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State of the Viral DNA in Rat Cells Transformed by Polyoma Virus

I. Virus Rescue and the Presence of Nonintegrated Viral DNA Molecules

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The interaction of polyoma virus with a continuous line of rat cells was studied. Infection of these cells with polyoma did not cause virus multiplication but induced transformation. Transformed cells did not produce infectious virus, but in all clones tested virus was rescuable upon fusion with permissive mouse cells. Transformed rat cells contained, in addition to integrated viral genomes, 20 to 50 copies of nonintegrated viral DNA equivalents per cell (average). "Free" viral DNA molecules were also found in cells transformed by the ts-a and ts-8 polyoma mutants and kept at 33 C. This was not due to a virus carrier state, since the number of nonintegrated viral DNA molecules was found to be unchanged when cells were grown in the presence of antipolyoma serum. Reclonning of the transformed cell lines produced subclones, which also contained free viral DNA. Most of these molecules were supercoiled and were found in the nuclei of the transformed cells. The nonintegrated viral DNA is infectious. Its specific infectivity is, however, about 100-fold lower than that of polyoma DNA extracted from productively infected cells, suggesting that these molecules contain a large proportion of defectives.

Infection with the oncogenic DNA viruses polyoma and simian virus 40 causes neoplastic transformation in cells that are nonpermissive to viral multiplication. Transformed cells do not generally produce infectious virus but can be shown to contain the viral DNA integrated into the host genome (20). Fusion of nonpermissive simian virus 40-transformed cells with permissive cells results in virus rescue (13, 22). In the case of polyoma. However, the most widely used host for transformation, hamster cells, usually fails to yield infectious virus, even after fusion with permissive mouse cells (2, 7, 22). This is not due to the fact that the heterokaryons are nonpermissive for viral multiplication (2). It is possible that the polyoma DNA fails to become excised or that a small degree of permissiveness in hamster cells causes selection for transformation by defective polyoma genomes. Hamster cells transformed by the ts-a mutant of polyoma virus at 33 C and kept at the nonpermissive temperature (39 C) can, in fact, produce virus upon fusion when shifted to permissive conditions (7).

Previous reports (5, 6) have shown that a clonal line of rat myoblasts transformed by large-plaque polyoma often produces a small amount of infectious virus. A large increase in virus yield is obtained when these cells are treated with several chemical and physical agents (6). Moreover, "virus-free" subclones of this line yield virus upon fusion with mouse cells (5). More recently, Kimura et al. (12) have shown that, in another line of rat cells transformed by polyoma virus, the virus can generally be rescued by fusion with mouse cells.

We have investigated in detail the interaction of polyoma virus with a continuous line of Fischer rat cells, F2408 (9). In this paper we report that virus can be rescued by fusion from all polyoma-transformed F2408 rat cells tested. These transformed cells do not produce infectious virus spontaneously but contain, in addition to integrated viral genomes, "free" viral DNA molecules.

MATERIALS AND METHODS

Cell lines. Swiss 3T3 mouse cells (clone D) and rat cells of the F2408 established line (9) were used. F2408 rat cells were kindly provided by G. DiMayorca. Primary rat cell cultures were prepared from Fischer's rat embryos. Cells were grown in Dulbecco-modified Eagle medium containing 10% calf serum.

Virus. Wild-type, small-plaque polyoma and the temperature-sensitive, large-plaque polyoma mutants ts-a (10) and ts-8 (4) were used. Wild-type

virus was grown at 37 C, and the ts mutants were grown at 33 C. Viruses were extracted, purified by cesium chloride density gradient centrifugation, and titered by plaque assay on monolayers of 3T3 cells.

Transformation. Transformation of rat cells by polyoma virus was determined by the ability of transformed cells to grow in suspension in soft agar, following the technique of MacPherson and Montagnier (14). Cells were infected with the virus at different multiplicites in TD buffer (0.8% NaCl, 0.038% KCl, 0.01% Na_2HPO_4, and 0.3% Tris-hydrochloride, pH 7.2). Adsorption was carried out in suspension for 1 h at room temperature. The final agar concentration in the medium was 0.34%. Wild-type polyoma-infected cells were incubated at 37 C and ts-a- and ts-8-infected cells were incubated at 33 C. After 2 to 4 weeks of incubation, transformed colonies were isolated. Uninfected cells never grew in agar medium, even when plated at a high concentration.

Virus rescue. Transformed cells (10^6) were mixed with mouse 3T3 cells (10^6) and exposed to β-propiolactone-inactivated Sendai virus as previously described (17). The fused cells were cultured for 24 h. The medium was changed, and the cells were incubated at 37 C for 4 days or at 32 C for 5 days. For cocultivation, the same procedure was used, but the cells were not exposed to the Sendai virus.

