Virus DNA Synthesizing Ability of T Antigen-forming Defective SV40 Produced by Successive Undiluted Passages

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SUMMARY

SV40 stocks prepared by successive undiluted passages in African green monkey kidney (GMK) cells contain particles which produce T antigen but not capsid antigen (T particles). The DNA of the T particles was shown to replicate during abortive infection of GMK cells, as indicated by immunofluorescence assay for antigen forming capacity of virus DNA. It was also shown that the synthesis of T particle DNA was induced in heterokaryons formed by fusion of 3T3 cells transformed by T particles with susceptible GMK cells.

INTRODUCTION

SV40 stocks prepared by successive undiluted passages in African green monkey kidney (GMK) cells consist mainly of heterogeneous, defective light virus particles (Uchida et al. 1968). These virus particles contain circular DNA molecules of various lengths and the maximum deletion of DNA amounts to at least 15% of the total genome of ’standard’ virus (plaque formers) (Yoshiike, 1968). The undiluted passage stocks have been found to contain three classes of defectives; T particles which produce T antigen but are unable to form capsid (V) antigen, V particles which produce V antigen in the nuclei of GMK cells without producing infectious progeny (Uchida et al. 1968), and i particles which are unable to produce even T antigen, but can interfere with propagation of plaque formers (unpublished data). Among these defectives, i particles exist most abundantly (unpublished data).

T particles cause tumours in hamsters and transform mouse 3T3 cells with the same efficiency as that obtained with plaque formers (Uchida & Watanabe, 1968, 1969). When 3T3 cells transformed by T particles were fused with GMK cells, an increase in intensity of T antigen-fluorescence and the SV40 specific nuclear alterations occurred in some heterokaryons similar to those seen in heterokaryons of plaque former transformants with GMK cells. As these changes were inhibited by cytosine arabinoside, they were considered to have resulted from autonomous replication of the virus genome that had been integrated (Uchida & Watanabe, 1969). This paper reports that T particles can direct the synthesis of virus DNA in GMK cells without the aid of other defectives and that the T particle DNA does in fact replicate in the heterokaryons.

METHODS

Cells. Secondary GMK cell cultures prepared from frozen stocks were used throughout this study. The method of culture was previously described (Uchida et al. 1968). Isolation
Table 1. 

<table>
<thead>
<tr>
<th>Sample</th>
<th>Estimated buoyant density (g/ml)</th>
<th>Virus particles/ml</th>
<th>p.f.u./ml</th>
<th>v.f.u./ml</th>
<th>t.f.u./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque former</td>
<td>1.34</td>
<td>$10^{15}$</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
</tr>
<tr>
<td>Defective virus particle</td>
<td>1.33</td>
<td>$10^{10}$</td>
<td>$10^{8}$</td>
<td>$10^{8}$</td>
<td>$10^{8}$</td>
</tr>
</tbody>
</table>

Estimated from extinction ($I_{358}$ unit = $6.4 \times 10^{12}$ virus particles).

and cultivation of the 3T3 cell lines transformed by the plaque former or T particle of SV40 were also described previously (Uchida & Watanabe, 1969).

**SV40 preparations.** A plaque former and a defective virus particle sample of SV40 (strain 777, small plaque type) were prepared by the same method as described previously (Uchida & Watanabe, 1968). Briefly, they were obtained from the appropriate fractions after CsCl density gradient sedimentation of the purified virus particles from the dilute and the third undiluted passage yields. Their titres, concentrations of particles and buoyant densities are listed in Table 1. The T and V antigen-forming activities were expressed as T or V antigen-forming units (t.f.u. or v.f.u.). The values have been normalized so that in the case of plaque formers the ratio t.f.u./v.f.u./p.f.u. becomes 1/1/1 (Uchida et al. 1968). The plaque former sample is a fraction at a slightly higher density than the peak of particles of the dilute passage material and is regarded as a homogeneous population of plaque formers. The defective particle sample is a fraction at a slightly lower density than the virus peak of the undiluted passage material, and the T antigen formers in this sample are largely composed of T particles as indicated in Table 1.

