Characterization of Simian Virus 40 Transformed
African Green Monkey Cells (CV-1)

I. Defective Virion and Viral Genome

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Abstract Several clones of SV40 transformed CV-1 cells have been characterized for the production of T- and V-antigens and for the state of viral genome. The transformed CV-1 cells failed to produce infectious virions as assayed after sonication or cocultivation and fusion with normal CV-1 cells, and were resistant to superinfection by SV40. Some clones of the transformed cells contained V-antigens. The population of V-antigen positive cells varied from 0 to 100% depending on the passage number while the T-antigen positive cells were always 100%. The virions isolated from the transformed cells were similar in morphology to complete SV40, but lighter in density than complete SV40. In one clone, a small amount of SV40 DNA was detectable in a free state while a large proportion of the DNA hybridizable with SV40 3H cRNA was linearly integrated into the cell DNA. The free SV40 DNA was noninfectious, closed circular DNA with a size smaller than infectious SV40 DNA component I. Since the cell extracts of the transformed cells contained an agent(s) which induced T- and V-antigens in normal CV-1 cells, it was suggested that the SV40 transformed CV-1 cells contained free as well as integrated defective SV40 genomes responsible for the synthesis of T- and V-antigens.

It has been reported that integration of SV40 genome occurs in permissive cells as well as in nonpermissive cells during primary infection (2, 5, 6, 10, 21). In order to confirm the integration of SV40 genome in permissive cells, the permissive transformed cell lines have been isolated from the permissive African green monkey cell lines, CV-1, infected with SV40 and examined for the presence of SV40 genomes. Several clones of permissive cells transformed by SV40 have been isolated in many laboratories (3, 14-16, 22). Since the SV40 infected permissive cells undergo almost exclusively a lytic cycle of SV40 replication and cell death, most of the SV40 transformed permissive cells were isolated from defective virus-infected cells. When African green monkey cells were transformed with the wild type of SV40 which consisted of infectious and defective virus, it was found that the transformed cells contained defective SV40 genome (1, 11, 13, 17).

In this paper, I report that SV40 transformed CV-1 cells used here contain

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SV40 genomes, mainly integrated into the cell DNA and that some clones of the transformed cells produce a small amount of SV40 which contain defective genomes.

MATERIALS AND METHODS

**Cell culture and virus.** SV40 (Rh911) was grown in CV-1 cells (12). Lines of CV-1 cells transformed by SV40 were obtained from Dr. Fred Jensen at the Wistar Institute, Philadelphia.

Normal and SV40 transformed CV-1 cells were routinely grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS).

**Cell fusion.** CV-1 cells ($5 \times 10^6$) and SV40 transformed cells ($5 \times 10^6$) were fused by 1,000 hemagglutinating units of β-propiolactone inactivated Sendai virus.

**Infectious activity.** For plaque assay, 0.1 ml of cell extracts were adsorbed to monolayers of primary African green monkey kidney cells or CV-1 cells in 60 mm petri dishes for 2 hr. The monolayers of infected cells were then overlaid with 2.5 ml of 1% agar in double strength Eagle's medium in Earle's balanced salt solution supplemented with lactalbumin hydrolysate and 5% FCS. At 6 days after infection (d.a.i.), 2 ml of the same medium was added as an additional overlay, and at 10 d.a.i. 2 ml of the overlay containing neutral red at a final dilution of 1:10,000 was added. The final plaque reading was done about 3 weeks after infection.

**Assay for SV40 T- and V-antigens.** The presence of SV40 T-antigen in SV40 transformed CV-1 cells was detected by indirect immunofluorescent (IF) staining using the serum collected from a tumor-bearing hamster and fluorescein conjugated anti-hamster globulin goat serum. A special care was taken for the detection of SV40 V-antigen by indirect IF staining using rabbit anti-SV40 and guinea pig anti-SV40 which were prepared against purified SV40 virions and tested for the presence of T-antibody on SV40 transformed human and monkey cells before assay.

