Analysis of Defective SV40 DNA by Agarose Gel Electrophoresis

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(Accepted 22 April 1975)

SUMMARY

The SV40 DNA that was generated by undiluted passaging of the virus was
analysed by agarose gel electrophoresis. Nine bands of virus DNA were
distinguished and each band contained a specific size class of DNA, all shorter than
the complete SV40 genome as was determined by electron microscopy measure-
ments. A difference of 2 % in length, about 100 base pairs, resulted in a clear
band splitting. Two sets of undiluted passaging were established and the defective
DNA in the two sets had both different and similar size classes varying in length
from 96 % to 73 % of the unit length SV40 DNA.

INTRODUCTION

Analysis of the DNA extracted from purified virus or from cell lysates after serial un-
diluted passage of SV40 or polyoma virus has revealed its heterogeneity in size because of
the presence of defective DNA molecules (Uchida et al. 1968; Tai et al. 1972; Fried, 1974).
The preparation of pure stocks of defective DNA molecules with specific deletions, substi-
tutions and/or duplications may be very useful for the mapping and identification of the
genes of the complete virus. A number of methods for growing defectives has been
described. Both for SV40 and polyoma virus an infectious centre method to obtain clonal
isolates of defectives has been employed (Brockman & Nathans, 1974; Fried, 1974). More
recently Mertz & Berg (1974) described a method of co-infecting a cell with defective SV40
DNA and a complementing ts mutant. Both methods resulted in virus stocks enriched
with only one homogeneous defective virus population. This method has, however, the
limitation that mixed stocks of a defective virus population plus the complementing helper
virus are always obtained, although the virus DNA's can be separated by agarose gel
electrophoresis. Agarose electrophoresis of DNA, prepared from serial undiluted passages
of SV40, has enabled us to distinguish a limited number of size classes of DNA among the
heterogeneous population of defective DNA molecules.

METHODS

Cells and media. For serial passage of the virus, BSC-1 cells were grown in Kimble roller
bottles. Cultures were initiated in Eagle's BME with Hanks' salts and supplemented with
10 % calf serum and antibiotics (100 U of penicillin/ml and 100 µg of streptomycin/ml).
Cultures were infected at subconfluency. At that time one bottle contained 1 x 10⁷ cells in
100 ml of medium. Cultures were then changed to Eagle's BME with Earle's salts and 5 %
calf serum. All powder media were purchased from Gibco.
**Virus.** Two sets of serial undiluted passages of SV40 were established. Set A was initiated at a multiplicity of 5 p.f.u./cell and a virus stock was used which probably contained defective virus particles for it had been passaged undiluted for several years. The second set (B) was initiated at a multiplicity of 100 p.f.u./cell and the virus stock used did not contain a detectable amount of defective virus particles (see below). After each passage the virus was harvested when 75% of the cells showed c.p.e. After freezing at -20 °C and thawing, 4 ml of the crude lysate was inoculated into a new roller bottle, 80 ml of the lysate was used for DNA isolation and the remaining lysate was stored at -20 °C. Wild type SV40 (strain VA 54-45) was used throughout all experiments. All virus preparations were titrated in monolayer cultures of BSC-1 cells in 5 cm Falcon Petri dishes; 0.2 ml of the appropriate virus dilutions in PBS was inoculated on to each of duplicate cultures. After an adsorption period of 1 h at room temperature 5 ml of 0.9% agar medium was added. Neutral red stain was added on the 14th day after inoculation and the plaques were counted the next day.

**Preparation of the non-defective virus stock.** Virus DNA prepared from passage 1 of Set A was subjected to agarose gel electrophoresis. The slowest migrating component I band (see Results) was excised from the gel and the DNA was extracted from the gel and re-electrophoresed. No other DNA species could be seen in the gel. This DNA-containing band was excised, the DNA was extracted from the agarose and ethidium bromide was removed. After dialysis against MEM-tris (3 vol. MEM without bicarbonate and 1 vol. 0.2 M-tris, pH 7.4; final pH 7.30 to 7.35) the DNA was inoculated in serial tenfold dilutions into tube cultures of BSC-1 cells that had been pre-treated with DEAE-dextran (100 µg/tube). The highest dilution of the DNA that resulted in c.p.e. was frozen. The highest dilution was chosen to eliminate a possible contamination with incomplete SV40 DNA from the gel. The infectivity of this lysate was 5 x 10^6 p.f.u./ml. The virus was further propagated by inoculation of roller bottle cultures at an input multiplicity of 0.0002. After harvesting when 75% of the cells showed c.p.e., the lysate was clarified by low-speed sedimentation and titrated; the titre was 4.2 x 10^7 p.f.u./ml. The virus DNA extracted from this lysate was tested electrophoretically and only one component I band was visible. The infectivity of the DNA was 1.3 x 10^6 p.f.u./µg when tested with the DEAE dextran method. Before the virus DNA was added, the BSC-1 cells were treated for 15 min with DEAE-dextran (500 µg/dish) at room temperature (McCutchan & Pagano, 1968). We define this virus as complete SV40 and the virus DNA extracted from it as the complete SV40 DNA. This DNA was further used as standard DNA in all our experiments.