**Cell fusion experiment.** Heterokaryon cultures were prepared by the method described previously (Uchida & Watanabe, 1969), except that the concentrations of cells and of u.v.-inactivated Sendai virus in the mixture for cell fusion were higher. Thus $7 \times 10^6$ transformed and $1.3 \times 10^7$ GMK cells were suspended in 1 ml of Eagle’s minimum essential medium containing 5% calf serum (MEM C5) and the suspension was mixed with 1 ml of MEM C5 containing u.v.-inactivated Sendai virus (2400 H.A.U.). This mixture was kept in an ice bath for 10 min with gentle shaking and incubated at 36 °C for 10 min. Then it was diluted 33-fold with medium and distributed into 90 mm plastic dishes ($4.5 \times 10^6$ cells/15 ml/dish) or small bottles (1.4 x 3.8 cm) with or without a coverslip ($3 \times 10^6$ cells/ml/bottle). The dishes or bottles were incubated at 36 °C in a CO2-flushed incubator and the medium was changed to MEM containing 1% calf serum (MEM C1) after 24 h incubation. The cultures were further incubated at 36 °C and used for the assays on day 3 or 6 after fusion.

**Extraction of DNA.** Purified virus, diluted with SSC (0.15 M-NaCl, 0.015 M-sodium citrate), was incubated at 36 °C for 3 h in the presence of 0.1 mg/ml Pronase P (KAKEN KAGAKU). DNA was extracted by phenol treatment and the phenol removed by extensive dialysis against PBS- (phosphate buffered saline without Ca++ and Mg++). Virus DNA was extracted also from infected GMK cells or heterokaryon cultures by the method of Hirt (1967), and the extract was further deproteinized with phenol. The cells in two or three 90 mm plastic dishes were washed twice with PBS- and lysed by adding to each culture 0.5 ml of PBS- (pH 7.5) containing 1.2% sodium lauryl sulphate (SLS: WAKO JUNYAKU) and 0.01 M-EDTA. After 15 min at room temperature, the viscous lysate was scraped from the dishes with a rubber policeman and poured into a plastic centrifuge tube. Five m-NaCl solution was added to make the final concentration 1 M, and the sample was mixed by slowly inverting the tube. The sample was stored at 4 °C for 8 h, and centrifuged.
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at 17000g for 30 min at the same temperature to remove the major portion of cellular DNA and protein. The supernatant fluid was treated twice with washed phenol, once with chloroform and dialysed against PBS-.

**Plaque assay of DNA.** The method of McCutchan & Pagano (1968) was employed. GMK cell monolayers 2 days after seeding were washed once with PBS- and inoculated with 0.1 ml of DNA serially diluted in PBS- containing 1 mg/ml diethylaminoethyl-dextran (DEAE-dextran: Pharmacia, Uppsala, mol. wt. 2 × 10⁶). After 30 min at room temperature, the inoculated cells were washed twice with PBS. The overlay with agar-medium, and staining and counting of plaques were carried out in the same way as the virus plaque assay (Uchida et al. 1968).

**Assay for antigen-forming activity of DNA.** Coverslips (1.2 x 3.2 cm) with GMK cell monolayers one day after seeding were washed once with PBS-, taken out of the bottles and inoculated with 0.03 ml of DNA serially diluted in PBS- containing 300 μg/ml DEAE-dextran. After 10 min at room temperature, each coverslip was replaced in the bottle, washed twice with PBS, and incubated at 36 °C in Earle’s Medium containing 0.5% lactalbumin hydrolysate (LE), supplemented with 0.1% anti-SV40 rabbit serum. On day 2 after infection, coverslip cultures were washed, fixed with acetone, and stained with the fluorescent SV40 V (green monkey serum) or T (hamster sera) antibodies. As described by Black & Rowe (1965), antigen was seen frequently in two or three nuclei in close proximity; such a focus was counted as one positive cell.

