A Specific Inhibitor of Polyoma Virus in Infected Rat and Hamster Cells and in Transformed Clones of Hamster Cells

By R. CRAMER AND H. YOSHIKURA*
Institut du Radium-Biologie, Orsay, Essonne
AND G. MEYER
Centre Régional de Lutte contre le Cancer – Marseille, France

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SUMMARY

Extracts of polyoma virus infected hamster cells made 8 to 10 hr after infection stimulated polyoma virus production in permissive mouse cells, whereas extracts made from 12 hr onwards inhibited virus production.

No such inhibitor was found in mouse embryo cells within 22 hr of infection under the same conditions. A genetic study of a mouse-hamster-hybrid cell line showed that non-permissiveness was not a dominant trait and seemed to depend on the inhibitor which could be expressed only in non-permissive cell lines. A specific inhibitor was found also in clones of hamster cells transformed by polyoma virus in vitro and in a clone of transformed hamster cells derived from a tumour induced by virus in vivo. This inhibitor did not reduce DNA synthesis or replication of non-infected permissive mouse cells. It reduced the number of infective centres and the yield of infective virus, infective DNA and of the polyoma DNA component I.

INTRODUCTION

A protein factor extracted from either SV40 virus—(Cassingena & Tournier, 1968; Suarez et al. 1971) or polyoma virus—transformed cells (Cramer, 1969) specifically inhibited the replication cycle of the corresponding viruses in permissive cells. Mouse cells are usually permissive for the replication cycle of polyoma virus in vitro, whereas hamster (Dulbecco & Vogt, 1960) and rat (Sachs & Medina, 1961) cells can be transformed specifically by this virus without formation of detectable progeny particles. As polyoma virus seems to penetrate mouse and hamster cells with equal efficiency (Fraser & Gharpure, 1962; Fraser & Crawford, 1965) the determination of the lytic or transformation cycle must occur at a later stage by a mechanism not yet understood. Cramer & Feinendegen (1966) have proposed that during the transformation cycle of polyoma virus in BHK21 hamster cells, virus replication may be limited by an autorepressor substance similar to that for phage P22 during establishment of lysogeny. This hypothesis is based on the observation that during the moderate virus–host interactions of either polyoma virus (Cramer & Feinendegen, 1966) or phage P22 (Smith & Levine, 1964), there is a transitory stimulation of thymidine incorporation. On the other hand, the moderate and lytic cycle of polyoma virus may be determined by a cellular factor, since the two interactions are observed in different host cells. Experiments have been made with polyoma virus to test these models (Cramer, 1969). An inhibitor was

* Present address: Department of Pathology National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo, Japan.
extracted from polyoma virus infected rat and hamster cells as well as from cells transformed by polyoma virus. The inducibility of the inhibitor in relation to permissiveness was also tested genetically in a hybrid cell-line obtained by fusion of cells from a permissive mouse cell line with those from a non-permissive hamster cell line. It was found that non-permissiveness was not dominant and may depend on the expression of the inhibitor. In this report on the mechanism of inhibition, previous results are extended and considered with new results.

METHODS

Viruses. A small plaque strain of polyoma virus was kindly provided by Dr M. Stoker. The virus was purified by treatment with fluorocarbon, differential centrifugation and equilibrium sedimentation in a CsCl density gradient (Cramer, Demerseman & Atanasiu, 1967). Haemagglutination was determined according to Cramer, Stewart & O'Connor (1963). Vesicular stomatitis virus was of the Indiana type and vaccinia virus of the Joklik strain.

Host cells. The cells used were as follows:—

- Hamster, BHK21 (MacPherson & Stoker, 1962); Melanoma 3460-3 (Davidson, Ephrussi & Yamamoto, 1968); Py/y (Jarret & MacPherson, 1968); STPy (Cesarini, 1971); MA40Cl2T4SV (Tournier et al. 1967); RS2Py4 (Montagnier, Meyer & Vigier, 1969); RB12 (Montagnier & Vigier, 1967).
- Mouse/embryo, L cells (Earle, 1943); LM(TK−)Cl1D (Kit et al. 1963).
- Human, HeLa cells (Gey, Coffman & Kubicek, 1952).
- Rat, Fibroblasts (De Maeyer-Guignard & De Maeyer, 1969).

