TEMPERATURE SENSITIVITY OF POLYOMA VIRUS, INDUCTION OF CELLULAR DNA SYNTHESIS, AND MULTIPLICATION OF TRANSFORMED CELLS AT HIGH TEMPERATURE

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Temperature-sensitive markers can be of value in order to elucidate the mechanism of virus-induced cell transformation, and to demonstrate the expression of virus-coded genes in transformed cells. It has been shown in previous studies with large and small plaque strains of polyoma virus (PV) that a small plaque mutant of PV is temperature-sensitive for virus replication at high temperature. Experiments on the time of the temperature-sensitive event suggested that this corresponded to the time of virus DNA synthesis. The present experiments were undertaken: (1) to obtain direct evidence on whether the temperature-sensitive step in the PV mutant involves a block in the synthesis of virus DNA, (2) to determine whether the temperature-sensitive mutant can induce the synthesis of cellular DNA at the high temperature, and if so (3) whether cells transformed by PV (and by simian virus 40) can be differentiated from normal cells and cells transformed by nonviral carcinogens by their ability to replicate at the high temperature.

Materials and Methods.—Isotope: H-thymidine (16,000-18,000 mc/mM) was obtained from the Radiochemical Centre, Amersham, England.

Cell cultures: Primary and secondary mouse embryo cultures used for plaque assays were grown in 0.5% lactalbumin hydrolyzate in Earle's saline (LA) with 10% horse serum. Primary mouse kidney cultures and secondary mouse embryo cultures used for experiments on virus replication were grown in Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) with 5% calf serum, and all other cells were grown in EM with 10% calf serum. Clones of hamster and rat cells transformed by the small plaque PV mutant SP2 and the large plaque PV strain LP11 (previously referred to as 1L11) were isolated from soft agar after infection of secondary embryo cells with 100 or 1000 plaque-forming units (PFU) per cell. A line of PV and a line of SV40-transformed 3T3 cells were kindly supplied by Drs. H. Green and G. J. Todaro, and a line of SV40-transformed hamster cells was obtained from Flow Laboratories. The two hamster cell lines transformed by nonviral carcinogens were obtained after transformation of hamster embryo cells by 3-methylcholanthrene, and by X irradiation with 300 R.

Virus: Stocks of the small plaque SP2 and the large plaque LP11 strain of PV were prepared from single plaques after three successive single plaque isolations. The virus stocks were used as lysates or purified as described previously.

Plaque and infectious DNA assay: Plaque assays were made on 2-day-old secondary mouse embryo monolayers in LA with 10% horse serum, and the plaques were finally counted on day 15. DNA was extracted and the infectious DNA assayed as described elsewhere. The cultures were incubated at 29°C for 30 min after addition of the DNA, and about 17 hr later 10e secondary mouse embryo cells were added to each plate before the addition of agar as in the usual plaque assay.

Hemagglutination: The hemagglutination test was carried out as previously described in 0.01 M Tris-saline buffer, pH 7.2. Samples were heated for 30 min at 56°C before the test.

Incubation at 41 and 41.5°C: In order to obtain better regulation of the temperature than that obtained in an incubator, cells were incubated in closed 125-ml conical beakers (Corning Works, catalogue no. 1080) in water baths whose temperature was maintained with a Thermomix II (Braun). The tops of the conical beakers were made circular, so that they could be
readily closed with stoppers. The cells were seeded and grown at 37°C before transfer to the high temperature.

**Infection of cultures:** Two-day-old secondary mouse embryo cultures or primary mouse kidney cultures were infected and the virus was adsorbed (2 hr) at 37°C before transfer to the high temperature. In the experiments on virus replication, the cultures were washed three times with medium after virus adsorption, and new medium was then added. In the experiments on the induction of cellular DNA synthesis, the cultures were not washed after virus adsorption, and the medium that was added after virus adsorption had previously been in contact with cultures of the same cells for 2 days. Controls were mock infected and maintained in the same medium.