The antigen-forming activities were expressed in cell-infecting units (Wheelock & Tamm, 1961) or antigen-forming units. The cell-infecting units (c.i.u.) are calculated as follows: c.i.u./ml = number of fluorescent cells/coverslip × dilution factor × 1/0.03, (0.03 ml: amount of inoculum). The antigen-forming units (t.f.u. and v.f.u.) are comparable units with p.f.u. as described for the activities of virus particles (Uchida et al. 1968). Assuming that the plaque former sample contains no defectives, we normalized the activities so that in the case of plaque formers the ratio t.f.u./v.f.u./p.f.u. becomes 1/1/1. The activities of the defective virus particle sample were normalized accordingly by the following equation, t.f.u, or v.f.u. (def) = c.i.u. (def) × p.f.u. (pl)/c.i.u. (pl) (def, defective virus particle sample; pl, plaque former sample).

**RESULTS**

**Fluorescence assay for antigen-forming capacity of defective virus DNA**

The antigen- and plaque-forming activities of DNA from an SV40 sample enriched with T particles were compared with those of DNA from a plaque former sample practically free of defective virus particles. The activities of the two virus samples used are shown in Table 1. The ratio t.f.u./v.f.u./p.f.u. of the defective virus sample is about 50/10/1.

DNAs were extracted from the samples diluted 20-fold with SSC. GMK cell monolayers grown on coverslips were infected with serial two- or threefold dilutions of each DNA and cultured in the presence of anti-SV40 serum. On day 2 after infection, when the number of fluorescent cells reached the peak, the coverslips were stained with the fluorescent V or T antibodies. The relationship between number of fluorescent cells and dilution of DNA was linear as shown in Fig. 1, indicating that the number was directly proportional to the DNA concentration, except when the dilution was low. The antigen- and plaque-forming titres of the DNAs are shown in Table 2. The fluorescence assay of DNA from the plaque former sample gave titres (c.i.u./ml) that were comparable to those obtained by the plaque method. The same result has been reported by Aaronson & Todaro (1970). The T antigen-forming titres of DNAs from the defective virus particle and the plaque former samples were
Capacity of T particles for virus DNA synthesis

We investigated the capacity of T particles to synthesize virus DNA, using the fluorescence assay for titration of virus DNA infectivity. GMK cell monolayers grown on 90 mm plastic dishes (5.5 × 10⁶ cells/dish) were inoculated with 0.5 ml of the defective virus sample (Table 1) at an input multiplicity of about 0.07 t.f.u. (0.014 v.f.u., 0.0014 p.f.u.) or 14 virus particles/cell. After a 2 h adsorption period at 36 °C, cultures were washed twice with PBS approx. equal, as those of the starting virus particle samples were. The ratio t.f.u./v.f.u./p.f.u. of DNA from the defective virus particle sample was, on the average, 30/6/1, which is similar to that of the starting defective virus particle sample.
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Fig. 2. Time course of synthesis of SV40 DNA in GMK cells. (a) Inoculated with plaque former sample. ▲—▲, p.f.u. (t.f.u., v.f.u.) (b) Inoculated with defective virus particle sample. ●—●, t.f.u.; ○—○, v.f.u.; ▲—▲, p.f.u.

and medium LE containing 0.1% anti-SV40 rabbit serum was added. At 4, 43, and 67 h, respectively, DNA was extracted from two cultures, and their biological activities were assayed. For comparison, the plaque former sample-infected cultures (at an input multiplicity of about 0.07 p.f.u. per cell) were treated in the same way (Fig. 2). As the T and V antigen-forming titres (c.i.u./ml) of extracts from cultures infected with the plaque former sample coincided with those expected from their plaque-forming capacity, they have been normalized so that the ratio t.f.u./v.f.u./p.f.u. is 1/1/1. The antigen-forming activities of the extracts from cultures infected with the defective virus sample have been normalized accordingly. As can be seen from Fig. 2, all the biological titres reached the highest levels within 43 h after infection. This is consistent with the result of Kit et al. (1969) showing that SV40 DNA replication begins at about 12 to 16 h and continues at a rapid rate until about 44 h after infection. Since the ratio of t.f.u./v.f.u./p.f.u. in defective SV40 infection was 75/41/1 (Fig. 2b), we conclude that the replication of defective DNA having T antigen-forming capacity resulted from the T particle infection. The yield of plaque former DNA (plaque-forming activity at 43 or 67 h) roughly paralleled the plaque-forming titres of each inoculum, and the T antigen-forming activity (at 43 or 67 h) of synthesized defective virus DNA was approx. equal to that of infectious DNA synthesized during a single cycle infection by the plaque former sample (Fig. 2).