Medium. The tris-buffered saline (TBS) solution, pH 7.6, contained MgCl2 6H2O, 0.1 g./l.; CaCl2, 0.1 g./l.; NaCl, 8 g./l.; KCl, 0.38 g./l.; Na2HPO4, 0.1 g./l.; SIGMA 7–9 tris (hydroxymethyl) aminomethane (99.0 to 99.5%), 3.0 g./l. Other components were DEAE dextran mol. wt 2 × 10^8 (Pharmacia) and thymidine-2[14C] of specific activity 30.4 mc/m-mole or [3H]-thymidine 15 c/m-mole or 9 c/m-mole (Amersham Radiochemical Centre, Buckinghamshire, England).

Induction of the inhibitor. Human, mouse, hamster or rat cells were infected by polyoma virus at 10 p.f.u./cell according to Cramer & Feinendegen (1966). The cells were then extracted after incubation for from 4 to 24 hr.

Extraction and purification of the inhibitor. Cell cultures in which the inhibitor was induced, as well as monolayers of normal and transformed cells at confluence, were washed with PBS, trypsinized and counted. The cell pellets obtained after centrifuging at low speed were resuspended in PBS and then centrifuged and again resuspended. The final cell pellets were resuspended at a concentration of 1 × 10^6 cells/ml. in Eagle's medium containing 10% horse serum. Cell suspensions together with Eagle's control medium were then chilled in dry ice and stored at −20° for 24 to 48 hr. The suspensions were then thawed and treated (2 × 2 min.) at maximum power in a magnetostrictive oscillator (Raytheon DF 101, 200 W at 10 kHz) at 4°. Disruption of the cells was checked microscopically. After ultrasonic treatment, the suspensions were centrifuged at 54,000 g for 3 hr using a type 30 rotor in a Spinco centrifuge. The supernatant fluids were the extracts for use in further experiments.

Titration of the inhibitor. Secondary cultures of mouse embryo cells in Falcon Petri dishes (60 × 15 mm.) were infected with polyoma virus (2 p.f.u./cell) previously treated with ultrasound for 3 min. at maximum power and heated at 56° for 30 min. Virus was adsorbed in volumes of 0.2 ml. for 60 min. at 37°. After withdrawal of the remaining virus suspension, the monolayers were washed 3 times with PBS and then 5 ml of the cell extract to be tested
A specific inhibitor of polyoma virus was added to 4 cultures. Also, 4 cultures were overlaid with an extract of control cells and 4 cultures with Eagle's medium only. After incubation at 37° for 36 to 48 hr the supernatant fluid was collected. The cells were removed by scraping with a rubber wedge and suspended in 3 ml. of nutrient medium containing 5% horse serum. Supernatant fluid and cell suspensions were sonicated for 3 min. and 4 min. respectively at maximum power and both were titrated by the plaque method (Winocour & Sachs, 1960) using 10 Petri dish cultures per sample. Alternatively, the inhibitor was titrated by determination of infective centres. Infected monolayers incubated for 8 to 10 hr with the extracts or control Eagle's medium were trypsinized and the infective centres determined as below.

Trypsin sensitivity of the inhibitor was determined according to the procedure of Cramer (1969).

Assay of polyoma DNA infectivity. The extract containing the virus DNA was boiled for 3 min. and assayed on secondary mouse embryo monolayers. The monolayers were washed twice with PBS and incubated for 15 min. at 37° with 0.1 ml. of a solution of DEAE-dextran (300 μg./ml. in TBS) before further incubation for 30 min. at 37° with 0.1 ml. of virus DNA diluted in TBS. Overlay medium (10 ml. at 1:3 strength) with 0.9% Difco Bacto Agar and 2.5% horse serum was added and the cultures were incubated at 37° for 13 days, when 3 ml. of overlay medium containing neutral red (0.008%) was added. The plaques were counted on the following day.

Action of inhibitor on replication of randomly growing cells. This was tested on secondary mouse embryo cell-cultures prepared by seeding 7 × 10⁵ cells in Petri dishes (6 cm.) and incubating in Eagle's medium with or without inhibitor. Cultures were trypsinized every 24 hr and the cells counted. Alternatively, non-confluent but established cell cultures were used in the same way.

