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

II. Identification of the Cells Containing Nonintegrated Viral DNA and the Effect of Viral Mutations

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F2408 rat cells transformed by polyoma virus contain integrated and nonintegrated viral DNA. The presence of nonintegrated viral DNA is under the control of the *A* early viral function. Polyoma *ts*-a-transformed rat cells lose the free viral DNA when grown at the nonpermissive temperature (40°C), but they reexpress it 1 to 3 days after they are shifted back to the permissive temperature. In contrast, rat cells transformed by a late viral mutant, *ts*-8, contain free viral DNA at both permissive and nonpermissive temperatures. Treatment of the transformed rat cells with mitomycin C produces a large increase in the quantity of free viral DNA and some production of infectious virus. Experiments of in situ hybridization, with ^{3}H-labeled polyoma complementary RNA as a probe, show that only a minority (~0.1%) of the transformed cells contain nonintegrated viral DNA at any given time. These results suggest that the presence of free viral DNA in polyoma-transformed rat cells is caused by a spontaneous induction of viral DNA replication, occurring with low but constant probability in the transformed cell population, and that the free viral DNA molecules originate from the integrated ones, probably through a phenomenon of excision and limited replication.

It is generally assumed that in cells transformed by the DNA oncogenic viruses polyoma and simian virus 40 (SV40) the viral DNA is integrated into the host genome in a stable manner (3, 10, 17, 22). Whereas the presence of integrated viral genomes in transformed cells has been studied extensively, the possible occurrence in such cells of nonintegrated viral DNA has not received equal attention.

In a previous paper (19) we reported that rat F2408 cells transformed by polyoma virus contain, in addition to viral DNA associated with host DNA, a small number (20 to 50 copies per cell average) of nonintegrated viral DNA molecules. This phenomenon was not due to a virus carrier state and was evident in all clones of polyoma-transformed rat cells tested. The cells did not produce detectable infectious virus, but virus could be rescued by fusion with permissive mouse cells (19).

In this paper, we show that the presence of free viral DNA in polyoma-transformed rat cells is probably due to a spontaneous and periodic induction of viral DNA replication in a minority of the cell population. We have also investigated the effect of viral temperature-sensitive mutations on the presence of free polyoma DNA in the transformed rat cells; apparently early viral functions are necessary to maintain the presence of nonintegrated viral DNA, whereas late functions are not.

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MATERIALS AND METHODS

Cell lines. Swiss mouse 3T3 cells (clone D), Fischer rat fibroblasts (F2408 line), wild-type polyoma-transformed F2408 cells (Py rat-12 and Py rat-13 lines), and rat cells transformed by the *ts*-a and *ts*-8 polyoma mutants (*ts*-a rat-13, *ts*-a rat-23, and *ts*-8 rat-11 lines) were used. These cell lines have been described previously (19). The cells were grown in Dulbecco modified Eagle medium containing 10% calf serum.

Virus. Wild-type small plaque polyoma and temperature-sensitive large plaque polyoma mutants, *ts*-8 and *ts*-a, were used (6, 9). Wild-type and temperature-sensitive mutants were grown in 3T3D cells at 37 and 33°C, respectively. Virus was purified by cesium chloride density gradient centrifugation. Polyoma virus infectivity was titered by plaque assay on monolayers of 3T3D cells (19). Hemagglutination was assayed as described previously (2).

Mitomycin C treatment. Semiconfluent cultures of polyoma-transformed cells were exposed to mitomycin C in the dark at four different concentrations

(0.5, 0.05, 0.03, and 0.01 μg/ml of medium). After 24 h, mitomycin C was removed and fresh medium was added. The cells were further incubated for 48 h. Then virus was extracted and titrated. The yield of virus was highest from the cells treated with 0.03 μg of the drug per ml. To determine lethality of mitomycin treatment, drug-treated cells were washed, trypsinized, and tested for plating efficiency. Plating efficiency decreased with increasing concentration of the drug. A concentration of 0.03 μg/ml caused 60% inhibition of colony formation. This concentration was chosen for use in all experiments.

Preparation of polyoma viral DNA. Unlabeled or ^{32}P-labeled polyoma viral DNA was prepared as previously reported (19).

Determination of the number of "free" viral DNA equivalents. Free viral DNA equivalents were estimated by measuring the effect of low-molecular-weight DNA, extracted from the cells by the Hirt method (12), on the rate of reassociation of ^{32}P-labeled polyoma DNA as previously described (19).