Infectivity of viral DNA. Confluent monolayers of 3T3 cells in 60-mm plates were washed with TD buffer. A 200-μg amount of DEAE-dextran (16) in 0.2 ml of TD buffer was spread over the cells. After 10 min, 0.1 ml of a solution containing DNA in 1× SSC (0.15 M NaCl and 0.015 M sodium citrate) was added to the cells. Twenty minutes later, the cells were gently washed with TD buffer and then overlaid with 7 ml of medium containing 0.9% agar. Cells were stained with neutral red at day 7 to 10 and incubated until the plaques were clearly countable.

V-antigen. The presence of polyoma V-antigen was determined by immunofluorescence, as previously described (1).

Chromosomes. The method for determining chromosomes has been described previously (1).

Autoradiography. Cells were grown on glass cover slips in petri dishes. After labeling with [^{3}H]thymidine, they were fixed with ethanol-acetic acid (9:1). After washing with 70% ethanol and drying, the cover slips were mounted on slides with Permount and dipped in nuclear track emulsion (NTB-2, Kodak). After the appropriate time of exposure the slides were developed and stained with Giemsa, prior to counting.

Preparation of polyoma viral DNA. 3T3D monolayers were infected with polyoma at 50 PFU/cell. When the cells were partially lysed, low-molecular-weight DNA was extracted following the Hirt procedure (11). The Hirt supernatant was extracted with saturated phenol and then with chloroform-isoamyl alcohol. Nucleic acid in the aqueous phase was precipitated by the addition of 2 volumes of ethanol. After 24 h at −20 C, the precipitate was centrifuged and suspended in 1× SSC. Form I DNA (covalently closed circular duplex DNA) was isolated after CsCl-ethidium bromide gradient centrifugation. For the preparation of [^{32}P]polyoma DNA, infected 3T3 cells were incubated in phosphate-free medium containing [^{32}P]orthophosphate (100 μCi/ml) for about 50 h. Viral DNA was extracted and purified by equilibrium density centrifugation in cesium chloride-ethidium bromide, followed by velocity sedimentation through neutral sucrose gradients. [^{32}P]polyoma DNA with a specific activity of 2×10^6 to 3×10^6 counts/min per μg was obtained.

Determination of the number of "free" viral DNA equivalents. Free viral DNA equivalents were estimated by measuring the effect of low-molecular-weight DNA preparations from transformed rat cells on the rate of reassociation of ^{32}P-labeled polyoma DNA. Cells were lysed in 0.6% sodium dodecyl sulfate-10^{-2} M EDTA, and low-molecular-weight DNA was extracted according to Hirt (11). The Hirt supernatant was extracted twice with phenol and once with chloroform-isoamyl alcohol (24:1). DNA was precipitated with ethanol at −20 C. The precipitate was dried and dissolved in a small volume of 0.01 M phosphate-0.001 M EDTA (pH 6.8) and dialyzed extensively against the same buffer. Before hybridization, DNA was fragmented by boiling together with the [^{32}P]polyoma DNA probe for 10 min in 0.3 M NaOH. DNA-DNA reassociation kinetics and hydroxyapatite chromatography were done according to Sharp et al. (18). Nonintegrated polyoma DNA equivalents per cell were calculated from the formula: number of polyoma DNA equivalents/cell = $a \times 2 \times 10^{11}/A$, where a = micrograms of viral DNA in the preparations, A = the number of cells, and 2×10^{11} = the number of polyoma DNA molecules per microgram.

Determination of the number of viral DNA equivalents associated with the cellular DNA. High-molecular-weight DNA was first resolved from low-molecular-weight DNA by Hirt extraction. The pellet was washed twice with 1× SSC at 4 C and DNA was extracted according to Marmur (15), with minor modifications. The DNA was further purified by neutral sucrose gradient centrifugation (10 to 30% sucrose in 1× SSC). Fractions containing DNA sedimenting faster than 50S were pooled, dialyzed against 1× SSC, and precipitated with ethanol. For DNA-DNA reassociation experiments the DNA was incubated in 0.3 M NaOH for 7 to 10 h at room temperature, neutralized with HCl, adjusted to 0.1 M NaCl, and precipitated with ethanol. The precipitate was dissolved in 0.01 M phosphate-0.001 M EDTA, pH 6.8, and dialyzed extensively against the same buffer. Before hybridization, cellular DNA was fragmented by sonication and subsequently boiled together with the [^{32}P]polyoma DNA probe for 10 min in 0.3 M NaOH. Samples were removed from the mixtures at intervals, and the fraction of ^{32}P-labeled single-stranded DNA (f_{ss}) was determined by chromatography on hydroxyapatite (18).