Action of inhibitor on cell replication in synchronized cultures. The mouse fibroblast cell line C3H2K (Yoshikura, Hirokawa & Yamada, 1967) was used. These cells stop growing in phase-G1 at very low saturation density and at the renewal of medium all cells divide synchronously once only. The factor inducing the cell replication is contained in calf serum. To cultures arrested in G1 phase was added new medium with or without inhibitor and the synchronous division was tested by thymidine incorporation, cell replication and the appearance of mitotic cells.

Thymidine incorporation. After medium change cells were labelled for 48 hr with 0.4 μC/ml. of [3H]-thymidine (9 c/m-mole); incorporation was stopped by washing the cultures twice with ice-cold PBS. The cells were trypsinized at 4° for 2 min. and then suspended in 2.5 ml. of ice-cold PBS. An equal volume of 20% TCA was added to the cell suspension and, after 45 min. on ice, 1 ml. of suspension (1/5 of the cultured cells) was collected on a Millipore filter and washed twice with ice cold 10% TCA and once with 70% ethanol. The filters were dried and placed in bottles with 5 ml. of scintillation liquid (1 l. toluene, 4 g. PPO, 0.049 g. POPOP) and counted in a Packard scintillation counter.

Estimation of cell replication. The medium was discarded 48 hr after replacement and 3 ml. of a solution containing 2% citric acid and 1% methylene blue was added to each culture. After incubation at 37° for 30 min. the cells were detached from the glass surface and the suspension transferred to a tube. The dishes were washed with 2 ml. of the same solution and the solution transferred to the same tube. After centrifuging at 3000 rev./min. for 10 min., the volume of the solution was adjusted to 2 ml. by careful aspiration and the cells counted in a Bürker haemocytometer.

Estimation of mitotic activity. At intervals the refractile cells in mitosis in a constant area (0.2 × 5 cm.²) were counted with an inverted microscope.
Infective centre determination. Cells (2 x 10^6) were suspended in polyoma virus culture fluid treated with ultrasound for 3 min. at maximum power. After removal of particle debris by centrifuging at 15,000 g for 10 min. (type 40 rotor of the Spinco ultracentrifuge) the virus–cell suspension was heated for 30 min. at 56°C in a water bath and virus adsorption allowed to proceed with magnetic stirring for 3 hr. For inhibitor titrations, Eagle’s medium with or without inhibitor was added during the 3 hr incubation period. The cells were then centrifuged, washed 4 times in PBS containing 0.1% horse serum, and counted. A volume of 0.1 ml. nutrient medium with 100 cells was plated on to a confluent layer of mouse embryo cells in 10 Petri dishes. After 15 min., 0.4 ml. of 0.9% agar was layered over the cells.

Selective extraction of polyoma virus DNA by the Hirt (1967) method. Infected mouse embryo cell monolayers in Petri dishes were incubated with Eagle’s medium containing the inhibitor which had been partially purified as above. From 10 to 38 hr after infection, the cultures were labelled with thymidine-2[14C] at a concentration of 1 μCi/Petri dish. The medium was then withdrawn, and new medium free of radioactive thymidine was added. The cultures were incubated for another 3 hr to exhaust the intracellular pool of radioactive thymidine. The monolayers were then lysed by adding to each culture 1 ml. of 0.6% sodium dodecyl sulphate (SDS) solution at pH 7.5 containing 0.01 EDTA. After 30 min. at room temperature, the viscous lysate was freed from the Petri dish by gentle movement. The lysate was poured into a plastic centrifuge tube of 8 mm. diameter, and 5 M-NaCl was added to a final concentration of 1 M. After mixing by slow inversion the sample was stored at 4°C for at least 24 hr and centrifuged at 17,000 g for 30 min. at the same temperature. This treatment removed the major portion of SDS and protein as well as most cellular DNA. The polyoma DNA remained in the supernatant fluid (Hirt-supernatant fluid I).