**Extraction and Purification of SV40 DNA.** The monolayer of infected BSC-1 cells was detached from the glass by freezing the medium and shaking during thawing of the medium. Virus and cells were spun down in the SW 27 rotor of a Beckman ultracentrifuge at 25000 rev/min for 2 h at 4 °C. Virus DNA was selectively extracted by the procedure of Hirt (1967); the pellet was suspended in 10 mM-Na-EDTA, pH 7.5 and sodium dodecyl sulphate (0.6%) and NaCl (1 M) was added. After storage overnight at 4 °C the lysate was centrifuged at 17000 g for 30 min at 4 °C. The supernatant fluid was mixed with CsCl to a density of 1.55 g/ml and ethidium bromide was added to a final concentration of 300 µg/ml. The mixture was centrifuged in the 50 rotor at 44000 rev/min for 40 h at 20 °C. The lower of the two visible bands was collected by puncturing the side of the tube. The ethidium bromide was removed by isoamyl alcohol and ether extraction. After dialysis against 10 mM-tris HCl, 1 mM-Na-EDTA, pH 7.5, the concentration of the DNA was determined in a Beckman spectrophotometer.

**Purification of the replicative form (RF) of φX 174 DNA.** For purification of φX 174 (RF) DNA the method of Jansz, Pouwels & Schiphorst (1966) was used with minor modifications.
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Instead of a Sephadex G-100 column we used a hydroxylapatite column. The φX 174 (RF) DNA containing fractions eluted from the column were still contaminated with Escherichia coli DNA. This contamination was removed by a second alkali denaturation step followed by hydroxylapatite chromatography.

PM2DNA was a gift of Mrs F. Fase-Fowler (Department of Medical Enzymology, Laboratory of Biochemistry, University of Amsterdam) and was prepared by the method of Espejo & Canello (1968).

Agarose gel electrophoresis. The preparation of the gels and the procedure for electrophoresis are essentially the same as described by Aay & Borst (1972). Ethidium bromide was added to gels and electrophoresis buffer (0.04 M-Tris, 0.02 M-sodium acetate, 0.002 M-EDTA adjusted with acetic acid to pH 7.6 at 20 °C) to a final concentration of 10 μg/ml. Electrophoresis was performed in a vertical tube apparatus (Poly analyst, Büchler) provided with a continuous buffer flow system. The quantity of DNA layered on the gels, the time of electrophoresis and the applied voltage are as described in the legends of the figures. The only difference from the original method of Aay and Borst is the use of a much lower voltage. After the run the DNA was visualized by illuminating the gels with an u.v. lamp (Sylvania Type F8T3-BLB) in a dark room. If photographs were to be taken the gels were first partially destained by soaking for 16 to 18 h in electrophoresis buffer without ethidium bromide. Photographs were taken with a Polaroid MP-3 land camera provided with a Kodak 23A filter, and Polaroid 4 × 5 land film type 55. Agarose was purchased from l'Industrie Biologique Française S.A. and from Biorad.

Preparation of DNA for electron microscopy. By irradiation with white light in the presence of ethidium bromide, about 70 % of component I of SV40, φX 174 (RF) and PM2DNA was converted to component II (Tai et al. 1972). After electrophoresis on agarose gels the component II bands were cut out. To isolate the DNA from the agarose we used the method developed in this laboratory by O. Zanen-Lim & C. Walig (unpublished experiments). The gel slice was sandwiched between two pieces of parafilm (American Can Company) and this was enclosed between two pieces of solid carbon dioxide. After 1 min the carbon dioxide was removed and the frozen gel slice between the parafilm was squeezed between finger and thumb. This, together with the thawing of the slice, resulted in the separation of buffer and DNA from the gel matrix. The ethidium bromide was removed by extraction with isoamyl alcohol.