RESULTS

Characteristics of the cells. Fischer rat fibroblasts derived from the established line F2408 were propagated in medium containing

10% calf serum. The mean doubling times of these cells were 16, 18, and 24 h at 40, 37, and 33 C, respectively. At saturation, these cells reached densities of about 1.5×10^6 cells/60-mm plate. The colony-forming ability of these cells at 37 and 33 C was ~45%, whereas at 40 C it was 25 to 30%. Karyological studies showed that these cells contained 42 chromosomes, including 12 telocentric, 14 metacentric, and 16 acrocentric. It appears that F2408 rat cells contain a diploid number of chromosomes.

Response of F2408 rat cells to polyoma infection. Rat cells are generally nonpermissive to polyoma multiplication but can be transformed by the virus (20). To determine the level of permissiveness of F2408 cells, semiconfluent cultures were infected with polyoma virus at a multiplicity of infection (MOI) of 50 PFU/cell. After adsorption infected cultures were kept in medium containing antipolyoma serum for 12 h to inactivate unadsorbed viral particles. After 3 days of incubation, no cytopathic effect was observed in the cultures. When virus was extracted and titered, there was no increase in virus titer above that determined at 0 time. Similar experiments were also done using infection with purified polyoma DNA. DNA infection (10^4 PFU/culture) was carried out as described in Materials and Methods. After 3 and 5 days of incubation virus was extracted and titered. In one experiment, DNA-infected rat cells failed to yield any infectious virus. In a second experiment, a yield of 10^2 PFU/culture was detected. The yield of similarly infected 3T3 mouse cultures was 1.6×10^6 and 10^7 PFU/culture at 3 and 5 days, respectively.

When infected rat cells were tested for the presence of polyoma V-antigen by immunofluorescence, we did not find any positive cells out of ~10^5 examined. If the MOI was raised to ~500 PFU/cell, a few (~0.1%) positive cells were observed. These results show that F2408 cells are nonpermissive for polyoma multiplication, although the use of very high MOI can lead to virus production in a small proportion of the infected cells.

Since polyoma induces cellular DNA synthesis in resting cells, it was of interest to determine whether it caused a similar effect on rat F2408 cells. Confluent cultures of rat cells were infected and the cultures were labeled with [^{3}H]dT at 12-h intervals. The frequency of the DNA-synthesizing cells was determined by autoradiography. In cultures infected at 200 PFU/cell, the frequency of DNA-synthesizing cells increased considerably with time, whereas it decreased gradually in uninfected cultures (Table 1).

Polyoma transformation. The technique used to transform rat cells with polyoma virus has been described in Materials and Methods. Figure 1 shows that the transformation frequency obeys one-hit kinetics. At an MOI of 1,000 PFU/cell, 1.7% of the infected cells were transformed. In comparison to wild-type virus, the ts-a and ts-8 mutants induced transformation at 33 C at a slightly reduced frequency. At the nonpermissive temperature (39 C) the polyoma ts-a mutant failed to transform rat cells (10).

Rat cells were transformed at low and high MOI using wild-type and mutant viruses. Transformed colonies were isolated. Cells from practically all of these colonies grew in a criss-

TABLE 1. *Induction of cellular DNA synthesis in rat F2408 cells after infection with polyoma virus*[a]

MOI	% Cells synthesizing DNA			
	0–12 h[b]	12–24 h	24–36 h	36–48 h
0	4.5	5	3.3	4.3
50	5.4	4.2	5.7	8.4
200	4.4	5.3	9.8	27.4

[a] Cells were grown on cover slips. When they reached confluence, fresh medium containing 10% calf serum was added. After 2 days, the cells were infected with polyoma virus and then received the old medium diluted 1:1 with fresh serum-free medium. Cells were labeled with [^{3}H]thymidine (2 μCi, 0.6 μg/ml) for the times indicated. They were then fixed and processed for autoradiography.

[b] Hours after infection.