Neutral CsCl velocity gradient. The supernatant fluid was tested by the CsCl band centrifugation method (Vinograd et al. 1968). A sample of 0.2 ml. of the Hirt-supernatant fluid I was layered on to 3 ml. of CsCl solution (ρ = 1.5 g./cm.3), covered with paraffin oil and centrifuged at 35,000 rev./min. for 3.5 hr at 25°C (Spinco Rotor SW 39). Two drops per fraction were collected from the bottom of the tube on to filter paper discs, and the radioactivity of the DNA was measured with a liquid scintillation counter.

Equilibrium sedimentation in CsCl ethidium bromide gradients. Closed superhelical DNA molecules can be separated from linear or nicked circular DNA molecules by equilibrium sedimentation in CsCl containing ethidium bromide (Radloff, Bauer & Vinograd, 1967). For this procedure CsCl was added (1 g./ml. of DNA solution) to the virus DNA solution (Hirt-supernatant fluid I) and the mixture centrifuged at 15,000 g for 20 min.; the surface layer of SDS and protein was removed and ethidium bromide was then added to the solution to a final concentration of 300 μg./ml. and the refractive index adjusted to 1.3900 ± 0.0010. The solution was then centrifuged (fixed-angle Spinco rotor 50.5 ml. per tube and filled to the cap with paraffin oil) for 48 to 60 hr at 41,000 rev./min. and 20°C. Fractions of 6 drops were collected from the bottom of the tubes into plastic tubes. One drop from each well mixed fraction was put on to filter paper discs, and the radioactivity of the DNA was measured with a liquid scintillation counter. Fractions corresponding to peak I (polyoma DNA component I and mitochondrial DNA I) to peak II (polyoma DNA II and III and linear DNA molecule of cellular origin) were harvested, as well as the bottom fractions containing most of the RNA. Ethidium-bromide was extracted either by three extractions with an equal volume of isopropanol, or by dialysis against 3 changes of 0.1 M-tris-HCl, pH 7.6, 0.005 M-EDTA for 12 hr. A solution of RNase A (Worthington) was prepared (4 mg. RNase/2 ml. buffer tris 0.01 M-EDTA 0.001 M, pH 7.6). The solution was boiled for 5 min. and 1 drop of 40 mg. RNase was added to the samples containing the peaks I and II.
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Fig. 1. Virus yield at 36 hr after infection of replicate mouse cell cultures incubated with extracts of BHK 21 cells prepared at different times after infection of the latter cells with polyoma virus. Control cultures were incubated in normal medium since stimulation of virus production was occasionally observed with extracts of non-infected BHK 21 cells.

of the CsCl-ethidium-bromide gradients. The samples were incubated for 1 hr at 37° and for a further hour at room temperature. The DNA concentration was measured by the ethidium-bromide-DNA fluorometric method (Le Pecq & Paoletti, 1966) with phage PM₂ DNA and calf thymus DNA as standards in a Zeiss spectrophotofluorometer. Excitation was obtained at a wavelength of 540 nm. and emission was recorded at 590 nm. The total amount of hypercoiled circular DNA was measured relatively since the quantitation by the ethidium-bromide-DNA fluorescence method depends on the binding constant of ethidium-bromide to DNA.

RESULTS

Specificity of the inhibitor

In established non-permissive cell lines of rat (De Maeyer-Guignard & De Maeyer, 1969) and hamster (BHK 21, Stoker, 1962) fibroblasts a specific inhibitor for the replication of polyoma virus appears 12 hr after infection (Table 1, Fig. 1). On the other hand no such inhibitor was induced within 22 hr of infection at the same multiplicity in either mouse embryo cells or different established lines of mouse cells or HeLa cells. An inhibitor of similar characteristics was found (Table 2) in hamster cell clones transformed either by polyoma virus alone or doubly transformed by polyoma and Rous sarcoma virus (Montagnier et al. 1969): an inhibition not found in clones of the same cells transformed by SV 40 virus (Tournier et al. 1967), or Rous sarcoma virus alone (Montagnier & Vigier, 1967) or in cells transformed spontaneously (Davidson, Ephrussi & Yamamoto, 1966). When a cell clone of
Table 1. *Inhibition of the replication of polyoma virus in mouse embryo cells by the inhibitor induced in cells of different species*