DNA-DNA reassociation kinetics. DNA-DNA hybridization kinetics and separation of single- and double-stranded DNA by hydroxyapatite chromatography were done essentially as described by Sharp et al. (21). The method of calculating the equivalents of viral DNA per diploid quantity of cell DNA was described by Gelb et al. (11). Before hybridization, cellular DNA was fragmented by sonic treatment and subsequently boiled together with the ^{32}P-labeled viral DNA probe for 10 min in 0.3 M NaOH.

Cellular DNA fractionation by "network formation." The procedure described by Varmus et al. (23) was followed. High-molecular-weight DNA was first resolved from low-molecular-weight DNA by Hirt (12) extraction. The pellet, which contains the bulk of chromosomal DNA, was washed with 0.01 M Tris (pH 7.5)-0.01 M EDTA at 4°C, dissolved in the same buffer, and treated with Pronase (1 mg/ml) at 37°C for 12 h. The viscous solution was extracted twice with phenol-chloroform (1:1) and then with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with ethanol at −20°C, dissolved in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate), and digested with pancreatic RNase (100 μg/ml) for 2 h at 37°C. The DNA was reextracted and again precipitated with ethanol. The DNA pellet was dissolved in 0.1× SSC and dialyzed extensively against the same buffer. The DNA solution (150 to 200 μg/ml) was denatured by heating at 100°C for 5 min, made 1 M in NaCl, and then incubated at 68°C for 1 h. The solution was chilled to 4°C and centrifuged at 40,000 × *g* for 40 min in a Sorvall SS34 rotor. The pellet was suspended in 0.01 M phosphate buffer-0.001 M EDTA and dialyzed extensively against the same buffer. The concentration of DNA was determined by its absorbance at 260 nm (A_{260}) in 10 mM NaOH.

In vitro synthesis of polyoma [^{3}H]cRNA. Polyoma tritiated complementary RNA ([^{3}H]cRNA) was synthesized in vitro by the method described by Kamen (13). The reaction mixture contained in 250 μl: 40 mM Tris-hydrochloride, pH 7.9; 10 mM $MgCl_2$; 2 mM $MnCl_2$; 16 mM KCl; 5 mM mercaptoethanol; 10 mmol each of tritium-labeled ATP (26 Ci/mmol), UTP (41.5 Ci/mmol), CTP (22 Ci/mmol), and GTP (8.1 Ci/mmol); 30 μg of supercoiled polyoma DNA; and 50 μg of *Escherichia coli* RNA polymerase holoenzyme (kindly provided by S. Leffler, Columbia University, New York, N.Y.). The synthesis was carried out at 37°C for 3 h. The reaction was stopped by incubation for 30 min at 37°C with DNase followed by addition of sodium dodecyl sulfate at a final concentration of 0.2%. The mixture was extracted with chloroform-isoamyl alcohol and then passed through a Sephadex G-75 column equilibrated with 0.1× SSC-0.1% sodium dodecyl sulfate. The excluded peak was kept at −20°C and used for the in situ hybridization experiment. The cRNA obtained was mostly the product of asymmetrical transcription (13), since only 17% of the [^{3}H]cRNA counts were still trichloroacetic acid precipitable after RNase treatment (30 μg/ml for 1 h at 37°C in 2× SSC). The specific activity of the cRNA preparation was 1.4×10^8 dpm per μg.

In situ hybridization. The method was essentially the same as that described by McDougall et al. (15) and Watkins (24). Cultures of cells on glass cover slips were rinsed with Tris buffer, fixed with methanol-acetic acid (1:1, vol/vol) for 10 min, and dehydrated in ethanol. Then the cover slips were air-dried and the DNA was denatured with 0.2 M HCl at room temperature for 20 min. After denaturation, the cover slips were rinsed with chilled 0.1× SSC and dehydrated in alcohol. A 25-μl portion of RNA (~2.5×10^6 dpm) in 5× SSC were placed on the bottom of a flat glass petri dish, and the dried cover slip was inverted onto it. The dish was incubated at 65°C in a covered water bath for 13 h. The cover slips were washed three times in 2× SSC, treated with bovine pancreatic RNase (50 μg/ml) for 45 min at 37°C, and washed extensively in 2× SSC at 4°C. After dehydration with alcohol, the cover slips were mounted on a slide, dipped in NTB-2 Kodak emulsion, exposed for 4 to 8 weeks, then developed in a Kodak D19 developer, and stained with Giemsa prior to counting.