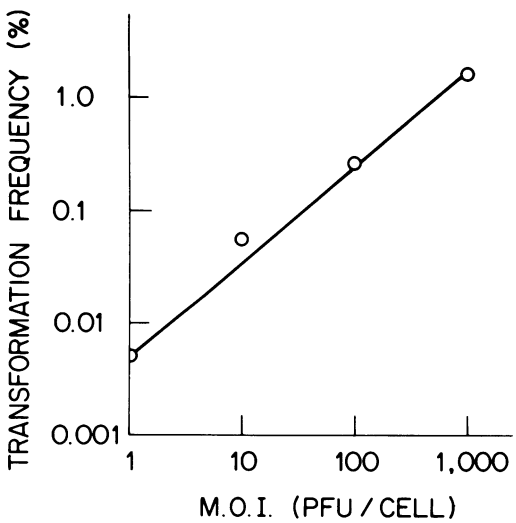

FIG. 1. *Relationship between the multiplicity of infection by polyoma virus (wild type) and the transformation frequency of rat F2408 cells. The experimental procedure has been described in Materials and Methods.*

cross manner to high saturation density (about 10 to 15 times higher than the normal cells). In addition, transformed cells were capable of sustained multiplication in medium containing a low serum concentration (0.5%), whereas normal cells ceased to grow.

Spontaneous yield of virus from the polyoma transformants. Cells of all the transformed lines tested did not usually produce infectious virus spontaneously (Table 2). Transformed clones polyoma Rat-11, polyoma Rat-12, and polyoma Rat-13 were serially passaged four times. Virus was extracted each time from 4×10^6 cells in 10 ml of culture fluid and titered. At passage 4 some virus (10 PFU/ml) was detected in the extracts from polyoma Rat-11 cells. Cells of the ts-a Rat-21, ts-a Rat-22, ts-a Rat-23, and ts-a Rat-24 lines did not produce infectious virus. However, when ts-a Rat-23 cells were maintained in culture for about 8 weeks, some virus was produced (15 PFU/ml). It thus appears that virus production in these cells is a very exceptional event.

On one occasion we detected in about 10^5 cells of the ts-a Rat-23 line, by immunofluorescence, two cells positive for V-antigen. Cells of other lines tested, such as polyoma Rat-12, polyoma Rat-13, and ts-a Rat-11, were always negative, and on a different occasion no positive cells were found in the ts-a Rat-23 line.

TABLE 2. *Viral production in rat cells transformed by polyoma (Py) virus*

Clone	PFU/culture		
	Spontaneous[a]	Co-cultivation[b]	Fusion[c]
Py Rat-11	0[d]	5×10^3	2×10^5
Py Rat-12	0	5×10^2	5×10^4
Py Rat-13	0	6.5×10^3	1×10^5
Py Rat-14	0	2.5×10^3	1.5×10^5
Py Rat-15	0	1×10^4	2.8×10^6
Py Rat-16	ND[e]	ND	4.6×10^6
Py Rat-17	ND	ND	8.5×10^4
Py Rat-18	ND	ND	1.5×10^4
Py Rat-19	ND	ND	1×10^4
ts-a Rat-21	0	ND	3.5×10^3
ts-a Rat-22	0	ND	5×10^3
ts-a Rat-23	0	ND	7.5×10^3
ts-a Rat-24	0	ND	2.5×10^4

[a] Cells from the Py rat and ts-a rat lines were grown at 37 and 33 C, respectively. Virus was extracted from confluent cultures (4×10^6 cells) and titered at 37 C for wild-type Py and at 33 C for ts-a.

[b] Transformed cells and 3T3D cells were mixed and then cultured for 4 days at 37 C. Virus was extracted and titered.

[c] The same procedure as in *b* except that the cells were fused by means of inactivated Sendai virus. Fused cells were incubated at 37 C for 4 days for the wild-type Py-transformed lines and at 32 C for 5 days for the ts-a-transformed lines. ts-a virus was titered at 33 C.

[d] 0, <10 PFU/culture.

[e] ND, Not done.

Virus rescue. It was of interest to determine whether infectious virus could be rescued from the polyoma-transformed rat cells. Rescue was attempted using two methods: (i) cells were fused with permissive 3T3D cells by means of inactivated Sendai virus; (ii) cells were co-cultivated with permissive cells. After 3 to 5 days the cells were extracted and the extracts were examined for infectious virus. Table 2 shows the results. In all the clones tested the virus was rescuable. The yield was 20- to 100-fold higher when the transformed cells were fused by Sendai virus as compared to co-cultivation alone (Table 2). It appears that the formation of heterokaryons with permissive cells is necessary to allow virus rescue.

Free viral DNA molecules in polyoma-transformed rat cells. We determined whether nonintegrated viral DNA molecules were by any chance present in the transformed F2408 rat cells. Low-molecular-weight DNA was extracted by the method of Hirt (11) and purified. The number of viral DNA equivalents was then determined by DNA-DNA reassociation kinetics. Table 3 shows the results. Cells of all the clones tested contained free viral DNA molecules.