<table>
<thead>
<tr>
<th>Cell line as origin of extract</th>
<th>Yield as p.f.u./cell in cell phase</th>
<th>Yield as p.f.u./cell in supernatant fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With extract</td>
<td>Without extract</td>
</tr>
<tr>
<td>Hamster – BHK 21</td>
<td>580 ± 38</td>
<td>248 ± 236</td>
</tr>
<tr>
<td></td>
<td>(76 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>Rat established fibroblasts</td>
<td>6.1 ± 0.9</td>
<td>19.2 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>(69 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>Mouse LM(TK−)Cl1D</td>
<td>284 ± 9</td>
<td>276 ± 10</td>
</tr>
<tr>
<td></td>
<td>(0 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>Man – HeLa cells</td>
<td>388 ± 14</td>
<td>67.6 ± 6</td>
</tr>
</tbody>
</table>

Table 2. *Inhibition of the infectious cycle of polyoma virus in mouse embryo cells by the inhibitor extracted from normal and virus transformed cells*

<table>
<thead>
<tr>
<th>Hamster cell lines as origin of extract</th>
<th>Transforming agent</th>
<th>Yield as p.f.u./cell in cell phase</th>
<th>Yield as p.f.u./cell in supernatant fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With extract</td>
<td>Without extract</td>
</tr>
<tr>
<td>BHK 21</td>
<td>Not transformed</td>
<td>7.7 ± 0.7</td>
<td>7 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>BHK 21 PyY</td>
<td>Polyoma virus</td>
<td>97.2 ± 8</td>
<td>264 ± 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(64 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>STPy</td>
<td>Polyoma virus</td>
<td>7.3 ± 0.9</td>
<td>26.6 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(73 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>RS4 Py 4</td>
<td>Rous sarcoma virus (SCHMIDT-RUPPIN strain + polyoma virus)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(74 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>RB18</td>
<td>Rous sarcoma virus (BRYAN strain)</td>
<td>1.6 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>MA 40</td>
<td>SV40</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0 % inhibition)</td>
<td></td>
</tr>
<tr>
<td>CL2 TSVS</td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

thymidine kinase negative L-cells (Davidson et al. 1966) was infected with polyoma virus and maintained for 2 years by weekly trypsinization and cloning in soft agar, polyoma virus was still released but no inhibitor could be extracted. The inhibitor is therefore specific to virus since it can be extracted only from non-permissive cells which have been infected or transformed by polyoma virus. The inhibitor was found to have no action on the replication cycle of SV40, vaccinia of vesicular stomatitis virus. An interferon-like activity is unlikely since the inhibitor extracted from infected and transformed hamster-cells had no action on the replication of vaccinia or vesicular stomatitis virus, even when tested in homologous hamster cells.

**Kinetics of inhibitor induction in non-permissive rat and hamster cells infected with polyoma virus**

Inhibitor was extracted from rat and hamster cells at different times after infection and its action tested on one cycle of polyoma virus replication in permissive mouse cells. All
extracts from non-infected cell species so far tested showed either no or slight stimulation
of polyoma virus replication. An exceptionally high stimulation of sixfold was observed
with extracts from HeLa cells grown in suspension cultures. Extracts of infected BHK 21
cells made 8 to 10 hr after infection stimulated virus replication by two to four-fold (Fig. 1).
Extracts prepared 12 hr or more after infection showed inhibition of 45 to 90%. The inhibi-
tion depended on the multiplicity of infection (1 to 10) of the BHK 21 cells. Thus after
incubation for 16 hr, the cell extracts at a multiplicity of 10 showed 60 to 87% inhibition,
whereas at a multiplicity of 1 the inhibition was only 34 to 47%. The stimulation detected
8 to 10 hr after infection must have been generated by the infective process although its
nature is unknown.

Inhibition as function of number of extracted transformed cells

Fig. 2 shows that the inhibition increased with the number of cells extracted until a satu-
ration plateau was reached. Less inhibition was obtained with much higher cell concentrations.
The ratio of the number of inhibitor containing cells used for the extract to the number of
polyoma virus infected mouse cells to which the inhibitor extract was added is called the
cell/cell multiplicity. Optimal inhibition was obtained at cell/cell multiplicities of 2 to 3.
When the inhibition was tested at constant inhibitor concentrations and at multiplicities
of infection of 1 to 20, comparable inhibition levels of approximately 60% were recorded.