Electron microscopy. Mixtures of the open circular forms of SV40 DNA and marker DNA (φX 174 (RF) DNA or PM2DNA) were spread by the aqueous method of Davis, Simon & Davidson (1971). The carbon-filmed copper grids were shadowed with platinum at an angle of 9° while rotating. The grids were examined in a Philips EM-300 electron microscope at 60 kV. Micrographs were taken at a magnification of 23,300 on a Kodak Eastman 5302 film. The negatives were enlarged 10 times and traced with a pencil on paper. The tracings were measured with a Hewlett Packard calculator and digitizer Model 9107 A.

RESULTS

Infectivity during serial passage

Two sets of undiluted passage of SV40 were established. Passage 1 (P1) of set A was inoculated with virus that probably contained defective virus because it had been passed undiluted for several years. The multiplicity of infection in the first passage was 5 p.f.u./cell. Each of the succeeding passages was infected with 4 ml of the frozen-thawed lysate of the preceding culture. Passage 1 (P1) of set B was inoculated at input multiplicity of 100 p.f.u./cell.
Table I. Infectivity of two sets of propagated undiluted SV4o

<table>
<thead>
<tr>
<th>Passage</th>
<th>Set A p.f.u./ml</th>
<th>Input multiplicity</th>
<th>Set B p.f.u./ml</th>
<th>Input multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0 x 10^6</td>
<td>5 x 10^8</td>
<td>4.2 x 10^7</td>
<td>1 x 10^2</td>
</tr>
<tr>
<td>2</td>
<td>9.0 x 10^4</td>
<td>8.0 x 10^-1</td>
<td>1.6 x 10^7</td>
<td>1.6 x 10^1</td>
</tr>
<tr>
<td>3</td>
<td>1.5 x 10^6</td>
<td>3.6 x 10^-2</td>
<td>3.8 x 10^6</td>
<td>6.0 x 10^6</td>
</tr>
<tr>
<td>4</td>
<td>4.0 x 10^4</td>
<td>6.0 x 10^-3</td>
<td>7.0 x 10^5</td>
<td>1.4 x 10^9</td>
</tr>
<tr>
<td>5</td>
<td>6.0 x 10^4</td>
<td>1.6 x 10^-3</td>
<td>1.8 x 10^4</td>
<td>2.8 x 10^-1</td>
</tr>
<tr>
<td>6</td>
<td>2.0 x 10^3</td>
<td>2.4 x 10^-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>5.0 x 10^4</td>
<td>8.0 x 10^-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2.0 x 10^5</td>
<td>2.0 x 10^-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.0 x 10^5</td>
<td>8.0 x 10^-3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

with virus containing no detectable amount of defective DNA (see Methods). Each of the succeeding passages in this set was also infected with 4 ml of the frozen-thawed lysate of the preceding culture.

Infectious virus content was determined in all passages of both series. These results, and the input multiplicity for each passage calculated from these data are summarized in Table I. For both sets a decrease in infectivity was found during the first few passages. In set A this continued up to the sixth passage and in the seventh passage there was a 200-fold rise in titre which again was followed by a decrease in titre in the eight and ninth passage. It was noted that the decline in infectivity was accompanied by a reduced yield of virus DNA in the first six passages. The yield of virus DNA after passage six was approx. 5% of the yield of passage one. From the lysate of passage seven an amount of virus DNA was isolated which was equivalent to that of passage one. In the following two passages there was again a decrease in the yield of virus DNA. For set B there was also a decrease in infectivity with increasing passage number and this was also accompanied by a lower yield of virus DNA.