**Rate of DNA synthesis and autoradiography:** To determine the rate of DNA synthesis, labeled thymidine was added to the culture medium in the specified amounts for the specified time periods, and the incorporation of radioactivity into DNA was determined as in previous experiments. The autoradiography of cells labeled with H³-thymidine was also carried out as in previous experiments.

**Chromatography of DNA:** H³-thymidine-labeled DNA from infected cells was extracted with sodium dodecyl sulfate-phenol, dialyzed, and then fractionated on a three-layered methylated albumin kieselgur (MAK) column with a linear elution gradient of 0.5-0.9 M NaCl and with the addition of a C³-thymidine-labeled virus DNA marker, as described. The extracts were fractionated either without or with prior heat treatment (boiling for 10 min in 0.015 M NaCl, 0.015 M Na-citrate, followed by rapid cooling in ice water).

**Results.—** **Virus multiplication at 37 and 41.5°C:** It has previously been concluded from experiments with the temperature control available in an incubator, that the small plaque PV mutant SP2 is temperature-sensitive for virus replication at 42°C. Results obtained with the more accurate temperature regulation in a water bath have indicated that at 41.5°C there was the same yield of LP11 as at 37°C, and 98-99.7 per cent inhibition of SP2 as measured by plaque assay and 100 per cent inhibition as measured by hemagglutination (Tables 1 and 2). Incubation in a water bath at 42°C gave some inhibition of LP11, and incubation at 41°C some growth of SP2.

**Inhibition of infectious DNA synthesis:** In order to determine whether the temperature-sensitive step in SP2 replication involves a block in the synthesis of DNA present at 2 hr p.i. and infectious DNA present at 4 hr p.i. for SP2 and 9.1 X 10⁴ for LP11.

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**TABLE 1**

<table>
<thead>
<tr>
<th>Virus assayed as</th>
<th>Virus strain</th>
<th>2 Hr p.i.</th>
<th>37°C</th>
<th>40 Hr p.i.</th>
<th>41.5°C</th>
<th>41.5°C (%)</th>
<th>Yield at 41.5°C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFU/ml</td>
<td>SP2</td>
<td>2.1 X 10⁶</td>
<td>1.1 X 10⁸</td>
<td>2.2 X 10⁸</td>
<td>2.0</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>LP11</td>
<td>5.4 X 10⁶</td>
<td>1.6 X 10⁷</td>
<td>1.6 X 10⁷</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemagglutination units</td>
<td>SP2</td>
<td>4</td>
<td>252</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LP11</td>
<td>2</td>
<td>1022</td>
<td>1022</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Contact-inhibited mouse kidney cultures were infected at a virus:cell ratio of 100 PFU/cell. Virus present at 2 hr p.i. has been subtracted from the yield at 40 hr p.i.; PFU/ml = PFU per ml of tissue culture fluid; p.i. = post infection.

**TABLE 2**

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>PFU/ml</th>
<th>DNA PFU/ml</th>
<th>PFU/ml</th>
<th>DNA PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP2</td>
<td>2.3 X 10⁶</td>
<td>1.5 X 10³</td>
<td>6.1 X 10⁷</td>
<td>1.7 X 10⁴</td>
</tr>
<tr>
<td>LP11</td>
<td>3.7 X 10⁶</td>
<td>3.0 X 10³</td>
<td>1.0 X 10⁷</td>
<td>1.1 X 10⁴</td>
</tr>
</tbody>
</table>

Mouse embryo cultures were infected at a virus:cell ratio of 100 PFU/cell. The amount of virus and infectious DNA present at 2 hr p.i. has been subtracted from the yield at 40 hr p.i. The efficiency of the infectious DNA assay at 37°C was 3.5 X 10⁴ for SP2 and 9.1 X 10⁴ for LP11.
virus DNA, infected cells were tested for infectious DNA at 40 hours post infection (p.i.). The results of these experiments have indicated that the synthesis of infectious DNA by SP2 is blocked at the high temperature (Table 2).