Inhibition as a function of time of addition of inhibitor

Inhibition was detected only when the inhibitor was added 1 or 2 hr after infection. No
effect was observed when the inhibitor was added from 3 to 15 hr after infection or added
24 hr before infection.

Physico-chemical characteristics of the inhibitor

The inhibitor was sensitive to trypsin and inactivated within 60 min. at 60°.
Fig. 3. Action of the inhibitor on synchronized cell growth. Cell count: ○—○, Eagle's medium containing the inhibitor; □—□, control Eagle's medium; △—△, not stimulated. Number of refractile cells in mitosis: ●—●, Eagle's medium containing the inhibitor; ■—■, Control Eagles's medium; ▲—▲, not stimulated. Thymidine incorporation was also determined by labelling cultures for 48 hr with 0.5 μC/mole [3H]-thymidine (5 C/mole). Cultures stimulated with Eagle's medium containing the inhibitor showed 28,000 counts/min.; cultures stimulated with Eagle's medium only 19,000 counts/min. and cultures not stimulated, 500 counts/min.

Mode of action of the inhibitor extracted from transformed cells

Effect of the inhibitor on the replication cycle of permissive mouse cells

H. Yoshikura & R. Cramer (in preparation) found that in synchronized cell cultures of permissive mouse cells, polyoma DNA synthesis was linked in time to host cell DNA synthesis. Thus any impairment of cell DNA synthesis would be expected to reduce the physiological competence of the host cell for the virus DNA replication cycle. For this reason, the inhibitor action on cell replication was tested in both randomly growing and synchronized cultures of permissive mouse cells. No inhibition of cell growth was recorded in the inhibitor treated cultures. The results for synchronized cultures are shown in Fig. 3. Whereas only 60% of the cells arrested in phase G1 were stimulated into phase S by medium alone, 100% of the cells were stimulated by the medium to which the inhibitor had been added. A higher incorporation of thymidine was also found in the inhibitor treated cultures. Furthermore the time course of mitosis was the same in cultures with or without inhibitor in the medium. These results suggest that the inhibitor acts specifically on some step of the infective cycle.
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The action of the inhibitor on the different expressions of polyoma virus infection was tested during one cycle of replication in mouse embryo cells. A 50 to 80% reduction in the yield of infectious virus in both the cell and the supernatant phase was shown (Tables 1, 2; Fig. 4). Similar inhibition was obtained for cultures infected with either infective virus or infective DNA. The yield of infectious polyoma DNA, the haemagglutination activity and the formation of infective centres were also reduced. Sedimentation in neutral CsCl velocity gradients (Fig. 5) showed that in the presence of the inhibitor, polyoma virus DNA-polymer formation did not occur to an extent sufficient to explain the reduced infectivity of polyoma DNA. Sedimentations in ethidium-bromide-CsCl equilibrium gradients allowed separation of the hypercoiled form I of polyoma DNA from the nicked circular form II and the linear form III, as well as from linear host DNA. It is shown in Fig. 6 that in cultures treated with
Fig. 6. Equilibrium banding in CsCl-ethidium-bromide of $^{14}$C-labelled DNA extracted from polyoma virus infected cultures in the presence (■---■) and absence (○---○) of the inhibitor and from non-infected control cultures (●---●). Infection and labelling conditions as described in ‘Methods’.

Table 3. Inhibition of the formation of polyoma DNA-component-I

<table>
<thead>
<tr>
<th>Gradient</th>
<th>Amount of DNA</th>
<th>With inhibitor</th>
<th>Without inhibitor</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.5 µg.</td>
<td>2.1 µg.</td>
<td></td>
<td>1238</td>
</tr>
<tr>
<td></td>
<td>(76 % inhibition)</td>
<td>(69 % inhibition)</td>
<td></td>
<td>3965</td>
</tr>
<tr>
<td>II</td>
<td>1.8 µg.</td>
<td>3.1 µg.</td>
<td></td>
<td>1638</td>
</tr>
<tr>
<td></td>
<td>(61 % inhibition)</td>
<td>(48 % inhibition)</td>
<td></td>
<td>2626</td>
</tr>
<tr>
<td>III</td>
<td>2.2 µg.</td>
<td>6.1 µg.</td>
<td></td>
<td>1040</td>
</tr>
<tr>
<td></td>
<td>(64 % inhibition)</td>
<td>(53 % inhibition)</td>
<td></td>
<td>2199</td>
</tr>
</tbody>
</table>