Characterization of the virus DNA by agarose gel electrophoresis

The virus-sized DNA isolated from the serial passages of set A was analysed by agarose gel electrophoresis. The electropherograms (Fig. 1a) show that the DNA migrates as several discrete bands. Electron microscopy of the DNA extracted from the gel showed that the fast migrating bands between 5 and 7 cm from the starting point of migration contained covalently closed superhelical DNA (component I) and the DNA in the region between 1.5 and 3 cm consisted of open circular DNA (component II). The band present at 1.25 cm contained linear DNA with a length of more than 2 μm probably representing cellular DNA. Gel a shows the result of the electrophoresis of SV4o DNA which was obtained from cells infected at a low input multiplicity (0.002). Only one component I band was visible and we have defined this band as the complete SV4o genome (see Methods). The electropherograms of the DNA samples of the passages of set A showed several bands migrating faster through the gel than the band of the complete genome. This band pattern was not a result of differences in superhelical density because the same pattern was observed with component II DNA. The band pattern is not due to a varying amount of protein bound to the DNA as was shown by pronase incubation and by chromatography on methylated albumin kieselguhr (MAK) of the DNA before electrophoresis: no change was observed in band pattern (results not shown).

It will be shown below that the separation of the DNA into distinct bands was due to
Gel electrophoresis of defective SV40 DNA

Fig. 1. (a) Electrophoresis of SV40 DNA through 1.5% agarose gels. Gels b to j were loaded with 1.5 to 2 μg DNA isolated from passage 1 up to passage 9 from the first set of undiluted passaged SV40 virus (set A). Gel a was loaded with 1 μg SV40 DNA isolated after a low multiplicity infection (0.002). (b) Gels after electrophoresis of 10 up to 100 ng complete SV40 DNA through 1.5% agarose gels. Electrophoresis was initiated at 2 V/cm gel and after 0.5 h continued with 1 V/cm gel during 40 h.

differences in size and therefore we believe that these faster migrating bands contain defective virus DNA species. The percentage of complete virus DNA, represented by the slowest migrating component I band, gradually decreased during passaging, and after the fourth passage it could not be seen in the gel. Approx. 2 μg DNA was loaded on the gel and since a band containing 20 ng DNA is visible (see Fig. 1b), this indicates that in P4, P5 and P6 less than 1% of the virus DNA is of complete size. In the seventh passage the band of
Fig. 2. Gels b to f show electrophoresis through 2% agarose of 1 to 1.5 μg SV40 DNA isolated from passage 1 up to passage 5 of the second set of undiluted passaged SV40 virus (set B). Gel a was loaded with 1 μg SV40 DNA isolated from the standard virus stock that was used to infect the first passage. Electrophoresis was initiated at 2 V/cm gel and after 0.5 h continued with 1 V/cm gel during 40 h.

Fig. 3. Co-electrophoresis of SV40 DNA from passage 9 of set A with SV40 DNA from passage 3 of set B through 2% agarose gels. Prior to electrophoresis about 70% of component I DNA was converted to component II form. Component I DNA had run off the gel. Gel a was loaded with 0.5 μg DNA of passage 9 of set A. Gel b was loaded with 0.5 μg DNA of passage 9 of set A and 0.25 μg DNA of passage 3 of set B. Gel c was loaded with 0.5 μg DNA of passage 9 of set A and 0.5 μg DNA of passage 3 of set B. Electrophoresis was initiated at 2 V/cm gel and after 0.5 h continued with 0.7 V/cm gel during 115 h.
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Fig. 4. Schematic representation of the various size classes present in both sets of undiluted passaged SV40 virus (set A and set B). The representation of set A plus set B mimics gel c from Fig. 3. Band A6 in set A is represented hatched, because this band is only present in the first three passages of set A.

The different classes of defective SV40 DNA are stable during serial undiluted passage with respect to their size, suggesting their presence in the inoculum derived from the preceding passage. Most likely the inoculum used for the first passage in set A already contained the observed size classes.

A second set (B) of serial undiluted passage was initiated to test whether selection for certain size classes exists. We started set B with a virus stock in which no defective virus could be detected by agarose gel electrophoresis of the DNA. Already in the first passage, which was inoculated at a multiplicity of 100 p.f.u./cell, defective virus DNA was present in a detectable amount. Fig. 2 shows the electropherograms of passages one to five. Again, distinct faster migrating bands of DNA were present. To test whether the same size classes had been generated in both sets we co-electrophoresed the DNA of set A and set B (Fig. 3). The bands were named in numerical order from the place they occupy with respect to the place of the complete SV40, as is graphically presented in Fig. 4. The character before the number refers to the set. Bands A2 and A3 were present in all passages of set A in a very low concentration. Band A6 was only present in a visible amount in the first three passages.

complete genome reappears with strong intensity and comprises as much as 90% of the virus DNA. In the eighth and ninth passage again a decrease in the relative amount of the complete SV40 genome can be seen.