**Preparation of SV40 virus and viral DNA.** SV40 virus was prepared from CV-1 cells infected at a multiplicity of infection (m.o.i.) of 0.001 and purified as described before (7).

SV40 DNA was isolated from the purified virus by the pH 9 sodium dodecyl sulfate (SDS) phenol method (8) and two successive ethidium bromide (Ethbr)-CsCl density gradient equilibrium centrifugations as described before (4).

**Integration of SV40 DNA into the DNA of SV40 transformed cells.** Cell DNA from the SV40 transformed CV-1 cells was isolated by velocity sedimentation in alkaline sucrose gradients as described before (6). The cell DNA immobilized on membrane filters was hybridized with SV40 ³H cRNA made in vitro by Escherichia coli DNA dependent RNA polymerase as described before (4).

When DNA isolated from sucrose gradient was hybridized with SV40 ³H cRNA, a precaution was taken for the concentration of sucrose solution. As shown on Table 1, the normal CV-1 cell DNA in sucrose solution was immobilized on the filter and hybridized with SV40 ³H cRNA ($1.6 \times 10^6$ cpm). The DNA in sucrose solution was hybridized with 2 to 30 times as efficiently as DNA which was not mixed with sucrose. The radioactivity bound to the filter varied when different batches of
commercial sucrose were used. Therefore, different batches of sucrose contained different amounts of contaminants which were nondialyzable. Since filtration of the sucrose solution was found to reduce the contaminants and result in a low background, the filtrated sucrose solution was used in all the experiments. Generally, glycerol did not affect the result of DNA-RNA hybridization although it was not used here.

**Labeling of SV40 infected and transformed CV-1 cells with a 14C amino acid mixture.**
Confluent monolayers of CV-1 cells were infected with SV40 at an m.o.i. of 10. At 24 hr post infection (p.i.), 10 μCi of a 14C amino acid mixture per ml was added to the culture. When cytopathic effect (CPE) became evident in most of the cells, the culture medium and cells were frozen and thawed three times.

Confluent monolayers of SV40 transformed CV-1 cells were split at a ratio of 1:4, and, after 24 and 48 hr, the cultures were exposed to 0.4 μCi of the 14C amino acid mixture per ml. At confluency, the medium and cells were frozen and thawed three times.

The thawed cultures of SV40 infected and transformed cells were centrifuged in a Spinco 30 angle rotor at 78,000×g for 3 hr. The sediments were suspended in phosphate buffered saline (PBS) by a sonifier cell disrupter (Heat System Co., Melville, N.Y.), and used for isolation of SV40 virus. The virions were purified by the method described before (7), using potassium bromide cushion and CsCl density gradient equilibrium centrifugation.

**Extraction of the closed circular DNA.** The confluent monolayers of SV40 transformed CV-1 cells (5×10^8) were lysed with 20 ml of 0.6% SDS, 0.01 M Tris (hydroxymethyl) aminomethane (Tris) buffer, pH 8.0, 0.01 M ethylene diamine tetraacetate (EDTA) (9). After complete lysis, 5 M NaCl was added to the lysate to a final

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**Table 1. Effect of sucrose solution on DNA-RNA hybridization**

<table>
<thead>
<tr>
<th>Solution for immobilization of CV-1 cell DNA (10 ml)</th>
<th>Weight of sucrose in 10 ml (g)</th>
<th>No. of filtration</th>
<th>Radioactivity bound (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6×SSC only</td>
<td>0</td>
<td>1</td>
<td>159</td>
</tr>
<tr>
<td>Sucrose #1</td>
<td>3</td>
<td>1</td>
<td>2,259</td>
</tr>
<tr>
<td>Sucrose #1</td>
<td>5</td>
<td>1</td>
<td>3,809</td>
</tr>
<tr>
<td>Sucrose #1</td>
<td>5</td>
<td>2</td>
<td>653</td>
</tr>
<tr>
<td>Sucrose #1, and dialyzed</td>
<td>5</td>
<td>1</td>
<td>3,205</td>
</tr>
<tr>
<td>Sucrose #2</td>
<td>3</td>
<td>1</td>
<td>2,259</td>
</tr>
<tr>
<td>Sucrose #3</td>
<td>3</td>
<td>1</td>
<td>314</td>
</tr>
<tr>
<td>Sucrose #4</td>
<td>3</td>
<td>1</td>
<td>2,729</td>
</tr>
<tr>
<td>30% of glycerol</td>
<td>0</td>
<td>1</td>
<td>174</td>
</tr>
</tbody>
</table>