Inhibitor, the counts for the heavy peak containing polyoma DNA form I amounts to only 40 % of the control. However, the rate of incorporation of a DNA precursor is not a sufficient measure of DNA synthesis since the uptake and transport of precursor, the precursor pool sizes and the kinase levels may all be influenced unpredictably in different biological situations. For this reason the DNA concentrations were determined also by the ethidium-bromide-DNA fluorometric method indicated above. The recorded values correspond to DNA concentrations because this method does not measure proteins and because RNA was digested by RNase. As is seen from Table 3, the DNA content of the heavy peak containing the circular hypercoiled form of polyoma DNA was again reduced. However, this peak may also contain circular hypercoiled mitochondrial DNA which, under our conditions, bands at about the same position as circular hypercoiled polyoma DNA. Non-infected cell cultures were therefore extracted and processed as controls to estimate the amount of mitochondrial DNA present in the heavy peak. Few counts were recorded (Fig. 6) at the
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Table 4. Induction of the specific inhibitor in mouse cells, hamster cells and mouse-hamster-hybrid cells and their competence for the infectious cycle of polyoma virus

<table>
<thead>
<tr>
<th>Cell species</th>
<th>Infective centres</th>
<th>Yield as p.f.u./cell in cell phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With inhibitor</td>
</tr>
<tr>
<td>Hamster – Melanosarcome 346o-3</td>
<td>0</td>
<td>118 ± 10 (60 % inhibition)</td>
</tr>
<tr>
<td>Hybrid</td>
<td>29 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Hamster – Mouse</td>
<td>27 ± 3.3</td>
<td>80.6 ± 5.6 (0 % inhibition)</td>
</tr>
<tr>
<td>Mouse thymidine deficient L cells</td>
<td>27 ± 3</td>
<td>284 ± 7</td>
</tr>
<tr>
<td></td>
<td>26 ± 2.8</td>
<td>(0 % inhibition)</td>
</tr>
</tbody>
</table>

Hamster, mouse and the mouse-hamster-hybrid cells were infected with polyoma virus and their permissiveness estimated by assay of infective centres. Comparable numbers of infective centres were obtained for the mouse and the mouse-hamster-hybrid cells showing that non-permissiveness was not dominant. Cells of the three lines were extracted 18 hr after infection with polyoma virus and the extracts tested for inhibition as for Table 1. Inhibition was found only in extracts from polyoma-virus infected non-permissive hamster cells.

The position of the heavy peak and these corresponded to DNA concentrations of less than 0.1 μg, as determined by the fluorescent method. These findings suggest that the reduced DNA content of the heavy peak for the inhibitor treated cultures corresponded to a reduced yield of circular hypercoiled form I of polyoma virus DNA. Further experiments are being made to test this hypothesis.

Origin of the inhibitor induced in non-permissive hamster cells

To test the dependence of the inhibitor on the virus genome, the cell genome or both, the following genetic experiments were made. Preliminary results have been reported by Cramer (1969, 1970). The competence of a hybrid mouse–hamster cell line (346o-3/C1D clone 1, and of the two parental mouse (LM(TK−)C1D), and hamster (hamster melanoma 346o-3); cell lines were tested for inhibitor induction and the lytic cycle of polyoma virus. The hybrid cell line was used at its 150th subcultivation and contained 45 to 50 metacentric and 40 telocentric chromosomes (D. R. Davidson, personal communication). This suggested that essentially the full chromosome set of the hamster partner was still present. This cell line was found to be as permissive as the parental mouse cell line (Table 4). On the other hand, the inhibitor could not be induced in either of the two permissive cell lines whereas it was induced upon infection of the non-permissive hamster cell line. Clones of both permissive cell lines infected with polyoma virus were maintained for 16 months and still released virus.

