp1 products sedimented as a 7S species corresponding to Vp1 pentamers, and by 3 h post-translation, the major peak was at 12S corresponding to multi-pentameric complexes (see Fig. 3*a*). The sedimentation profile clearly demonstrates the progression of Vp1 multimerization from monomer to pentamer to multi-pentamer *in vitro*. The non-covalently interacting pentameric complexes are not detectable in non-reducing SDS–PAGE further confirming that covalent interactions are inter-pentameric. These results are consistent with our previous report that inter-pentamer disulfide linkage(s) stabilize 12S complexes of SV40 Vp1 in cell-free lysates (Gharakhanian *et al.*, 1995).

### $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ of Vp1 are essential and sufficient for formation of inter-pentamer disulfide bonds *in vitro*

To directly test the role of cysteine residues in stabilization of Vp1 inter-pentamer complexes, a septuple mutant at all seven cysteine codons (pSp6Vp1–7X) was generated and was assayed for multimerization as described above. After 5 h of post-translational incubation, all of the gene products of pSp6Vp1–7X migrated as Vp1 monomers in non-reducing SDS–PAGE assays (Fig. 2*b*). This implies involvement of cysteines in disulfide-linked multimerization. In sucrose-gradient sedimentation, > 90% of total precipitable radioactivity was associated with the pentameric 7S species, but no multi-pentamer 12S species was detectable (Fig. 3*b*). Thus, Vp1 assembles into 7S pentamers independent of cysteine residues and disulfide linkage, but one or more cysteines must participate in disulfide-linked stabilization of multi-pentamer Vp1 complexes in cell-free lysates.

$Cys^{49}$, $Cys^{87}$, $Cys^{254}$ and $Cys^{267}$ were labelled in the mercury labelling experiment of Liddington *et al.* (1991) and were thus inferred not to be involved in disulfide bond formation. A quadruple mutant at those residues was constructed (pSp6Vp1–4X) and its protein product was subjected to multimerization assays as described above. Vp1–4X continued to form disulfide-linked 12S species similar to wild-type Vp1 as assayed by both non-reducing SDS–PAGE analysis (Fig. 2*c*) and by sucrose-gradient sedimentation (Fig. 3*c*).

Next, a Cys-to-Ser triple mutant Vp1 (at $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$) was assayed for multimerization as described above. In contrast to wild-type and quadruple mutant Vp1 and similar to septuple mutant Vp1, the triple mutant did not form disulfide-linked post-pentameric complexes in both non-reducing SDS–PAGE (Fig. 2*d*) and in sucrose gradients (Fig. 3*d*), yet continued to form non-disulfide linked 7S pentameric complexes by 1 h post-translation as assessed by sucrose-gradient sedimentation (Fig. 3*d*).

The results suggest that one or two or all three cysteine residues at positions 9, 104 and 207 play a role in inter-pentamer Vp1 disulfide interactions *in vitro*. To distinguish between these possibilities, Cys-to-Ser single and double mutants at $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ were assayed for multimerization as described above. The single mutant proteins at $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ continued to form S–S bonds in non-reducing SDS–PAGE (Fig. 2*e–g*) and sedimented as 12S species by 3 h post-translation (Fig. 3*e–g*). In all three single mutants, a small peak of 7S pentamers remained after 3 h as assayed by sedimentation (Fig. 3*e–g*), and densitometric analysis of the non-reducing SDS–PAGE showed slightly higher persistence of non-disulfide linked Vp1 at 5 h post-translation (data not shown).

Similar to wild-type Vp1, all three double mutants at $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ formed disulfide-linked 12S complexes by 3 h post-translation (Fig. 2*h–j* and 3*h–j*). However, both non-reducing SDS–PAGE and sucrose-gradient analysis of time-dependent multimerization revealed a decrease in disulfide-linked multimerization relative to wild-type.

These results show that cysteine residues at positions 9, 104 and 207 are essential and sufficient for disulfide-linked stabilization of inter-pentamer complexes *in vitro*, and that while all three are necessary for maximum complex formation, involvement of any one of the three is sufficient for the process.