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Table 2. Ratios between the measured lengths of a marker DNA and complete or defective SV40 DNA

<table>
<thead>
<tr>
<th>Mixture of DNA molecules</th>
<th>Number of pairs</th>
<th>Length ratio (mean ± s.e.*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM2, ϕX174 (RF)</td>
<td>18</td>
<td>1.873 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>1.890 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>1.892 ± 0.023</td>
</tr>
<tr>
<td></td>
<td>54†</td>
<td>1.885 ± 0.008</td>
</tr>
<tr>
<td>PM2, SV40</td>
<td>20</td>
<td>1.917 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>1.927 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.912 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.896 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>65‡</td>
<td>1.913 ± 0.005</td>
</tr>
<tr>
<td>PM2, A1</td>
<td>21</td>
<td>2.103 ± 0.013</td>
</tr>
<tr>
<td>ϕX174 (RF), A5</td>
<td>15</td>
<td>1.297 ± 0.007</td>
</tr>
<tr>
<td>ϕX174 (RF), A7</td>
<td>18</td>
<td>1.378 ± 0.011</td>
</tr>
</tbody>
</table>

* Two neighbouring molecules of different species formed a pair. Pairs with one or two molecules, whose measured length differed more than three standard deviations from the mean of the size class to which they apparently belonged, were excluded in the calculation of the ratio.

† There was no significant difference between the three separately determined length ratios of PM2 and ϕX174 (RF) DNA and therefore the data were pooled.

‡ There was no significant difference between the four separately determined length ratios of PM2 and SV40 and therefore the data were pooled.

Fig. 5. Histograms of the measured contour lengths of complete SV40 DNA and the defective SV40 DNA classes A1, A5 and A7. As internal standards were used PM2 DNA or ϕX174 (RF) DNA. Correlation of all results was obtained by measuring the two internal standards together on one grid. (a) PM2 and ϕX174 (RF) DNA; (b) PM2 and SV40 DNA; (c) PM2 and A1 DNA; (d) ϕX174 (RF) and A5 DNA; (e) ϕX174 (RF) and A7 DNA. The black squares present measurements that differ more than three standard deviations from the mean and were excluded from the calculation of the length ratios presented in Table 2.
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Table 3. Mol. wt. of SV40 and the defective virus DNA

<table>
<thead>
<tr>
<th>DNA species</th>
<th>Mol. wt. in megadalton* (mean ± s.e.)</th>
<th>Percentage length of SV40 (mean ± s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40</td>
<td>3.35 ± 0.02</td>
<td>100</td>
</tr>
<tr>
<td>B1</td>
<td>3.21</td>
<td>95.8</td>
</tr>
<tr>
<td>A1</td>
<td>3.05 ± 0.02</td>
<td>91.0 ± 0.6</td>
</tr>
<tr>
<td>A2, B2</td>
<td>3.00</td>
<td>89.5</td>
</tr>
<tr>
<td>A3, B3</td>
<td>2.84</td>
<td>84.8</td>
</tr>
<tr>
<td>A4, B4</td>
<td>2.74</td>
<td>81.8</td>
</tr>
<tr>
<td>A5</td>
<td>2.62 ± 0.02</td>
<td>78.2 ± 0.6</td>
</tr>
<tr>
<td>B5</td>
<td>2.54</td>
<td>75.8</td>
</tr>
<tr>
<td>A7</td>
<td>2.47 ± 0.02</td>
<td>73.7 ± 0.7</td>
</tr>
<tr>
<td>B6</td>
<td>2.44</td>
<td>72.8</td>
</tr>
</tbody>
</table>

* Mol. wt. presented with a standard deviation were calculated from the results of electron microscopic length measurements presented in Table 2. The value of 3.40 megadalton for the internal standard ϕX 174 (RF) (Jansz & Pouwels, 1965) was used for the calculation. From Table 2 the length ratio between ϕX 174 (RF) and SV40 DNA is found to be 1.015 with a 95% confidence interval of 0.010. The mol. wt. presented without standard deviation are estimated from the electrophoretic mobilities (Fig. 7).

(Fig. 1). At least two size classes in each of the two sets were not present in such a quantity that it could be seen in the other series. These are the bands A1 and A5, B5 and B6. No difference in electrophoretic mobility between band B5 and A6 was detected.