Polyoma Rat-11, polyoma Rat-12, and polyoma Rat-13 were independently isolated transformants from cells infected at 1,000 PFU with wild-type virus. Rat cells transformed by the ts-a mutant at 1 PFU (ts-a Rat-13) and at 200 PFU (ts-a Rat-21) also contained free viral DNA. When polyoma Rat-12 and ts-a Rat-13 cells were kept in culture for over 2 months, the number of free viral DNA molecules did not change appreciably. Thus, it appears that transformed rat cells contained nonintegrated polyoma DNA, regardless of the type of virus or the multiplicity used for transformation.

Origin of the free viral DNA in rat transformed cells. Since all polyoma-transformed rat cells contained free viral DNA molecules, it was important to verify whether or not the cells carried over some infectious virus from the initial infection or whether a few cells in the population made a small amount of virus which constantly caused reinfection, producing free viral DNA molecules. To rule out the possibility of a virus "carrier state," polyoma Rat-12 cells were grown in the presence or absence of antipolyoma serum for 8 days. (The concentration of antiserum that we used was found to be able to reduce virus titer by 4 logs in 60 min at

TABLE 3. *Free viral DNA molecules in rat cells transformed by polyoma (Py) virus*

Transformed line[a]	Viral DNA equivalents/cell[b]
Py Rat-11	11
Py Rat-11b[c]	8
Py Rat-11c[c]	22
Py Rat-11f[c]	21
Py Rat-12	22
Py Rat-13	11
ts-a Rat-13	52
ts-a Rat-21	20
ts-8 Rat-11	14
Py Rat P-1[d]	18.5

[a] Wild-type Py-transformed lines were grown at 37 C, ts-a- and ts-8-transformed lines at 33 C.

[b] Determined as described in Materials and Methods.

[c] Subclones of Py Rat-11.

[d] Derived from primary rat embryo cultures.

37 C). Low-molecular-weight DNA was isolated. The number of viral DNA equivalents was then determined and found to be 33 and 39 copies/cell (Fig. 2). It therefore appears that reinfection did not play a significant role in determining the presence of nonintegrated viral DNA in polyoma-transformed rat cells.

Since we used a large number of cells in all of these experiments, the possibility existed that production of free viral DNA was a function of only a specific minority of the cell population. To answer this question, cells of polyoma Rat-11 were recloned in the presence of antipolyoma serum and three clones were tested. Cells of each clone contained free viral DNA (Table 3). In summary, the presence of nonintegrated viral DNA molecules in these cells is not due to a virus carrier state, and every cell in the population appears capable of producing or carrying free viral DNA.

Localization of the free viral DNA. Nuclear and cytoplasmic fractions were prepared from polyoma Rat-12 cells as previously described (19). The number of viral DNA molecules present in the Hirt supernatant of these fractions was determined. We found that ~13 copies of viral DNA equivalents were present in the nuclear fraction and ~4 copies were present in the cytoplasmic fraction. It is likely that during fractionation some nuclei might have been broken, thus contaminating the cytoplasmic fraction. We therefore interpret these results as indicating that the bulk of free viral DNA in these cells is nuclear.

Physical state of the free viral DNA. Low-molecular-weight DNA extracted from polyoma-transformed rat cells was subjected to ethidium bromide-cesium chloride density gradient centrifugation in the presence of simian virus 40 DNA, which was added as a marker. Polyoma viral DNA sequences were then detected by DNA-DNA reassociation in the fractions containing supercoiled DNA and in the fractions containing linear and open circular DNA. Ninety percent of the polyoma DNA sequences were found in the supercoiled fraction (Fig. 3). Thus it appears that most of the free polyoma DNA in transformed rat cells is present as supercoiled circular molecules. This experiment also shows that the appearance of viral DNA in the Hirt supernatant was most likely not due to the fact that integrated viral DNA molecules were randomly excised during extraction.

Infectivity of the free viral DNA. The specific infectivity of the free viral DNA molecules was determined by plaque assay on 3T3 cells. The viral DNA isolated from the polyoma Rat-12 and ts-a Rat-13 lines was infectious but formed plaques at a much lower efficiency than polyoma DNA extracted from infected 3T3 cells (Table 4).