## Discussion

A series of early and recent studies has suggested that Vp1 disulfide bonds, along with calcium ions, may play a role in stabilizing the SV40 capsid (Walter & Deppert, 1974; Brady *et al.*, 1977, 1978; Ng & Bina, 1981; Liddington *et al.*, 1991; Kosukegawa *et al.*, 1996; Stehle *et al.*, 1996). We have previously reported the involvement of disulfide linkage in the stabilization of multi-pentamer complexes of SV40 Vp1 in cell-free lysates (Gharakhanian *et al.*, 1995). In order to identify the specific cysteine residue(s) involved in disulfide bonding, various Cys-to-Ser site-directed mutant Vp1 molecules were assayed for disulfide-linked interactions in rabbit reticulocyte lysates. In such assays, wild-type Vp1 is monomeric (4–5S) immediately after translation, is pentameric (7S) by 1 h post-translation, and is multi-pentameric (12S) by 3 h post-translation. The low yield of Vp1 molecules in this system

precipitable radioactivity was determined by liquid-scintillation counting. BSA (4·5S) peaked at fraction 12, native IgG (7S) peaked at fraction 10 and catalase B (11·3S) peaked at fraction 7. The arrow indicates direction of increasing sedimentation values. A slightly different scale was used in panels (*b*), (*d*) and (*g*).

(Gharakhanian *et al.*, 1988) precludes formation of the larger multimeric complexes seen in high-yield expression systems. Multimerization involves formation of a Vp1 pentamer via non-covalent interactions, followed by stabilization of 12S complexes via inter-pentamer disulfide bonds. The 7S and 12S sedimentation values are slower than those expected from globular proteins of the same mass. This is most likely due to the unfolded amino- and/or carboxy-terminal arms of Vp1 which increase the frictional coefficient and reduce the sedimentation rate relative to a compact molecule of same mass (Liddington *et al.*, 1991; Stehle *et al.*, 1996).

A septuple mutant at all seven cysteine residues of Vp1 formed no disulfide-linked multi-pentamer complexes as assayed by non-reducing SDS–PAGE and by sucrose-gradient fractionations, directly implicating cysteine-mediated disulfide linkage in Vp1 inter-pentamer stabilization. In the same assays, a quadruple mutant at $Cys^{49}$, $Cys^{87}$, $Cys^{254}$ and $Cys^{267}$ continued to form 12S complexes. The triple mutant protein Vp1.C9S.C104S.C207S, however, was severely defective in formation of inter-pentamer disulfides and sedimented as pentameric 7S species at both 1 and 3 h post-translation. Collectively, the results suggest that inter-pentamer disulfides at $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ are essential and sufficient for inter-pentamer disulfide-linked stabilization of Vp1 molecules in cell-free lysates. This result is in complete agreement with the structural mercury-labelling experiments that led to the labelling of the four unaffected cysteines, suggesting that only $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ can be occupied in disulfide linkage (Liddington *et al.*, 1991). The size and S value of disulfide-linked complexes seen in this study show that disulfide bonds play a role in the stability of Vp1 complexes at the inter-pentamer level. This is consistent with the Liddington *et al.* study where disulfide bonds were not detected at Vp1 monomer level nor at pentamer level by X-ray crystallography; inter-pentamer complexes were not studied in that report. More recently, Stehle *et al.* (1996) actually detected inter-pentamer disulfides between B and B′ Vp1 molecules at $Cys^{104}$. Cys-to-Ser single and double mutant Vp1 proteins at $Cys^{9}$, $Cys^{104}$ and $Cys^{207}$ were only partially affected and continued to form disulfide-linked 12S complexes. The fact that mutant proteins containing only one of the three critical cysteines continue to form disulfide-linked 12S complexes strongly suggests that the disulfide bridges are between identical cysteines (e.g. $Cys^{104}$–$Cys^{104}$). In fact, the only structurally visualized SV40 Vp1 disulfides are between $Cys^{104}$ residues (Stehle *et al.*, 1996). Lack of drastic changes from wild-type in the assembly of double mutants precluded meaningful results from mixed-mutant experiments.