RESULTS

Effect of viral mutations on the presence of free viral DNA in polyoma-transformed rat cells. In a previous paper (19), we reported that all clones of polyoma-transformed rat cells tested contained a small number of nonintegrated viral DNA molecules. This was also the case for cells transformed by two temperature-sensitive polyoma mutants, *ts*-8 (6) and *ts*-a (9), when growing at the permissive temperature (33°C). To determine whether the viral functions affected in these mutants were involved in the maintenance of free viral DNA, *ts*-8- and *ts*-a-transformed rat cells were grown at the nonpermissive temperature of 40°C, and the presence of nonintegrated viral DNA was determined as a function of time at this temperature. *ts*-8-transformed rat cells contained nonintegrated viral DNA even after 2 months of

growth at 40°C (data not shown). Since the *ts*-8 mutation is a late mutation, presumably affecting virus assembly (6), these results show that virion production is not required to maintain the presence of free viral DNA in transformed rat cells. The results also confirm previous data (19), which showed that the presence of free viral DNA in these cells was not due to a virus carrier state.

The results with *ts*-a-transformed cells, on the other hand, showed clearly that the *A* viral function was necessary to maintain the presence of free viral DNA in polyoma-transformed rat cells. *ts*-a-transformed rat cells lost the free viral DNA within 2 to 4 days after shift to 40°C (Fig. 1). Since the *A* polyoma function is necessary for viral DNA synthesis (10), these results suggest that independent viral DNA replication is required for expression of this phenomenon. The possibility that the *ts*-a mutation also affects excision of integrated viral DNA cannot, however, be entirely ruled out (see below).

To test whether *ts*-a-transformed rat cells would have been permanently "cured" after growth at 40°C, the cells were grown at the nonpermissive temperature for variable times

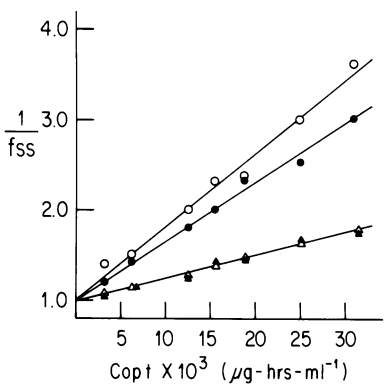

FIG. 1. *Reassociation kinetics of polyoma [^{32}P]DNA in the presence of low-molecular-weight DNA from ts-a rat-23 cells grown at the permissive (33°C) and nonpermissive (40°C) temperature. Hybridization was carried out as previously described (19). Each reaction mixture contained 3 × 10^{-3} μg of polyoma [^{32}P]DNA per ml (specific activity, 1.5 × 10^6 cpm/μg). The data are plotted as $1/f_{ss}$ versus $C_{op} \times t$, where f_{ss} represents the fraction of [^{32}P]DNA that is single stranded, C_{op} the input of probe in micrograms per milliliter, and t the time of sampling in hours. Symbols: (△) renaturation of polyoma [^{32}P]DNA in the presence of low-molecular-weight DNA from untransformed rat cells; (○) DNA from ts-a rat-23 cells grown at 33°C; (▲) DNA from ts-a rat-23 cells grown at 40°C for 8 days; (●) DNA from ts-a rat-23 cells grown at 40°C for 16 days and then shifted to 33°C for 10 days.*

(in one case over 2 months) and then shifted to the permissive temperature. In all cases, free viral DNA became again detectable in the cells within 2 to 4 days (Table 1, Fig. 1). Although the kinetics of reappearance of nonintegrated viral DNA varied slightly from experiment to experiment, in most cases, by 3 days after shift-down the cell had reached the steady-state number of free viral DNA copies (Table 1). These experiments show that the free viral molecules in polyoma-transformed rat cells can originate from the integrated ones. As discussed later, the number of copies of integrated viral genomes in the *ts*-a-transformed rat cells did not vary even after long periods of growth at 40°C. The dilution factor obtained by keeping the cells in constant growth at 40°C for about 2 months (average generation time, 16 h) is very high (~26 logs), and, thus, it is unlikely that the free viral DNA molecules that reappear in the *ts*-a-transformed cells upon shift to 33°C can originate from a few remaining copies of nonintegrated viral genomes.