To confirm that T particles can synthesize virus DNA without the aid of other particles which are not able to produce even T antigen, we examined the relationship between yields of T particle DNA and input multiplicities of virus particles per cell (Fig. 3). GMK cell monolayers grown on 90 mm plastic dishes and coverslips were inoculated with the defective virus particle sample at input multiplicities of about 3.6, 1.2, and 0.4 virus particles per cell, and cultured in the presence of anti-SV40 serum as the previous experiment. At 43 h after infection, virus DNA was extracted from two dish cultures. The number of cells infected with T particle was counted on coverslip cultures treated with anti-T fluorescent antibodies at the time of DNA extraction. The T antigen-forming activity of extracted DNA paralleled
the input multiplicity of virus particles or the number of T particle infected (fluorescent) cells. These results suggest that most T particles are able to multiply their progeny DNA without the aid of other virus particles in GMK cells, with an average yield per cell similar to that of infectious DNA in plaque former infection.

**Synthesis of defective SV40 DNA in heterokaryons**

We had reported previously indirect evidence (increase of intensity of T antigen-fluorescence) suggesting the induction of synthesis of virus genome in heterokaryons produced by fusion of 3T3 cells transformed by T particles with GMK cells (Uchida & Watanabe, 1969). In the following experiment we attempted to obtain more direct evidence, using the fluorescence assay for infectivity of virus DNA.

3T3 cell lines transformed by plaque former or T particle stocks were fused with GMK cells. DNA was extracted from three 90 mm plastic dish cultures 3 days after fusion by the method used in the preceding section. At the same time, we treated parallel fused cultures on coverslips with fluorescent antibodies to determine the number of heterokaryons containing V or strongly fluorescent T antigen. The production of infectious virus or antigen-forming defective virus was examined on day 6 after fusion by the plaque or immunofluorescence test (Uchida et al., 1968), following the release of virus by ultrasonic treatment of the cells suspended in the culture fluid.

In the fused cultures of plaque former transformants, infectious virus was detected, and the heterokaryons containing V antigen roughly corresponded to the number of those containing strongly fluorescent T antigen. In the fused cultures of T particle transformants, neither infectious virus nor antigen-forming defective virus was detected, but the intensity of T antigen-fluorescence increased in some heterokaryons. These results are identical with those reported previously (Uchida & Watanabe, 1969).
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Table 3. Plaque and fluorescence assay of SV40 DNA extracted from heterokaryon cultures produced by fusion of 3T3 cells transformed by SV40 with GMK cells

<table>
<thead>
<tr>
<th>Transformed cell line</th>
<th>u.v.-inactivated Sendai virus</th>
<th>Expt no.</th>
<th>Total activities of DNA†</th>
<th>Number of heterokaryons containing strongly fluorescent T antigen per three dish cultures‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Plaque forming titres (p.f.u.)</td>
<td>V antigen forming titres (c.i.u.)</td>
</tr>
<tr>
<td>Plaque former</td>
<td>+</td>
<td>+</td>
<td>1</td>
<td>$10^{5.8}$</td>
</tr>
<tr>
<td>d19</td>
<td></td>
<td></td>
<td></td>
<td>$10^{4.0}$</td>
</tr>
<tr>
<td>T particle</td>
<td>+</td>
<td>+</td>
<td>1</td>
<td>$10^{6.0}$</td>
</tr>
<tr>
<td>transformant M</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>$10^{5.0}$</td>
</tr>
<tr>
<td>W3</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>$10^{5.0}$</td>
</tr>
<tr>
<td>W4</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>$10^{6.0}$</td>
</tr>
<tr>
<td>W5</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>$10^{6.0}$</td>
</tr>
<tr>
<td>c6</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>$10^{5.0}$</td>
</tr>
<tr>
<td>Control W3</td>
<td>−</td>
<td>−</td>
<td>2</td>
<td>$10^{5.0}$</td>
</tr>
<tr>
<td>W3</td>
<td>−</td>
<td>+</td>
<td>2</td>
<td>$10^{5.0}$</td>
</tr>
<tr>
<td>W3 + CA*</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>$10^{5.0}$</td>
</tr>
</tbody>
</table>

* Heterokaryon cultures incubated in the presence of 10 μg/ml cytosine arabinoside.
† DNA was extracted from three 90 mm plastic dish cultures.
‡ Calculated from the number assayed on a coverslip with heterokaryon culture prepared simultaneously.