Of the three cysteine residues discussed, $Cys^{104}$ may indeed be the preferred residue for disulfide bond formation since this residue is highly conserved between SV40 and polyomavirus (Liddington *et al.*, 1991; Gharakhanian, 1988), and is located in a highly conserved hydrophobic region among related Vp1 proteins (Gharakhanian, 1988). The SV40 Vp1 $Cys^{104}$ is located in a loop on the Vp1 pentamer that is in contact with other $Cys^{104}$-containing loops on neighbouring pentamers (Liddington *et al.*, 1991), making it an ideal cysteine residue for disulfide bonding in the post-pentameric Vp1 complex as detected by Stehle *et al.* (1996). $Cys^{9}$ is also highly conserved between SV40 and polyomavirus (Gharakhanian, 1988; Liddington *et al.*, 1991); it is located on the amino-terminal arm of Vp1 and is exposed on a Vp1 pentamer. The position of $Cys^{9}$ in the capsid has not been visualized by crystallography, so this free amino-terminal arm on the pentamer may be an alternative site for disulfide bonding with other pentamers. $Cys^{207}$ is located on an exposed loop on the pentamer (Liddington *et al.*, 1991), making it another possible alternative site for disulfide bond formation with other pentamers. While a single disulfide bond may be sufficient, all three may be involved to enhance stabilization of the complex. The fact that out of three possible disulfide bonds, any one is sufficient for Vp1 multimerization may also be an evolutionary advantage for the virus; if mutations occur in any two of the three cysteines, the potential for formation of one stabilizing disulfide bond is maintained. Alternatively, potential involvement of each of the three cysteines in inter-pentamer disulfides may be limited in the capsid where minor viral structural proteins, Vp2 and Vp3, and the SV40 minichromosome are present and higher-order configurations may be more constrictive. Additionally, within the capsid, the three cysteines may find themselves in differential redox states, and different cysteines may be favoured in the three differentially interactive clusters of Vp1 (Yan *et al.*, 1996).

At what point the disulfide bonds form during the assembly of SV40 virus remains speculative. Whether the disulfide bonds stabilizing the SV40 virus form during its assembly in the cell or sometime after completion of assembly and after cell lysis remains unclear. Our results show SV40 Vp1 inter-pentamer disulfide bond formation in the cytoplasmic environment of cell-free lysates where 0·5–1 mM DTT is present, suggesting that Vp1 disulfides may form in the reducing intracellular environment. Disulfide bonds are most commonly seen in the proteins of the secretory pathway due to the non-reducing environment found there. Recently, however, disulfide bonds have also been reported outside of the secretory pathway, especially among viral proteins (Monastryskaya *et al.*, 1994; Volpers *et al.*, 1994; Kosukegawa *et al.*, 1996; Chang *et al.*, 1997; Sandalon & Oppenheim, 1997). These observations are supported by recent reports which have established the ability of cytoplasmic and nuclear compartments to direct redox regulation of various proteins at cysteine residues (Schreck *et al.*, 1991; Pognonec *et al.*, 1992; Xanthoudakis *et al.*, 1992; Meyer *et al.*, 1993; Benerza, 1994; Chinenov *et al.*, 1998). Our results, together with the cited reports, suggest that disulfides and other redox regulation of cysteines can occur in cytoplasmic or nuclear environments that are reducing overall. Non-reducing micro-environments may be found at the interface of interacting complexes due to inaccessible

hydrophobic pockets, or to localized oxidative activities. To further study the spatial and temporal involvement of specific cysteines in disulfide-linked stabilization of the SV40 capsid, *in vivo* studies are in progress. The combination of *in vitro* and *in vivo* studies can increase our understanding of specific SV40 Vp1 cysteine-mediated interactions and their significance.

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