Effect of mitomycin C. It has been reported that the LPT line of polyoma-transformed rat myoblasts, which spontaneously produces small amounts of virus, yields a large amount of virus and/or viral DNA after treatment with mitomycin C (7, 16). A similar effect has been described in some SV40-transformed hamster cell lines treated with this drug (4). Therefore, the effect of mitomycin C on the presence of free viral genomes in polyoma-transformed rat cells was tested. Cells of Py rat-12 and *ts*-a rat-23 lines were exposed to mitomycin C at 37 and 33°C, respectively, for 24 h. In addition, cells of the *ts*-a rat-23 line that had been first grown at 40°C and then shifted to 33°C were also exposed to the drug for the 24 h immediately after shift-down. Two days later, small-molecular-weight DNA was extracted and the number of viral DNA equivalents was determined. Mitomycin

TABLE 1. *Induction of free viral genomes in ts-a rat-23 cells after shift from 40 to 33°C* [a]

Hours after shift to 33°C	Free viral DNA equivalents/cell
0	0[b]
24	0.6
48	2
72	20
240	20

[a] *ts*-a rat-23 cells were grown at 40°C for 2 weeks. Then they were shifted to 33°C. Low-molecular-weight DNA was extracted from cells after different periods of growth at 33°C. The number of free viral DNA equivalents was then determined as described in the text.

[b] <0.5.

treatment increased the amount of free viral DNA by a factor of 30 to 200 (Table 2).

Since mitomycin C at higher doses is known to inhibit DNA synthesis (14) while our results showed an increase in the number of free viral genomes, the effect of mitomycin C on the replication of viral DNA molecules was determined during polyoma lytic infection. 3T3D cells were either treated with mitomycin C (0.03 μg/ml) for 24 h before infection or exposed to the drug after virus adsorption. In both cases, treatment with mitomycin C inhibited polyoma viral DNA production by about 50% (data not shown). Thus, though the mechanism of action of mitomycin in our system is not clear, it appears unlikely that it acts by stimulating the replication of the free viral DNA molecules.

Virus production. We previously reported that polyoma-transformed rat cells did not generally produce any infectious virus (19). However, on a few occasions cells were found to release small amounts (1 to 10 PFU/10^6 cells) of infectious polyoma virus, and, again, occasionally we detected a few (<0.01%) V-antigen-positive cells in the cultures (19). These observations and the finding that most of the free viral DNA molecules present in these cells were noninfectious (19) prompted us to investigate whether these lines produced noninfectious virus. Large numbers of cells were grown, and virus was extracted and purified by cesium chloride density gradient centrifugation. In some experiments, SV40 was mixed with the crude cell lysate to serve as an internal density marker and to control recovery of the experiment. We tested cells of the Py rat-12 line growing at 37°C and *ts*-a rat-23 cells, which after growth at 40°C had been shifted to 33°C for 3 days.

Purified virus, isolated from the position in the gradient identified by the SV40 marker, was titrated by hemagglutination (HA) and by infectivity. Since HA is a function of polyoma virions, irrespective of whether they contain viral DNA, the ratio PFU/HA gives an indication of the degree of infectivity of the viral preparations (1, 5, 22). This ratio was about 10^5 for our control virus, which had been grown in mouse 3T3 cells. The PFU/HA ratio was much lower for the two preparations of virus extracted from polyoma-transformed rat cells (Table 3).

These results, therefore, show that polyoma-transformed rat cells produce small amounts of virus, which is, however, largely noninfectious.

Since mitomycin C causes a considerable increase in the number of free genome equivalents in polyoma-transformed rat cells, we tested whether mitomycin C also increased virus production. Table 4 shows that mitomycin C substantially increases virus production.

TABLE 2. *Effect of mitomycin C on the amount of free viral DNA in polyoma-transformed rat cells*

Transformed lines	Free viral DNA equivalents/cell	
	Cells exposed to mitomycin C	Cells not exposed to mitomycin C
Py rat-12[a]	1,674	8.6
ts-a rat-23[a]	1,500	53
ts-a rat-23[b] (40 → 33°C)	148	5

[a] Py rat-12 and *ts*-a rat-23 cells were grown at 37 and 33°C, respectively, and exposed to mitomycin C (0.03 μg/ml) for 24 h. Then they were washed free of the drug and incubated in fresh medium for 48 h. At that time, low-molecular-weight DNA was extracted, and the number of viral DNA equivalents was determined as described in the text.

[b] *ts*-a rat-23 cells growing at 40°C were shifted to 33°C. At the same time they were treated with mitomycin (0.03 μg/ml), which was kept in the medium for 24 h. After washing and incubation in fresh medium for 48 h, low-molecular-weight DNA was extracted from the cultures, and the number of viral DNA equivalents was determined.