* Solid sucrose or fluid glycerol was added to the alkali-denatured CV-1 cell DNA (50 μg) solution and adjusted to 10 ml of 6×SSC (a final volume and concentration), pH 7.5 and slowly passed through a nitrocellulose membrane filter (BAC-T-FLEX, B-6, Schleicher and Schnell, Keene, N.H.) which had been soaked in 6×SSC and hybridized with SV40 3H cRNA (1.6×10^6) as described before (7). Sucrose #1 was purchased from Nutritional Biochemical Corp., #2 from Mann Research, #3 from E. Merck, #4 from Baker.

* The CV-1 cell DNA solution (50 μg) containing 5 g of sucrose was dialyzed against distilled water, alkali-denatured, and adjusted to 6×SSC (a final concentration), pH 7.5 before immobilization on the filter.
concentration of 1 M, and the mixture was kept at 4 C overnight. The sample was centrifuged in a Spinco 30 angle rotor for 30 min at 34,000 \( \times g \) at 4 C. The DNA was extracted from the supernatant and the sediment by pH 9 SDS phenol method as described before (4), and designated as Hirt supernatant DNA and Hirt sedimented DNA, respectively. The DNA was solubilized in a solution of 0.01 M Tris, pH 8.0, 0.01 M EDTA, and 200 \( \mu g \) of Ethbr per ml and mixed with solid CsCl to a density of 1.566 g per cm\(^3\), and then centrifuged in a Spinco 50 angle rotor for 72 hr at 109,000 \( \times g \) at 20 C. The fractions were collected from the bottom of the tube, and the density was determined from refractive indices measured in an Abbe refractometer (Bausch&Lomb Inc., Rochester, N.Y.). Water (0.8 ml) was then added to each fraction and the optical density at 260 nm was measured. The closed circular DNA fractions were pooled. Ethbr in the solution was eliminated by passing through a Dowex 50 column (0.6 \( \times \) 5 cm) and dialyzing against 0.1 \( \times \) SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate).

**Transfection.** Confluent monolayers of CV-1 cells in a petri dish (6 \( \times \) 10\(^5\) of cells) were incubated with 0.3 ml of the closed circular DNA in PBS containing 100 \( \mu g \) of DEAE-dextran for 30 min at 37 C, then washed three times with PBS and cultured in a fresh medium supplemented with 2% FCS.

**Negative staining.** Purified virus preparations were spread on carbon-coated grids and stained with 2% potassium phosphotungstate (PTA), pH 7.0, and observed under an electron microscope (Hitachi, HS-8) at magnification of 24,000- and 33,000-fold.

**Materials.** Cytidine-5'-triphosphate 5-\(^3\)H (24.5 Ci per mm) and L-amino acid mixture-14C were all purchased from New England Nuclear, Boston, Mass.

**RESULTS**

**V-Antigen in SV40 Transformed CV-1 Cells**

Confluent monolayers of CV-1 cells at the 21st passage were infected with SV40 at an m.o.i. of 0.001. They were subcultured at 24 hr p.i. After three subcultures, most of the cells displayed a typical cytopathic effect (CPE) and the virus titers associated with the cells and in supernatant fluid of the infected culture were \( 10^{5.5} - 10^{6.0} \) PFU per 10\(^6\) cells and \( 10^8 - 10^4 \) PFU per ml. However, at the 20th passage after infection (p.a.i.), no CPE was detected in the cultures, but 100% of the cell population were T-antigen positive (parental CV-67SV). Ten morphologically different clones were established from the culture of parental CV-67SV. At the 5th passage after cloning, each clone was tested for infectious virus by direct assay of sonicated cells (5 \( \times \) 10\(^7\)). There was no infectious virus in the cell extract of any SV40 transformed CV-1 cell clone. Experimental results of superinfection by SV40 and virus rescue by cocultivation with normal CV-1 cells or by fusion with normal CV-1 cells in the presence of Sendai virus were all negative.