**Induction of cellular DNA synthesis:** Contact-inhibited mouse kidney cultures were used to determine whether there can be an induction of cellular DNA synthesis at 41.5°C. Results on the rate of DNA synthesis after infection with SP2 and LP11 have indicated that both strains of PV can induce the synthesis of DNA at the high temperature, and that there was a peak of synthesis at 24 hours p.i. (Table 3). Determinations on the per cent of labeled cells have also shown an induction of DNA synthesis at the high temperature (Table 4). The per cent of labeled cells at 41.5°C after infection with SP2 was similar to the per cent of labeled cells at 37°C (Table 4). Chromatography of the nucleic acid extract from SP2-infected cells labeled from 23 to 25 hours p.i. has indicated that the induced DNA synthesized was cellular (Fig. 1).

Incubation of noninfected cultures of normal cells at 41.5°C resulted in a rapid inhibition of DNA synthesis. When sparse cultures of normal mouse embryo cells were incubated at 41.5°C, DNA synthesis was inhibited by 78 and 93 per cent after two and four hours, respectively. In experiments with contact-inhibited mouse kidney cultures, the noninfected control cultures incubated at 41.5°C for 24 hours showed about a 75 per cent reduction in their DNA synthesis compared to similar cultures incubated at 37°C. Cells in contact-inhibited kidney cultures at 41.5°C thus presumably contained both the contact inhibition and the high temperature blocks for DNA synthesis.

**Multiplication of nontransformed and transformed cells at 41°C:** Since the above results have shown that DNA synthesis of normal cells was inhibited at 41.5°C and that PV can induce the synthesis of cellular DNA at 41.5°C, the question arose whether cells transformed by PV and by SV40 can multiply at the high temperature. The results of a preliminary experiment indicated that, although cultures of nontransformed 3T3 cells showed a decrease in cell number after two days at 41.5°C, the PV-transformed 3T3 cultures showed an increase equivalent to less than one cell division after two days at this temperature, and there was no increase in cell number in cultures of the SV40-transformed 3T3 cells. However, when the cultures were tested at 41 instead of 41.5°C, multiplication of the nontransformed 3T3 was still inhibited, and both transformed cell lines showed a considerable increase in cell number after two days. Subsequent experiments on cell multipli-
Fig. 1.—MAK column chromatography of DNA extracted from SP2-infected mouse kidney cells. The infected cultures were labeled from 23 to 25 hr p.i. with 10 μc H3-thymidine (±0.45 μg unlabeled thymidine). (A) With heating. An aliquot of H3-labeled infected cell DNA was mixed with C14-labeled PV marker DNA and boiled for 10 min. After quick cooling in an ice bath, the mixture was chromatographed on a MAK column as described in Methods. The recoveries were H3, 2.5%; and C14, 80%. ○ = H3; ● = C14. The recovery of a small per cent of heated H3-labeled DNA as seen in (A) has also been obtained after heating of DNA extracted from cultures of normal uninfected mouse cells. (B) Without heating. The same mixture as used in (A) was chromatographed without boiling. The recoveries were H3, 78%; and C14, 100%. ○ = H3; ● = C14.

cation at high temperature were therefore carried out at 41°C. The results of these experiments have indicated that all the nontransformed cells tested and two cell lines transformed by 3-methylcholanthrene and by X irradiation were inhibited from multiplying at 41°C, whereas cells transformed by both strains of PV and by SV40 were able to multiply at the high temperature (Fig. 2, Table 5).

Discussion.—The present experiments have provided direct evidence that the temperature-sensitive step in the replication of the small plaque PV mutant SP2 at high temperature involves a block in the synthesis of virus DNA. This indicates that there is a virus-coded gene product required for virus DNA synthesis. It will be of interest to determine the nature of this gene product, presumably an enzyme, and whether SP2 can synthesize at the high temperature some form of
virus DNA that is not detected by the infectious DNA assay or by chromatography on the MAK column.