DISCUSSION

Mechanisms of the action of inhibitor extracted from transformed cells

A non-specific effect due to cell contaminants, such as PPLO organisms, is considered to be unlikely. Extracts of a mouse L-cell line harbouring PPLO organisms did not inhibit polyoma virus replication. On the other hand the TSPy cell line, derived from a hamster tumour obtained by polyoma virus in vivo, contained the inhibitor and was found to be free of PPLO organisms (J. P. Cesarini, personal communication). The TSPy cell line was also free of the virus particles (Cesarini, 1971) sometimes found in hamster cell lines (Bernhard & Tournier, 1964). As the inhibitor did not repress DNA synthesis or cell replication of non-infected permissive mouse cells, its action is probably not due to an altered physio-
logical cell competence for the replication of polyoma virus DNA. The inhibitory effect of polyoma virus release which has been observed under special nutritional conditions (Wincour & Sachs, 1960) can be excluded because a comparable reduction of virus yield was observed in both the supernatant fluid and the cell phases (Table 1). We therefore assume a specific inhibition during formation of infectious polyoma virus particles. A direct neutralization of virus particles can be excluded since no reduction of infectivity was observed after incubation for 3 hr at 37° of the inhibitor with polyoma virus. The observation that the inhibitor is active only when given 1 or 2 hr after infection, suggests that its action is concerned with an early event of the replication cycle. An inhibition of polyoma virus adsorption onto mouse cells can be excluded, since similar inhibition was observed when the inhibitor was added either immediately after virus adsorption for 60 min. or 1 hr later. An effect on the uncoating of the virus particles can be excluded since the inhibition was also observed when cells were infected with infective polyoma DNA. The reduced yield of infectious polyoma DNA suggests that the inhibitor acts at the point of polyoma DNA synthesis, either in reducing the yield or in altering the conformation of the DNA molecules as in polymer formation. The possibility must be considered that the inhibitor may favour the formation of non-infectious, host cell DNA containing pseudovirus (Michel, Hirt & Weil, 1967; Cramer et al. 1967). The results from neutral CsCl-velocity-gradients exclude the hypothesis of polymer formation (Fig. 5), whereas the results of CsCl-ethidium-bromide equilibrium gradients show that the DNA content of the heavier peak containing circular hypercoiled polyoma DNA amounts to only 30 to 50% of the control. The inhibition is therefore most likely to be due to an inhibition of polyoma DNA synthesis, followed by a reduction in capsid formation. The latter is known to depend on replicated polyoma virus particles (Urbano, 1967). Alternatively, the reduced yield of circular hypercoiled polyoma DNA particles may be due to an accelerated destruction of the polyoma DNA particles, resulting either from a raised nuclease activity or an increased vulnerability of non-encapsulated polyoma DNA particles. In the latter case, a specific block in capsid antigen formation may be expected. We are testing these possibilities.

Origin of the inhibitor induced in infected hamster cells

The results of the genetic experiment with the mouse–hamster-hybrid cell line and their implication concerning the mode of action and origin of the inhibitor have been discussed (Cramer, 1969). The non-permissiveness of the parental hamster cell line may be due simply to a lack of factors needed for polyoma virus replication and supplemented in the hybrid cell line by the mouse cell genome. Alternatively, non-permissiveness may be due to the specific inhibitor which cannot express itself in the hybrid cell line because of the presence of the mouse cell genome. The specificity of the inhibitor suggests that it is coded for by the polyoma genome. It is hoped to test the possible cellular origin of the inhibitor by experiments with hamster cells transformed by mutants of polyoma virus. We have shown that cell extracts made up to 10 hr after infection of non-permissive hamster cells with polyoma virus stimulate virus replication in permissive mouse cells, whereas extracts made after 12 hr are inhibitory. Under the same experimental conditions a transitory stimulation of thymidine incorporation was found in earlier work (Cramer & Feinendegen, 1966) and it was suggested that an autoinhibitor may determine the abortive infectious cycle of polyoma virus in non-permissive hamster cells. We are testing the relationship between the two mechanisms of stimulation. It is not yet established that the inhibitors extracted from infected and transformed hamster cells are identical. More must be known of the chemical characteristics, origin and mode of action of these inhibitors before they can be compared.
A specific inhibitor of polyoma virus with restriction enzymes (Messelson & Yuan, 1968) exclusion enzymes (Darlington & Levine, 1971) or repressor proteins (Ptashne, 1967) of bacterial systems.

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