TABLE 3. *Spontaneous yield of virus from polyoma-transformed rat cells*

Cells	HA	PFU/ml	PFU/HA
Py rat-12[a]	80	1×10^3	12
ts-a rat-23[b] (40 → 33°C)	200	5×10^4	250
3T3[c]	6,400	7×10^8	1.1×10^5

[a] Virus was extracted from 7×10^8 cells grown at 37°C, concentrated, and purified as described in the text. Its infectivity was then determined by plaque assay on 3T3 cells, and it is expressed in plaque-forming units per milliliter. HA activity was determined on guinea pig erythrocytes and is expressed as the reciprocal of the dilution giving complete agglutination. Total yield from 7×10^8 cells was 2×10^3 PFU.

[b] Virus was extracted from 5×10^8 cells that had been first grown at 40°C for 15 days and then shifted for 3 days to 33°C, purified, and titrated as in *a*. Total yield was 5×10^4 PFU.

[c] 3T3 cells were infected with polyoma at a multiplicity of infection of 50 PFU/cell and incubated for 3 days at 37°C. Virus was extracted, purified, and titrated as in *a*.

Presence of integrated viral genomes. The presence of nonintegrated viral genomes in polyoma-transformed rat cells makes it more difficult to determine the number of genome equivalents associated with the host cell genome (integrated). We attempted two methods to obtain pure preparations of chromosomal DNA free of contaminating free viral DNA molecules. In

TABLE 4. *Effect of mitomycin on production of infectious virus in polyoma-transformed rat cells*[a]

Transformed line	PFU/10^6 cells	
	Exposed to mitomycin C	Not exposed to mitomycin C
Py rat-12	4×10^3	<5
ts-a rat-23	3×10^4	50
ts-a rat-23 (40 → 33°C)	10^3	20

[a] Py rat-12 and *ts*-a rat-23 cultures were exposed to mitomycin C (0.03 μg/ml) for 24 h at 37 and 33°C. The cells were then washed and incubated in fresh medium for 48 h. *ts*-a rat-23 (40 → 33°C) cells were first incubated at 40°C and then shifted to 33°C. The cells were exposed to mitomycin C for 24 h starting at the time of shiftdown and then were incubated in mitomycin C-free medium for 48 h. Virus was extracted, and its infectivity was titrated by plaque assay on 3T3 mouse cells.

the first method, the Hirt pellet was extracted and high-molecular-weight DNA was then purified by sucrose density gradient sedimentation at neutral pH. DNA sedimenting faster than 50S was then analyzed for the presence of polyoma viral DNA sequences (19). In the second method, we used cellular DNA sequences prepared by the network technique (23) as described above. The cellular DNA preparations should contain highly repetitive DNA sequences and viral DNA, if any, covalently joined with them (23). Cell DNA purified in this manner was analyzed for its ability to influence the rate of reassociation of polyoma [^{32}P]DNA. Chromosomal DNA from Py rat-12 cells grown at 37°C and *ts*-a rat-23 grown at 40°C produced a similar enhancement of the reassociation rate of the probe. Calculations made as described by Gelb et al. (11) (assuming the molecular weight of polyoma DNA to be 3×10^6) reveal that the number of polyoma DNA equivalents (per diploid cell genome) associated with chromosomal DNA was 7.5 for Py rat-12 and 8 for *ts*-a rat-23 (Fig. 2).

Py rat-13 cells also appear to contain approximately six viral genome equivalents per diploid quantity of cell DNA (19). Whereas some degree of contamination of the high-molecular-weight cell DNA from free viral DNA in the wild type-transformed lines cannot be excluded, the fact that the values obtained are similar to those obtained for *ts*-a-transformed cells kept at 40°C, conditions under which these cells do not contain detectable free viral DNA, makes us confident that the values obtained for these lines reflect a stable association of more than one genome equivalent of polyoma DNA with the genome of the transformed rat cells. It is worth mentioning that F. Birg, M. Fried, R. Dulbecco, and R. Kamen (manuscript in preparation) have also identified in other clones of independently transformed rat F2408 cells a relatively large number (8 to 10) of integrated polyoma DNA molecules. In addition, these authors have obtained data suggesting that in many of these transformants viral DNA molecules are integrated in a tandem fashion.