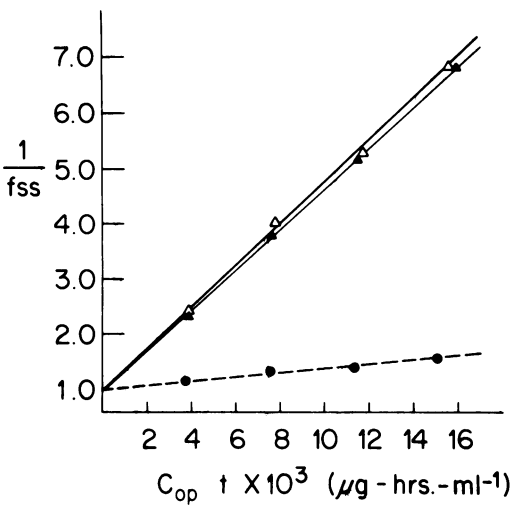

FIG. 2. *Reassociation kinetics of [^{32}P]polyoma DNA in the presence of low-molecular-weight DNA from polyoma Rat-11 cells grown in the presence or absence of antipolyoma serum for 8 days. Medium, containing fresh antiserum, was changed every 3 days. Hybridization was carried out as described in Materials and Methods. The data are plotted as $1/f_{ss}$ versus $C_{op} \times t$, where f_{ss} represents the fraction of ^{32}P-labeled single-stranded DNA, C_{op} the input of probe in micrograms per milliliter, and t the time of sampling in hours. Symbols: (●) renaturation of [^{32}P]polyoma DNA in the presence of low-molecular-weight DNA from untransformed rat cells; (▲) DNA from polyoma Rat-11 cells grown in the absence of antiserum; (△) DNA from polyoma Rat-11 cells grown in the presence of antiserum.*

Viral genomes associated with chromosomal DNA. To determine whether the polyoma-transformed rat cells also contained viral DNA molecules associated with the chromosomal DNA and therefore presumably integrated, purified high-molecular-weight DNA from transformed cells was analyzed for its ability to influence the rate of reassociation of [^{32}P]polyoma DNA (Fig. 4). On the basis of the reassociation kinetics, polyoma Rat-13 and ts-a Rat-23 contained 6 and 10 copies of viral DNA equivalents associated with the host genome, respectively. It is important to note that the value for ts-a Rat-23 cells was obtained with DNA extracted from cells grown at 39.5 C. Under these conditions these cells did not contain detectable free viral DNA (manuscript in preparation).

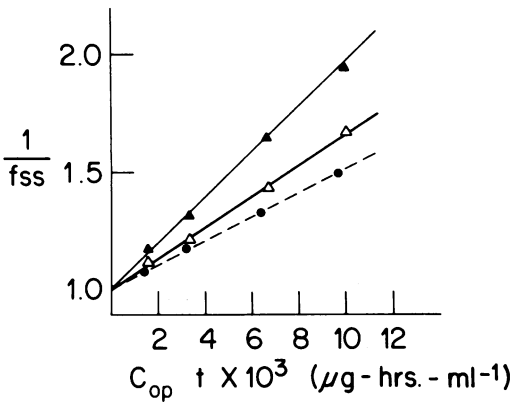

FIG. 3. *Reassociation kinetics of [^{32}P]polyoma DNA in the presence of form I and form II (linear or nicked circular) low-molecular-weight DNA from polyoma Rat-12 cells. Hybridization was done as described in Materials and Methods and the data are expressed as in Figure 2. Symbols: (●) reassociation of [^{32}P]polyoma DNA by itself (control); (△) reassociation of [^{32}P]polyoma DNA in the presence of form II DNA; (▲) reassociation of [^{32}P]polyoma DNA in the presence of form I DNA.*

Virus rescue from cells containing integrated and free viral DNA molecules. We wanted to determine whether the virus rescued from transformed rat cells upon fusion with mouse cells originated from free, integrated, or both types of viral DNA molecules.

The thermosensitive polyoma mutant, ts-a, does not multiply at the nonpermissive temperature (40 C) but does multiply at the permissive temperature (33 C) (10). ts-a-transformed rat cells at 33 C contain approximately 20 copies of free viral DNA molecules per cell. When these cells were incubated at the nonpermissive temperature, viral DNA disappeared from the Hirt supernatant, but 10 copies of viral DNA were still found associated with the host DNA (manuscript in preparation). ts-a-transformed cells were incubated for 10 days at the nonpermissive temperature. When these cells were shifted to the permissive temperature and incubated for 3 days, free viral DNA molecules reappeared (20 copies/cell).

Cells from the ts-a Rat-23 and ts-a Rat-21 lines grown at 33 and 40 C were fused separately with 3T3 cells in the presence of Sendai virus. Cells were then incubated at the permissive temperature. Virus was extracted and titered. If the presence of free viral DNA had been necessary for virus rescue, the viral yield from the fusion using cells from 33 C should have been higher and earlier than that from the fusion using cells from 40 C, since the latter cells did not contain free viral DNA at the time of fusion. However, similar amounts of virus were rescued from the cells grown at 33 and 40 C, either after 3 or 5 days (Table 5).