Electron-microscopy length measurements

For a further characterization of the defective DNA, length measurements were performed. Besides the complete SV40 genome three defective classes of DNA were chosen. These were A1, A5 and A7. These classes became the dominant species upon further undiluted passaging in set A. Band A4 vanished and A2 and A3 remained present in a low concentration. The virus DNA component I was converted to component II by irradiation with white light (see Methods), and the DNA was separated into the several size classes by agarose electrophoresis. The component II bands were cut out and the ethidium was removed from the DNA after its extraction from the gel (see Methods). The DNA was mixed with approx. the same number of molecules of a marker DNA that had been treated in the same way. For the complete SV40 DNA and the DNA of band A1, the DNA of phage PM2 was used as a marker. For the DNA extracted from band A5 and A7 ϕX 174 (RF) DNA was used as a marker. A mixture of ϕX 174 (RF) DNA and PM2DNA was also measured. An approx. equal number of molecules of both DNA's present in a mixture were photographed and measured.

For the calculation of the mol. wt., pairs of molecules that were in close vicinity on the grid were chosen and the ratio between their measured length was calculated. From a number of pairs the mean ratio was then calculated. By applying this method, we believe that we diminish the influence of the carbon film, which is not of uniform quality on one grid. The method resulted in a smaller standard deviation, compared with the method of random measured molecules, which is a demonstration of the validity of the method. The influence of different grids on length ratios was determined by preparing several grids with the same two sorts of molecules: from the data in Table 2 it was concluded that this effect is not significant and therefore the data were pooled. The results of the measurements of one grid with the mixtures PM2/SV40 DNA and PM2/ϕX 174 (RF) DNA are graphically presented in Fig. 5 together with the results of the defective SV40 DNA and the calculated mol. wt. are
Fig. 6. Co-electrophoresis of SV40 DNA from passage 9 of set A and φX 174 (RF) DNA through 2% agarose. Prior to electrophoresis about 70% of both DNA's were converted to the component II form. Component I DNA has run off the gel. Gel a was loaded with 1 μg SV40 DNA from passage 9 of set A. Gel b was loaded with 1 μg SV40 DNA from passage 9 of set A and 0.3 μg of φX 174 (RF) DNA. Electrophoresis was initiated at 2 V/cm gel and after 0.5 h continued with 0.7 V/cm gel during 11.5 h.

summarized in Table 3. As standard we took the value of $3.40 \times 10^6$ for φX 174 (RF) DNA (Jansz & Pouwels, 1965). The calculated mol. wt. of $6.37 \times 10^6$ for PM$_2$DNA is in good agreement with the value of $6.40 \times 10^6$ reported by Pettersson et al. (1973). According to the measurements φX 174 (RF) DNA is slightly longer than complete SV40 DNA; this is in agreement with the somewhat slower electrophoretic mobility as is shown in Fig. 6. The standard deviations of the lengths of the defective DNA's are of the same magnitude as
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Fig. 7. Electrophoretic mobilities of the component II form of SV40 DNA, A1 DNA, A5 DNA and A7 DNA through 2% agarose plotted against the natural logarithm of their mol. wt. The mobilities are shown relative to the mobility of SV40 DNA. The relative mobilities were calculated from the gel presented in Fig. 3b. The mol. wt. were determined by electron microscopy length measurements. The limits of the 95% confidence interval of these measurements are indicated by horizontal bars.

those for φX 174 (RF) DNA and complete SV40 DNA. This indicates also the homogeneity in size of these DNA's. The calculated mol. wt. of the DNA present in bands A1, A5 and A7 were 9, 22 and 26% less than the mol. wt. of the complete SV40. The mol. wt. of the remaining size classes were estimated from their electrophoretic mobility. Fig. 3b shows all size classes present in both sets. Complete SV40 and the variants A1, A5 and A7 were used as internal standards for the plot of the logarithm of the mol. wt. against electrophoretic mobility, as shown in Fig. 7. From this plot the mol. wt. of the remaining variants have been calculated. These results are depicted in Table 3.

DISCUSSION

Earlier-reports (Uchida, Watanabe & Kato, 1966) that serial undiluted passage of SV40 leads to a decrease in the production of infectious virus have been confirmed. Two independent sets of serial undiluted passages were established and in both sets a fall in infectivity occurred in the early passages. It was further demonstrated by Uchida et al. (1968) that this drop in infectivity was accompanied by an increased production of non-infectious defective virus. The defective virus contained shorter sized DNA as was shown by equilibrium density and sucrose gradient sedimentation of the virus or virus DNA and electron microscopic length measurements of the DNA (Yoshiike, 1968; Tai et al. 1972).