The results of biological assays of extracts are summarized in Table 3. Antigen-forming titres of extracts from heterokaryon cultures of plaque former transformants agreed approximately with titres expected from their plaque-forming ability, indicating that the virus DNA synthesized was non-defective. In the extracts from heterokaryon cultures of T particle transformants, only T antigen-forming activity was detected. No activities were detected in the extracts from control cultures (transformed cells, homokaryons of transformants fused by u.v.-inactivated Sendai virus, heterokaryons incubated in the presence of 10 μg/ml cytosine arabinoside). Samples of the extract from fused cultures of a T particle transformed cell line M were treated as follows: at 100 °C for 10 min; at 33 °C for 20 min with 50 μg/ml RNase (Worthington Biochemical Corporation, Freehold, New Jersey) previously boiled for 10 min to inactivate traces of DNase; at 33 °C for 20 min with 50 μg/ml DNase (Worthington Biochemical Corporation) in the presence of 0.005 M-MgSO4. Although the antigen-forming activity was not changed by the former two treatments, it was completely destroyed with DNase. Thus, the synthesis of T antigen-forming defective SV40 DNA was demonstrated to be induced in the fused cultures of T particle-transformed and GMK cells.

As indicated in Table 3, the T antigen-forming titres of DNA roughly paralleled the number of heterokaryons containing strongly fluorescent T antigen. The suggestion that the increase of intensity of T antigen-fluorescence resulted from the induction of synthesis of virus genome is supported also by this correlation.

DISCUSSION

In the preceding paper (Kato et al. 1974), we suggested that the T antigen-forming defective virus particles (T particles) had the capacity to induce the synthesis of cell DNA and to direct the synthesis of virus DNA. However, we could not determine then whether or not complementation between defective mutants had played a significant role in those activities,
since the results were obtained at high multiplicities of infection of virus particles (1400 to 140 particles or 10 to 1 T antigen-forming units per cell). In the present investigation, we studied the synthesis of T particle DNA in GMK cells infected with a defective virus particle sample at low multiplicities of infection (14 to 0.4 particles or 0.07 to 0.002 T antigen-forming units per cell), using the fluorescence assay of the DNA. The results indicated that most T particles were able to synthesize their progeny DNA apparently without the aid of other virus particles, with an average yield per cell similar to that of infectious DNA in plaque former infection.

It was shown that the increase in intensity of T antigen-fluorescence in heterokaryons produced by fusion of 3T3 cells transformed by T particles with GMK cells (Uchida & Watanabe, 1969) was the result of the induction of synthesis of T particle DNA by using the fluorescence assay for antigen-forming activity of virus DNA. That the increase in intensity of T antigen-fluorescence has been observed in all of nine T particle transformed cell lines tested (Uchida & Watanabe, 1969, and an additional cell line included in this article) is consistent with the conclusion in this report that most T particles are able to synthesize their progeny DNA.

Since the T particle stocks contain V particles, V particle transformed cells could have been detected in the cell lines transformed by T particle stocks, but these are not detected in the nine transformed cell lines tested. Perhaps V particle transformed cells are detected when more cell lines transformed by T particle stocks are tested.

Many of the 3T3 cell lines transformed with u.v.-irradiated SV40 samples did not show the increase of intensity of T antigen-fluorescence in heterokaryons with GMK cells, and T antigen-forming activity was not demonstrated in DNA fractions extracted from the heterokaryon cultures (unpublished data). These results suggest that the defective viruses which produce T antigen but are unable to multiply virus DNA occur frequently by u.v. irradiation, and that such defectives can transform 3T3 cells. The fact that the defective deletion mutants which produce T antigen but not virus DNA were not detected in the third undiluted passage stock suggests that either the deletion does not occur at random or that such mutants are weeded out during successive undiluted passages.

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REFERENCES


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