Identification of the cells containing free viral DNA. The finding that polyoma-transformed rat cells contained, on the average, 20 to 50 copies of nonintegrated viral DNA molecules per cell (19) did not indicate whether every cell in the transformed populations contained 20 to 50 free polyoma DNA molecules, or whether only a minority of the cells contained a larger number of viral DNA copies. To distinguish between these two hypotheses, we performed experiments of in situ hybridization (18), using as a probe polyoma [^{3}H]cRNA, transcribed in vitro with *E. coli* RNA polymerase. Cells that had been growing on cover slips were fixed, and hybridization was performed as described above. The autoradiographs were exposed for

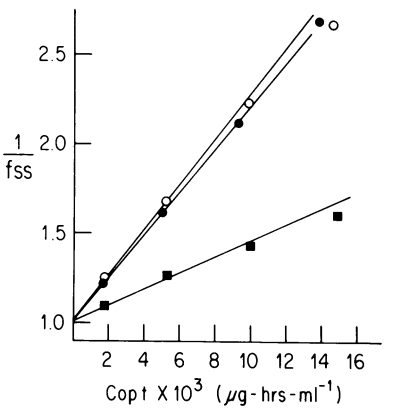

FIG. 2. *Renaturation of polyoma [^{32}P]DNA in the presence of unlabeled DNA prepared by "network formation" from polyoma-transformed rat cells. Each reaction mixture contained 500 μg of cellular DNA per ml and 2.5×10^{-3} to 2.7×10^{-3} μg of polyoma [^{32}P]DNA per ml (specific activity, 2.0×10^6 cpm/μg). The hybridizations were carried out at 68°C essentially as in (21). Before hybridization, cellular DNA was fragmented by sonic treatment and subsequently boiled together with the probe for 10 min in 0.3 M NaOH. Samples were removed from the mixtures at intervals, and the fraction of single-stranded [^{32}P]DNA (f_{ss}) was determined by chromatography on hydroxyapatite. The data are plotted as $1/f_{ss}$ versus $C_{op} \times t$, where C_{op} represents the input of probe in micrograms and t the time of sampling in hours. Symbols: (■) renaturation of polyoma [^{32}P]DNA in the presence of DNA from normal rat cells (control); (○) DNA from ts-a rat-23 grown at 40°C; (●) DNA from Py rat-12 cells.*

different periods of time and developed, and the number of cells containing a significant number (over the background) of silver grains was counted. 3T3 mouse cells productively infected by polyoma virus at a multiplicity of infection of 10 PFU/cell and fixed at 30 h after infection were used as positive controls. Since we did not expect the sensitivity of the assay to be high enough to detect integrated viral DNA, we also tested *ts*-a-transformed cells kept at 40°C in addition to untransformed rat cells as negative controls. The results of these experiments (Table 5, Fig. 3), showed clearly that only a small number of cells in the populations of *ts*-a rat-13 cells at 33°C or Py rat-12 cells at 37°C contained an amount of viral DNA sequences sufficient to give detectable hybridization with polyoma cRNA. Thus we conclude from these experiments that the presence of free viral DNA in polyoma-transformed rat cells is restricted to a minority of the cell population at any given time.

DISCUSSION

In a previous paper (19) we showed that rat cells transformed by polyoma virus contained a small number of nonintegrated viral DNA molecules. These molecules were mostly in the form of superhelical closed circles and had low infectivity. We have now investigated the effect of viral mutations on the viral DNA state in the polyoma-transformed rat cells, and we also studied possible mechanisms leading to production of free viral DNA in these cells.

The results showed that in cells transformed by a late temperature-sensitive mutant of polyoma virus, growth at the nonpermissive temperature did not have appreciable effect on the presence of free viral DNA. In cells transformed by the *ts*-a early mutant, on the other hand, 3 to 4 days of growth at the nonpermissive temperature were sufficient to bring the quantity of free viral DNA below detectable levels. Cells growing at 40°C for as long as 2 months reacquired free viral DNA within 2 to 4 days after shift to the permissive temperature.

TABLE 5. *Frequency of polyoma-transformed rat cells producing free viral DNA as detected by in situ hybridization with polyoma cRNA*

Transformed lines and controls	No. of cells scored[a]	No. of positive nuclei	%
Py rat-12	4×10^4	31	0.08
ts-a rat-13 (40°C)	5×10^4	0	–
ts-a rat-13 (33°C)	8×10^4	158	0.2
Polyoma-infected 3T3[b]	690	171	24.8
Rat cells	5×10^4	0	–

[a] This number (except in the case of the infected 3T3 cells) is approximate. It was calculated on the basis of the average cell density on the cover slip and the total cover slip area scored.

[b] Mouse 3T3 cells infected with polyoma virus at 10 PFU/cell and fixed for in situ hybridization 30 h after infection.