We have analysed the defective DNA by low-voltage agarose gel electrophoresis. The
DNA isolated from the undiluted passaged virus splits into several distinct component I bands. However, only one component I band could be detected after agarose gel electrophoresis of SV40 DNA isolated after a low multiplicity infection.

All the extra bands migrated faster than standard SV40 DNA and contained shorter DNA as was shown by electron microscopy length measurements. The length of the DNA in the extra bands varied between 96 and 73% of the length of complete SV40 DNA.

Two independent sets of undiluted passaging of SV40 virus were analysed by means of electrophoresis. The first set was started with a virus stock that probably already contained defective variants and the second was initiated with a virus stock containing no detectable defective virus. In the first set a total of seven extra bands could be detected during serial passage and in the second set six extra bands were found. From the DNA present in three of the extra bands obtained after electrophoresis of the DNA present in the passages of the first set we determined the length by electron microscopy. These lengths were 91.78 and 74% of complete SV40 DNA.

From the result of a co-electrophoresis of the DNA of both sets (Fig. 3) two conclusions can be drawn. The first concerns the sensitivity of the low-voltage agarose gel electrophoresis technique. Prior to electrophoresis the DNA was converted to the component II form to exclude differences in superhelix density that could influence the electrophoretic mobility. On co-electrophoresis a DNA band (B5) migrated between the bands A5 (78% of normal genome length) and A7 (74%). From the plot of the natural logarithm of the mol. wt. against the relative mobility, the length of this DNA was estimated to be 76% of complete SV40 DNA. Thus a difference of only 2% in length, representing about 100 base pairs, resulted in a clear band splitting. The second conclusion is that in the two sets of undiluted passaged virus both similar and different size classes of defective SV40 DNA are generated.

The DNA isolated from passage nine of set A which contained six classes of defective DNA was mixed with the DNA of passage three of set B which also contained six defective size classes. After co-electrophoresis nine defective size classes could be seen (Fig. 3). Thus at least three size classes were distinctly different, and the others had no detectable differences in electrophoretic mobility. It remains to be determined whether the observed similarities in size classes of virus DNA in both sets are merely a coincidence or a consequence of the mechanism by which they are formed.

The results depicted in Table 1 warrant a comment. The decrease in infectivity, accompanied by a lower yield of virus DNA, observed in set A and B and the relatively decreased amount of complete SV40 DNA in the passages of both set A and B is probably due to interference and to an advantage in the replication rate of defective SV40 compared to complete SV40. In set A this decrease in titre was followed by a temporary rise in infectivity accompanied by a higher virus DNA yield. The sudden rise of the infectivity in passage seven of set A and the reappearance of a strong complete SV40 DNA band in the electropherogram (Fig. 1) probably occurred because the yield of defective and complete SV40 in passage six was very low. In passage seven the chance of double infection of a cell with complete and defective SV40 was very low and nearly all the defectives were lost since complete SV40 is in all likelihood required as a helper. This phenomenon, which is known for other viruses (Huang, 1973), was not observed again in later passages of set A or set B. We assume that an equilibrium has been reached based on a complicated interaction between the factors described.

Under the conditions used, nine distinct size classes of SV40 DNA have been found after serial undiluted passage of wild-type SV40. Restriction enzyme analysis of the DNA of some size classes present in one of the sets, suggests a genetic homogeneity. The agarose gel
Gel electrophoresis of defective SV40 DNA

electrophoresis technique could therefore serve as a method for the purification of a great number of defective variants of SV40.

Our present investigation on the presence of host cell DNA in the variants may lead to a better understanding of the mechanisms by which the defectives are formed. By determining the biological properties of the variants a better insight into the mechanism of transformation by SV40 may be obtained and experiments along these lines are in progress.

We thank Ine Hassing for her excellent technical assistance. We are also grateful to Dr W. Voorn for his statistical compilation of the electron microscopic length measurements. We are indebted to R. van Dillen for his help with the electron microscopy. The investigations were supported (in part) by the Foundation for Medical Research (FUNGO) which is subsidized by the Netherlands Organization for the Advancement of pure Research (Z.W.O.).

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(Received 21 March 1975)