The results have indicated that the temperature-sensitive mutant can overcome the block in normal cellular DNA synthesis that occurs at high temperature. This is another block in cellular DNA synthesis that can be overcome by PV infection, in addition to the block produced by contact inhibition\textsuperscript{10–13, 15, 16} and X irradiation.\textsuperscript{11, 13} The induction of cellular DNA synthesis also appears to be due to a virus-coded gene product.\textsuperscript{11} The results on multiplication of the virus trans-

\begin{table}
\centering
\caption{Multiplication at 37 and 41\textdegree C of Nontransformed Cells and of Cells Transformed by Polyoma, SV40, and Nonviral Carcinogens}
\begin{tabular}{|c|c|c|c|}
\hline
Cell type & Ham. normal & Ham. XR & Ham. MC & Ham. SP2 clone 1 & Ham. SP2 clone 2 & Ham. SP2 clone 3 & Ham. LP11 clone 1 & Ham. SV40 & 3T3 not transformed & 3T3 SV40 & 3T3 PV & 3T3 PV & Rat normal & Rat SP2 clone 1 \\
& 0 time & 2 days at 41\textdegree C & 2 days at 37\textdegree C & 0 time & 2 days at 41\textdegree C & 2 days at 37\textdegree C & 0 time & 2 days at 41\textdegree C & 2 days at 37\textdegree C & 0 time & 2 days at 41\textdegree C & 2 days at 37\textdegree C & 0 time & 2 days at 41\textdegree C & 2 days at 37\textdegree C \\
\hline
Ham. normal & 1.1 & 1.3 & 4.5 & 1.2 & 1.2 & 4.7 & 1.0 & 1.0 & 5.2 & 1.2 & 7.0 & 9.5 & 0.9 & 7.0 & 8.2 \\
Ham. XR & 1.2 & 1.2 & 4.7 & 1.0 & 1.0 & 5.2 & 1.2 & 7.0 & 9.5 & 1.2 & 7.0 & 9.5 & 1.2 & 7.0 & 9.5 \\
Ham. MC & 1.0 & 1.0 & 5.2 & 1.2 & 7.0 & 9.5 & 0.9 & 7.0 & 8.2 & 1.2 & 7.0 & 9.5 & 1.2 & 7.0 & 9.5 \\
Ham. SP2 clone 1 & 1.2 & 5.0 & 8.5 & 1.2 & 5.0 & 8.5 & 1.3 & 7.2 & 7.5 & 1.2 & 7.0 & 10.0 & 1.2 & 7.0 & 10.0 \\
Ham. SP2 clone 2 & 0.9 & 7.0 & 8.2 & 1.2 & 7.0 & 9.5 & 0.9 & 7.0 & 8.2 & 1.2 & 7.0 & 9.5 & 0.9 & 7.0 & 8.2 \\
Ham. SP2 clone 3 & 1.2 & 5.0 & 8.5 & 1.3 & 7.2 & 7.5 & 1.2 & 7.0 & 9.5 & 1.2 & 7.0 & 9.5 & 1.2 & 7.0 & 9.5 \\
Ham. LP11 clone 1 & 1.3 & 7.2 & 7.5 & 1.3 & 7.2 & 7.5 & 1.3 & 7.2 & 7.5 & 1.3 & 7.2 & 7.5 & 1.3 & 7.2 & 7.5 \\
Ham. SV40 & 1.2 & 7.0 & 10.0 & 1.2 & 7.0 & 10.0 & 1.2 & 7.0 & 10.0 & 1.2 & 7.0 & 10.0 & 1.2 & 7.0 & 10.0 \\
3T3 not transformed & 0.4 & 0.2 & 1.0 & 0.4 & 0.2 & 1.0 & 0.4 & 0.2 & 1.0 & 0.4 & 0.2 & 1.0 & 0.4 & 0.2 & 1.0 \\
3T3 SV40 & 0.4 & 1.5 & 1.8 & 0.4 & 1.5 & 1.8 & 0.4 & 1.5 & 1.8 & 0.4 & 1.5 & 1.8 & 0.4 & 1.5 & 1.8 \\
3T3 PV & 0.5 & 2.3 & 3.0 & 0.5 & 2.3 & 3.0 & 0.5 & 2.3 & 3.0 & 0.5 & 2.3 & 3.0 & 0.5 & 2.3 & 3.0 \\
3T3 PV & 1.6 & 4.6 & 7.2 & 1.6 & 4.6 & 7.2 & 1.6 & 4.6 & 7.2 & 1.6 & 4.6 & 7.2 & 1.6 & 4.6 & 7.2 \\
Rat normal & 1.6 & 1.3 & 3.6 & 1.6 & 1.3 & 3.6 & 1.6 & 1.3 & 3.6 & 1.6 & 1.3 & 3.6 & 1.6 & 1.3 & 3.6 \\
Rat SP2 clone 1 & 1.5 & 9.0 & 12.0 & 1.5 & 9.0 & 12.0 & 1.5 & 9.0 & 12.0 & 1.5 & 9.0 & 12.0 & 1.5 & 9.0 & 12.0 \\
\hline
\end{tabular}
\end{table}