The fact that the virus yield from cells that contained or did not contain measurable amounts of free viral DNA molecules at the time of fusion is about the same suggests that the presence of nonintegrated viral DNA does

TABLE 4. *Infectivity of the "free" viral DNA extracted from polyoma (Py)-transformed rat cells*

Expt	DNA source[a]	Concn[b] (μg/ml)	Infectivity[c] (PFU/ml)	Specific infectivity (PFU/μg)
I	3T3D lytically infected with wild-type Py (control)	0.2	3.6×10^4	1.8×10^5
	Py Rat-12	0.011	20	1.8×10^3
	ts-a Rat-13	0.013	20	1.6×10^3
II	3T3D lytically infected with wild-type Py	12	1.5×10^6	1.2×10^5
	Py Rat-12	0.04	30	7.5×10^2
	ts-a Rat-13	0.07	50	7.1×10^2

[a] ts-a Rat-13 cells were maintained at 33 C; the other cells were maintained at 37 C.
[b] Determined by DNA-DNA reassociation kinetics.
[c] DNA from polyoma wild type-infected or -transformed cells was titered at 37 C, DNA from polyoma ts-a-transformed cells at 33 C.

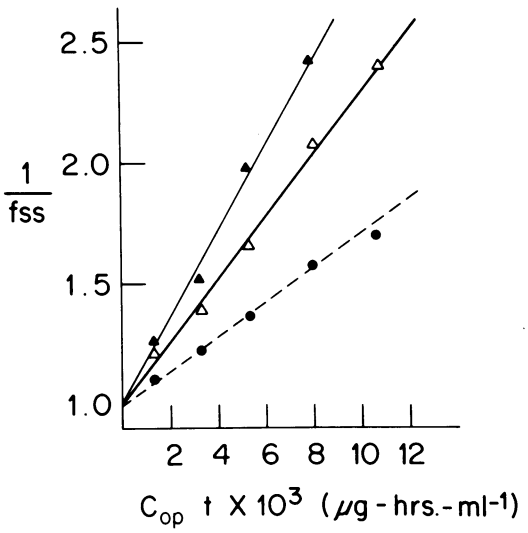

FIG. 4. *Renaturation of [^{32}P]polyoma DNA in the presence of high-molecular-weight chromosomal DNA from polyoma-transformed rat cells. Each reaction mixture contained 500 μg of cellular DNA per ml and 2.5 × 10^{-3} to 3.0 × 10^{-3} μg of [^{32}P]polyoma DNA per ml (specific activity, 2.3 × 10^{6} counts/min per μg). The data are plotted as $1/f_{ss}$ versus $C_{op} \times t$, where C_{op} represents the input of probe in micrograms per milliliter and t the time of sampling in hours. Symbols: (●) renaturation of [^{32}P]polyoma DNA in the presence of DNA from untransformed rat cells; (△) DNA from polyoma Rat-13 cells; (▲) DNA from ts-a Rat-23 cells (growing at 39.5 C).*

not significantly increase the efficiency of virus rescue by fusion.

Free viral DNA molecules in rat embryo cells transformed by polyoma. Primary rat cultures were prepared from Fischer's rat embryos. Cells of secondary cultures were transformed by polyoma at an MOI of 500 PFU in soft agar. After 5 to 6 weeks of incubation, transformed colonies were isolated. Transformed cells grew slowly, yet attained high saturation density. When these cells were inoculated into soft agar, they again formed colonies. Cells were analyzed for the presence of free viral DNA molecules. The Hirt supernatant from these cells (polyoma Rat P-1) contained 18.5 copies of free viral DNA molecules/cell (average) (Table 3).

DISCUSSION

The system of polyoma-transformed rat cells described in this paper has a number of interesting features. F2408 cells grow easily in culture, have a good efficiency of plating and a diploid chromosome number, and easily withstand high incubation temperatures. They can be transformed with good efficiency by polyoma virus. Untransformed cells do not grow at all in agar medium, thus making it very easy to detect even very low frequencies of transformants in a transformation assay.

The rat cells used are for all practical purposes nonpermissive for polyoma virus multiplication, independent of whether they are infected with virus or viral DNA. The nonpermissiveness is, however, not absolute, as infection at a very high MOI (>1,000 PFU/cell) results in the appearance of a few (~0.1%) virus-producing cells. This situation is very similar to what is found in BHK-21 hamster cells (1, 7, 8). In contrast to BHK-21 cells, however, F2408 rat cells always produce virus upon fusion with mouse 3T3 cells. The transformed cells per se do not generally produce virus, and in our case only an extensive search for virus production revealed that occasionally these cells spontaneously produce small amounts of infectious virus.