When several clones of SV40 transformed CV-1 cells were examined for V-antigen by an IF method, it was found that some cell lines were V-antigen positive. The number of V-antigen positive cells was different not only among the cell lines,
but also depending on the passage number of the culture, while T-antigen positive cells were always 100%. During a period of over 3 years, the proportion of V-antigen positive cells (parental CV-67SV and Cl-15a cells) varied from 0 to 100% depending on the passage number after infection (p.a.i.) (Fig. 1). The percentage of V-antigen positive cells in Cl-15a cell culture was 20% when the cells were cloned (32 p.a.i.), changed to 0% at 87 p.a.i., to 100% at 129 p.a.i. and then declined during further passage of the culture. A similar pattern of V-antigen positive cells was found in the cultures of parental CV-67SV cells. It appears that there is a cycle for appearance of V-antigen positive cells in the cultures of SV40 transformed CV-1 cells, while the number of T-antigen positive cells was 100% at any time during cell passage.

*Isolation of Virions from the SV40 Transformed CV-1 Cells*

When thin sections of the SV40 transformed CV-1 cells (parental CV-67SV at 148 p.a.i. and Cl-15a at 99 p.a.i.) were examined electron microscopically, it was found that 10 to 15% of the examined nuclei contained a few dense bodies (1–5 particles per nucleus section) with the size and shape of SV40 particles (not shown). They were not clustered in groups, but scattered among the dispersed chromatin and were not found in the cytoplasm of both cell lines.

Since the SV40 transformed CV-1 cell cultures contained V-antigen positive cells at high percentages, there should have been many empty virus particles or capsid proteins which could not be detected by thin sectioning. Therefore, $8 \times 10^7$ of Cl-15a cells at 129 p.a.i. were labeled with a $^{14}$C amino acid mixture, and virus particles
were isolated and analyzed in CsCl density gradient equilibrium centrifugation. Standard SV40 virions labeled with the $^{14}$C-amino acid mixture were isolated from $5 \times 10^6$ CV-1 cells infected with SV40 at an m.o.i. of 10. As shown on Fig. 2A, the SV40 infected CV-1 cells contained full particles and empty particles with a density of 1.34 and 1.30 g per cm$^3$, respectively, while C1-15a cells at 129 p.a.i. gave a single peak at a density of 1.31 g per cm$^3$ (Fig. 2B).
Electron microscopic observations of the fraction with a density of 1.31 g per cm$^3$ from C1-15a cells revealed the presence of particles with an average diameter of 42 nm. Most of the particles were severely degraded or definitely empty particles and 15 to 20% thereof showed a complete virion structure (Fig. 3B). In contrast, 95% of the particles from SV40 infected CV-1 cells were complete virions with an average diameter of 45 nm (Fig. 3A).
Detection of SV40 Genome in the SV40 Transformed CV-1 Cells

Since the SV40 transformed CV-1 cells contained SV40 specific T-antigen in the nucleus, the presence of SV40 genome in the transformed cells was expected. It was therefore examined whether the SV40 genome was integrated into the DNA of SV40 transformed CV-1 cells. In order to separate free SV40 DNA from cellular DNA, sedimentation in an alkaline sucrose density gradient was employed (Table 2).