Cells were seeded and grown for 20 hr at 37\textdegree C (0 time), and then incubated at 41 or 37\textdegree C for 2 days. The hamster PV-transformed clones were tested at 12–33 passages after transformation, and the other transformed cells after they had been in culture for many months. Ham. and rat normal = secondary embryo cultures. Cells transformed by: XR = X irradiation; MC = 3-methylcholanthrene; SP2 = small plaque PV; LP11 = large plaque PV.

**Fig. 2.—** Multiplication of SP2-transformed clone 2 (A), LP11-transformed clone 1 (B), and normal hamster embryo cells (C), at 37 and 41\textdegree C. Cells were seeded and grown for 20 hr at 37\textdegree C (0 time), and then incubated at 37 or 41\textdegree C. $\circ = 37\textdegree C$; $\bullet = 41\textdegree C$. 

No. of days at 37$^\circ$C or 41$^\circ$C 

No. of cells per culture ($\times 10^6$) 

0 1 2 0 1 2 

0 1 2 0 1 2 

0 1 2 0 1 2 

A B C
formed cells at 41°C therefore suggest that the virus-coded gene that induces the synthesis of cellular DNA at high temperature is expressed in cells transformed by PV. Since the temperature-sensitive step in SP2 replication involves a block in the synthesis of virus DNA and the virus-transformed cells can synthesize cellular DNA at the high temperature, experiments are in progress to determine whether the culture of transformed cells at the high temperature can result in the reversion of transformed cells to normal cells.

The results obtained with SV40-transformed cells suggest that SV40 also has a virus-coded gene for the induction of cellular DNA synthesis at high temperature that is expressed in transformed cells. It will be of interest to determine whether this applies to cells transformed by other viruses.

Summary.—A small plaque mutant of polyoma virus (PV) is temperature-sensitive for virus replication at 41.5°C. It has been shown that the temperature-sensitive step in virus replication involves a block in the synthesis of virus DNA. Infection of normal cells with the temperature-sensitive mutant can induce the synthesis of cellular DNA at the high temperature. Cells transformed by PV (and by simian virus 40) are able to multiply at 41°C whereas the normal cells and cells transformed by nonviral carcinogens that have been tested, are inhibited from multiplying at this temperature. The results indicate that a virus-coded gene is required for the synthesis of virus DNA, and that a virus-coded gene that induces the synthesis of cellular DNA at the high temperature is expressed in transformed cells.

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