This finding shows that the *A* polyoma function, which is necessary for polyoma DNA replication (10), is also necessary for the maintenance of the free viral DNA in polyoma-transformed rat cells. The simplest interpretation of this result is that free viral DNA molecules have to undergo a certain degree of independent replication to reach detectable numbers. The observation that *ts*-a-transformed cells kept at 40°C quickly reacquire free viral DNA when shifted to 33°C suggests that free viral DNA can originate from integrated molecules, presumably by a mechanism of excision and replication. It is thus possible that in the *ts*-a-transformed cells at 40°C not only is viral DNA replication defective, but there may be also an inhibition of the excision process. Alternatively, viral DNA replication and excision may be coupled. Folk and Bancuk (8) have described a similar phenomenon occurring in polyoma *ts*-a-transformed BHK hamster cells continuously grown at 39.5°C when the cells are shifted to the permissive temperature.

To understand whether all the cells in the transformed population carried a small number of free viral DNA molecules or whether a minority of the cells had larger amounts of viral DNA, we performed experiments of in situ hybridization (18). The results obtained show that only a small proportion of the cells contain a detectable amount of free viral DNA at any given time. The exact frequency is difficult to assess, however, as it is likely that the cells containing free viral DNA will have a wide range of amounts of viral DNA molecules. It is possible that by our technique we could not detect cells having a small number of molecules; the frequency we measured will have to be considered a minimal estimate. In any case, however, it is unlikely that the total frequency of induced cells exceeds 1% at any given time.

In view of these results, it is likely that the presence of free viral DNA in polyoma-transformed rat cells is caused by a temporary induction of viral DNA replication occurring with a low but constant probability in the cell population. These data, together with the results on *ts*-a-transformed cells upon shift from 40 to 33°C, suggest that free viral DNA originates from integrated molecules by a phenomenon of excision and replication similar, at least superficially, to lysogenic induction. What determines the probability of this event is not clear.