In the Cl-15a cells at 45 p.a.i., a large proportion of the DNA hybridizable with SV40 $^3$H cRNA was integrated into the cell DNA. At 98 p.a.i., when V-antigen positive cells were 62%, a small amount of free SV40 DNA was detected in the Cl-15a cells. The presence of free SV40 DNA was not a result of fragmentation of host cell DNA since the amount of the slowly sedimented DNA in the alkaline sucrose density gradient was less than 5% of the fast sedimented cell DNA. In addition, there was no detectable amount of free SV40 DNA when the V-antigen positive cells were less than 5%. The proportion of the free SV40 DNA in Cl-15a cells varied in proportion to the percentage of V-antigen positive cells while the amount of hybridizable viral DNA integrated into the cell DNA was fairly constant during the passages (8).

Characterization of Free SV40 DNA Isolated from Cl-15a Cells

When the Hirt supernatant and sedimented DNAs of Cl-15a cells ($5 \times 10^7$) were analyzed in Ethbr-CsCl density gradient equilibrium centrifugation, it was found that most of the DNA hybridized with SV40 $^3$H cRNA in the supernatant fraction was closed circular DNA while the hybridizable DNA of the Hirt sedimented fraction was mostly linear DNA (Fig. 4). This result shows that SV40 DNA is linearly integrated into the DNA of Cl-15a cells.

In order to examine the size of free SV40 DNA in the Cl-15a cells, the Hirt supernatant DNA from $1 \times 10^7$ cells was analyzed by sedimentation in an alkaline sucrose density gradient (Fig. 5). Since the amount of free DNA was too small to be examined under an electron microscope, the viral DNA was detected by DNA-RNA hybridization. While most of the DNA isolated from purified virions with a density of 1.34 g per cm$^3$ sedimented at 53 S, the free SV40 DNA from Cl-15a cells sedimented consistently slower than 53 S of SV40 DNA in three separated experiments.
Fig. 4. Ethidium bromide-CsCl density gradient equilibrium centrifugation of Hirt supernatant and sedimented DNAs isolated from Cl-15a cells. The Hirt supernatant and sedimented DNAs were isolated from confluent monolayers of Cl-15a cells (5 × 10⁷) by the selective method of Hirt (9). The total amount of Hirt supernatant DNA (A) and 100 μg of Hirt sedimented DNA (B) were analyzed in a Spinco 50 angle rotor for 72 hr at 109,000 × g at 20 C. The fractions were collected from the bottom of the tube. Water (0.8 ml) was then added to each fraction of Hirt sedimented DNA to measure the optical density at 260 nm (●). The DNA of each fraction was dialyzed against 0.1 × SSC, and then hybridized with SV40 ³H cRNA (1.6 × 10⁶ cpm) as described before (7). Arrows indicate the positions of (a) a closed circular DNA and (b) an open circular and linear DNA, respectively. The radioactivity (cpm) of hybridized SV40 ³H cRNA is indicated by bars.

Fig. 5. Alkaline sucrose gradient centrifugation of SV40 DNA and Hirt supernatant DNA isolated from Cl-15a cells. (A) SV40 DNA (0.1 μg) and (B) Hirt supernatant DNA isolated from Cl-15a cells (1 × 10⁷) were layered on alkaline sucrose gradient (10 to 30%) in 0.3 N NaOH, 0.01 M EDTA and 0.5 M NaCl, and centrifuged in a Spinco SW25.1 rotor for 8 hr at 84,000 × g at 4 C, as described before (6). The fractions (1 ml per tube) were collected from the top of the gradient by an ISCO density gradient fractionator. The DNA of each fraction was hybridized with SV40 ³H cRNA (1.6 × 10⁶ cpm) as described before (7). The radioactivity (cpm) of hybridized SV40 ³H cRNA is indicated by bars.
It appears, therefore, that Cl-15a cells contain closed circular DNA with a size smaller than the normal SV40 DNA component I.

When the free SV40 DNA in the Cl-15a cells was tested for the presence of infectious DNA, it was found that the free SV40 DNA from $4 \times 10^8$ of Cl-15a cells did not give any CPE in $6 \times 10^5$ CV-1 cells during one month after transfection, while 0.005 µg of normal SV40 DNA component I, corresponding to about 2 viral genome equivalents per cell, gave typical CPE with vacuolation and inclusions in the CV-1 cells ($6 \times 10^5$) within 3 weeks after transfection. These results suggested that the free SV40 DNA in the Cl-15a cells is defective.