This situation superficially resembles that of the LPT line of polyoma-transformed rat myoblasts described by Fogel and Sachs (5). There are, however, two major differences. (i) In their case only cells transformed by large-plaque polyoma virus, and only one clone in particular, could be induced to make virus. In our case, all transformed clones tested produced virus upon fusion, irrespective of the type of virus used for infection. (ii) Spontaneous virus production did not generally occur in our transformed lines or was, at best, a very exceptional event.

It should be noted that Kimura et al. (12) reported that most polyoma-transformed rat cells they isolated were virus-free and produced virus upon fusion with mouse cells. The situa-

TABLE 5. *Rescue of virus from ts-a-transformed rat cells by fusion with mouse 3T3 cells*

Clone[a]	Time of incubation of the fused cells (days)	Yield of virus (PFU/culture)
ts-a Rat-21 (40 C)	5	1 × 10^{3}
ts-a Rat-21 (33 C)	5	1.8 × 10^{3}
ts-a Rat-21 (40 C)	3	0[b]
ts-a Rat-21 (33 C)	3	0
ts-a Rat-23 (40 C)	5	7.5 × 10^{4}
ts-a Rat-23 (33 C)	5	6.5 × 10^{4}
ts-a Rat-23 (40 C)	3	2 × 10^{3}
ts-a Rat-23 (33 C)	3	1 × 10^{3}

[a] Cells from ts-a Rat-21 and ts-a Rat-23 lines which had been growing at 33 or 40 C for 10 days were fused with 3T3 cells by means of inactivated Sendai virus. Fused cells were incubated at 33 C. Virus was extracted and titered at 33 C.

[b] <10 PFU/culture.

tion appears to be very similar to what we found in rat F2408 cells.

The most interesting finding in our study was that, in all cases tested, transformed rat cells contained, in addition to presumably integrated viral DNA, a small number of nonintegrated viral DNA molecules. This number remains rather constant even with long times in culture, and the variation among different clones is not very high.

Although no infectious virus can generally be found in these cultures, it was still important to rule out the possibility of a virus-carrier state (below detectable levels) being responsible for this phenomenon. This was conclusively excluded by the fact that incubation in the presence of antipolyoma serum did not affect the presence of free viral DNA. In addition, subcloning in the presence of antipolyoma serum produced cell lines that still contained free viral DNA.

The latter experiment is also important in that it showed that every cell in the transformed population was capable of carrying or at least producing free viral DNA molecules. It is not clear at present whether all cells in the transformed population contain a limited number of nonintegrated polyoma DNA molecules, or whether only a minority of the cell population contains a large number of viral DNA molecules per cell at any given time. The former situation would be analogous to that of a cell carrying a plasmid DNA (3), whereas the latter would be likely to be caused by some mechanism of induction of viral DNA replication continuously occurring with a low but constant probability in every cell. The free viral DNA would, in the latter case, originate from the integrated one and replicate to some extent without concomitant virus production. Work is in progress to distinguish between these two hypotheses.

With regard to the origin of the free viral DNA, the data presented in this paper suggest that it originates from an integrated template. Thus, we were not able to establish the presence of free viral DNA in a majority of cells at 1 week after infection, and, more importantly, cells transformed at an MOI ranging from 1 to 1,000 PFU/cell contained approximately the same number of free viral DNA molecules. In a paper now in preparation we will show conclusively that free viral DNA can originate from integrated genomes.

It is interesting to note that the presence of free viral DNA does not seem to influence the efficiency of the virus rescue by fusion. In our opinion there are two possible explanations of this finding. (i) Fusion of the transformed cells with permissive mouse cells causes immediate excision of the integrated viral DNA. Thus, cells that contain nonintegrated viral DNA or cells that do not contain it would be in practice in the same state shortly after fusion. (ii) The number of cells that contain free viral DNA could be quite low (e.g., <0.1%). Since fusion probably does not involve more than 10% of the cells, most of which do not become virus producers anyway (21, 22), the contribution of these cells to the total virus production would be negligible.

A final point of interest resides in the nature of the free viral DNA. As shown in this paper, it is generally present as nuclear, supercoiled form I DNA, which is infectious. Its specific infectivity is, however, very low. This finding strongly suggests that these molecules contain a large proportion of defectives. It is tempting to speculate that this results from improper excision of integrated viral DNA.

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