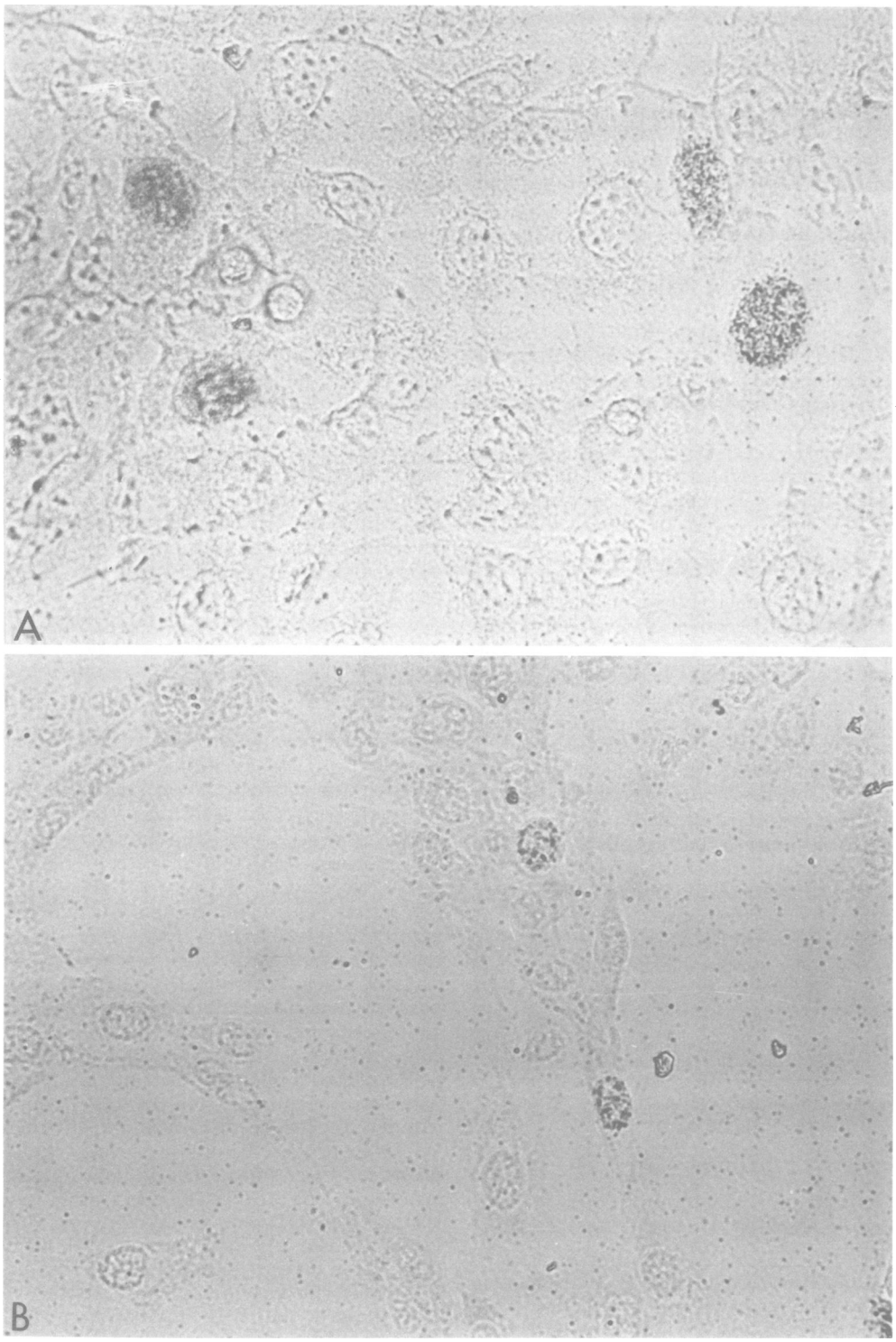

FIG. 3. *Autoradiographs of cells showing in situ hybridization of polyoma [^{3}H]cRNA. Exposure was carried out for 8 weeks. (A) 3T3 cells infected with polyoma virus and fixed at 30 h after infection; (B) cells of the Py rat-12 line. Although this autoradiograph shows two positive cells, the finding of more than one positive cell in a single microscopic field was exceptional.*

It is worth noting that a similar situation has been found to occur in various lines of human cells transformed by SV40 (M. Botchan, J. McDougall, and J. Sambrook, manuscript in preparation). It would be also interesting to ascertain the fate of cells that have undergone induction of viral DNA synthesis. If they are not killed, a certain proportion of them may be cured of viral DNA.

The similarity of the phenomenon in polyoma-transformed rat cells with the situation described by other authors in certain clones of polyoma- or SV40-transformed cells (4, 7, 16) prompted us to investigate the effects of mitomycin C in our system. Treatment with mitomycin C for 24 h produces a considerable increase (30- to 100-fold) in the quantity of free viral DNA and some infectious virus. The mechanism of action of mitomycin is unclear. It is unlikely that it stimulates directly the replication of viral DNA, and our experiments with mitomycin treatment of lytically infected cells showed, in fact, a certain degree of inhibition of polyoma DNA replication. Thus, it is possible that mitomycin directly enhances excision. This has been suggested by Rakusanova et al. (20) on the basis of experiments in which SV40-transformed cells were treated with mitomycin and DNA synthesis inhibitors, and the distribution of the viral DNA from high (presumably associated with host DNA) to low molecular weight (presumably free) was followed. In our case, excision in a larger proportion of the cells followed by the same extent of replication would produce a higher average number of free viral DNA copies. We, thus, tested the possibility that mitomycin stimulated excision in our system in an experiment similar to that described by Rakusanova et al. (20), using *ts*-a-transformed rat cells treated with mitomycin at 40°C. Since the *ts*-a mutation inhibits polyoma DNA replication, any free viral DNA detected after mitomycin treatment would have probably been produced by excision of the integrated genomes. The results, however, were negative; i.e., we did not detect any free viral DNA in the *ts*-a-transformed rat cells at 40°C even after mitomycin treatment.

That mitomycin induced production of some infectious virus and the fact that the free viral DNA molecules found in the transformed rat cells were mostly noninfectious (19) suggested the possibility that these cells produced noninfectious virus. It was found, in fact, that these cells produce small amounts of virus, which on the basis of the PFU/HA ratio also seems to be mostly noninfectious. The exact reduction of infectivity of this virus is, however, difficult to determine since the amounts produced are quite small.

In conclusion, our results suggest that the association of polyoma viral DNA with the host DNA in transformed rat cells is not absolutely stable, so that with low but constant probability a small number of cells in the transformed population is "induced," producing a certain amount of free viral DNA and a small amount of virions. The "induction" requires the *A* viral function; this requirement could be simply due to the fact that this function is necessary for viral DNA replication, but it is possible that the *A* function is also necessary for excision or that some viral DNA replication actually preceeds and causes excision. In this respect, it is worth mentioning that most of the free viral DNA in polyoma-transformed rat cells seems to be defective (19). The study of the nature of these defective molecules, which is now in progress, may, thus, help in understanding their production and the mechanisms maintaining the association between the viral and the cellular DNA in polyoma-transformed cells.

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