Similar results were obtained for the DNA isolated from SV40-like particles with a density of 1.31 g per cm$^3$ (Fig. 2B) which contained the closed circular DNA hybridizable with SV40 $^3$H cRNA.

**T- and V-Antigen Forming Agent in the SV40 Transformed CV-1 Cells**

From the fact that infectious virus was undetectable in any clone of SV40 transformed CV-1 cells, it was examined whether the transformed cells produced any agent which induced T- and V-antigens in normal CV-1 cells.

Several clones of SV40 transformed CV-1 cells ($2 \times 10^7$) were sonicated and inoculated to normal CV-1 cells ($5 \times 10^6$). As shown in Table 3, a small number of cells positive for T- and V-antigens was detected by the IF method. However, CPE was not seen in the culture of CV-1 cells even at one month after inoculation, while the CV-1 cells infected with SV40 at an m.o.i. of 0.001 showed CPE in 100% of cells at 2 weeks after infection.

<table>
<thead>
<tr>
<th>Table 3. Induction of T- and V-antigens in CV-1 cells incubated with the cell extracts from SV40 transformed CV-1 cells$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>CV-67 SV (parental)</td>
</tr>
<tr>
<td>C1-6</td>
</tr>
<tr>
<td>C1-15a</td>
</tr>
</tbody>
</table>

$^a$ Percentage of T- and V-antigen (Ag) positive cells was based on counts of $10^4$ to $10^5$ cells.

$^b$ Irregular foci.

**DISCUSSION**

The present paper has shown that, while several clones of SV40 transformed CV-1 cells failed to produce infectious virus when assayed with the cell extract prepared by sonication of the cells or by cocultivation with permissive cells, some clones of the transformed cells contained V-antigens, which varied in amount from passage to passage. The virion-like particles isolated from the transformed cells had a lighter
density in CsCl than infectious SV40 virions. Most of the particles in a clone of the
transformed cells, Cl-15a, were empty and a small fraction of them contained DNA
hybridizable with SV40 3H cRNA. In addition, the extract of the transformed cells
contained an agent(s) which induced T- and V-antigens in normal CV-1 cells.
These results suggest that the SV40 transformed CV-1 cells produced defective SV40
virions as have been found in serial undiluted passage of SV40 in African green
monkey cells (18).

The experiments concerning the state of SV40 DNA in one clone of transformed
cells, Cl-15a, indicated that, while a large proportion of SV40 DNA was integrated
into the cell DNA, a small amount of SV40 DNA was present in a free form. The
amount of the free viral DNA changed from passage to passage. Since this clone is
resistant to superinfection with SV40 virus, the free viral DNA may be originated
from the integrated viral DNA, presumably by excision from the host cell DNA.
Yoshiike (19, 20) has reported that successive undiluted passages of SV40 in African
green monkey kidney cells results in the production of heterogenous defective par-
ticles with densities lower than that of infectious virions, and that the light virions
contain circular DNA molecules smaller than those from plaque formers. The
situation is similar in the SV40 transformed CV-1 cells in that the free viral DNA was
closed circular DNA with a size smaller than normal SV40 DNA component I. If
the free viral DNA was originated from the integrated viral DNA, the latter may
have been defective viral genome. This interpretation may explain why infectious
virions were not rescued from the transformed CV-1 cells by fusion with normal
permissive cells. If this was the case, the defective viral genome must contain genes
for V-antigen as well as T-antigen. It cannot be ruled out, however, that the free
viral DNA replicates autonomously and independently of the integrated viral DNA.

In order to investigate the defectiveness of the free and integrated viral genomes,
experiments by reassociation kinetics between a specific fragment of SV40 DNA and
the DNA from the transformed cells are needed.

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