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Multiple Free Viral DNA Copies in Polyoma Virus-Transformed Mouse Cells Surviving Productive Infection

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Mouse 3T6 cells were infected with polyoma virus at high multiplicity, and survivors were isolated. Clones from single cells were then established and were found to be resistant to a second infection. However, in some clones viral functions could at least be partially expressed during reinfection, as judged from a stimulation of nuclear tumor antigen expression. One such clone was studied in detail. These cells were transformed and produced low amounts of virus (less than 1 PFU per cell per generation). The persistent infection did not seem to be a carrier-state phenomenon, since infectious-center assays showed that most cells produced virus. The resistance of the cells to reinfection can be explained by interference from viral DNA present in the cells, averaging about 1,500 "free" copies per cell. This DNA had the normal physical characteristics of polyoma DNA. However, it had a slightly larger size than authentic polyoma DNA. Mapping with restriction endonucleases showed that the addition to the DNA was about 5% of the wild-type genome and was located close to the origin of DNA replication. This DNA was infectious, although it had a 10-fold lower infectivity than wild-type polyoma DNA. Both virus and DNA from the polyoma-resistant cells had a small-plaque morphology, as opposed to the large-plaque morphology of the virus used for the initial selection of cells.

Polyoma virus can interact with infected cells in two ways (21). Mouse cells in culture are permissive and support a productive infection cycle. A large number of viral DNA molecules are synthesized in the cells. Within 2 days, the infected cells die and progeny virus is released. In contrast, infection of semi- or nonpermissive hamster or rat cells in culture results in an abortive infection cycle. Only little viral DNA and progeny virus is produced (3), and the majority of the cells survive the infection. In this case, some of the infected cells undergo malignant transformation. The transformed cells show a number of new characteristics, of which the most easily studied relate to the growth properties in vitro, e.g., a lowered serum requirement for growth and ability of anchorage-independent multiplication. In polyoma- and simian virus 40-transformed cells, a few viral genomes are present (7, 11). Normally these are found in association with cellular DNA, probably linearly integrated in the host genome.

In polyoma-transformed nonpermissive cells, only a part of the viral genome is expressed (11). It is unclear what factors limit the expression of viral genes in these cells. However, the restriction is not absolute, since such cells can be induced to produce virus (4, 5). The occurrence of transformed permissive cells is a rare event, since there is no restriction on the expression of viral genes and therefore the cells do not normally survive the infection. However, polyoma-transformed mouse cells can be obtained. Vogt and Dulbecco (22) isolated mouse embryo cells surviving polyoma infection. The survivors, some of which showed transformed growth characteristics, were resistant to reinfection with polyoma, probably as a result of the isolation procedure. Benjamin (1), using another selection procedure, isolated polyoma-transformed mouse 3T3 cells that could support a lytic infection cycle of polyoma after reinfection.

Here we report on the properties of clonal isolates of mouse 3T6 cells surviving polyoma infection, and particularly on the amounts and characteristics of viral DNA isolated from these cells.

MATERIALS AND METHODS

For many of the details concerning general methodology, previous publications should be consulted (15, 24).

Cells and media. Mouse fibroblast 3T6 cells were grown in Dulbecco's modification of Eagle medium supplemented with 5% fetal calf serum (Bio-Cult).

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For virus assays, secondary whole mouse embryo cells were used. These were grown in Dulbecco medium containing 10% fetal calf serum. After infection, the cells were overlaid with Dulbecco medium containing 0.9% agar and 3% horse serum. Assays for growth of cells in soft agar were done according to Macpherson (14). The cells were plated in 0.33% agarose (SeaKem) on an underlayer of 0.5% agarose in Dulbecco medium supplemented with 5% fetal calf serum and 10% tryptose phosphate broth.

Virus. The polyoma virus was of the A-2 type of the Pasadena large-plaque strain (9). Virus stocks were prepared by infecting cultures of primary mouse kidney cells at a multiplicity of 0.01 PFU/cell with virus from a single plaque.

Infection of cells. Infection of monolayer cell cultures was done according to standard procedures. Transfection of cells with polyoma DNA was done as described for polyoma by Miller and Fried (17), using the DEAE-dextran technique. Infectious-center assays were done according to Folk (5) as follows. Indicator cells grown in 50-mm petri dishes were overlayed with 1.5 ml of 0.9% agar medium. When the agar had solidified, another 1.5 ml of 0.9% agar medium containing the cells was added. Finally, the second layer was overlaid with 5 ml of 0.9% agar medium.

Immunofluorescent staining. Cells were grown on cover slips, washed in Tris-buffered saline (5), and briefly swelled in distilled water before fixation in acetone. The cover slips were stored at −20°C. T-antigen staining was done using hamster T-antiserum obtained from M. Fogel and fluorescein-conjugated rabbit anti-hamster globulin. The cells were immediately examined by fluorescence microscopy.

Preparation of viral DNA. The DNA was labeled with [^{3}H]thymidine (20 Ci/mmol; New England Nuclear Corp.) added directly to the culture medium at a concentration of 20 μCi/ml. The cells were lysed in 0.7% sodium dodecyl sulfate (SDS), and low-molecular-weight DNA was extracted according to Hirt (10). High-molecular-weight DNA was recovered from the Hirt pellet by dissolving it in distilled water at 50°C. DNA was then purified by phenol extraction and concentrated by precipitation in 70% ethanol at −20°C. Viral DNA was further purified by equilibrium centrifugation in CsCl-propidium diiodide (PDI) density gradients (δ = 1.54 g/cm^3, 0.2 mg of PDI per ml). The covalently closed circular fraction of the DNA was collected, and the PDI was removed by extraction with isopropanol (equilibrated with an aqueous CsCl solution). The DNA was again concentrated by ethanol precipitation and sedimented in 5 to 20% neutral sucrose gradients. Material sedimenting around 20*S* in these gradients was used for further experiments.

Restriction endonuclease digestion of DNA. The restriction endonuclease from *Haemophilis parainfluenza* (endo R·*Hpa* II) was purified as described by Sharp et al. (20). The restriction endonucleases from *Escherichia coli* RY-13 (endo R·*Eco*RI), *Bacillus amyloliquefaciens* H (endo R·*Bam*HI), and *Haemophilus haemolyticus* (endo R·*Hha* I) were obtained from U. Pettersson and B. Griffin. Enzyme digestions were carried out at 37°C for 2 h in 10 mM Tris-chloride (pH 7.5), 10 mM $MgCl_2$, and 1 mM dithiothreitol. For endo R·*Eco*RI digestions, 0.1 M NaCl was included. The reactions were stopped by the addition of SDS to 0.5%. The samples were stored frozen and were heated to 50°C for 15 min before gel electrophoresis.

Gel electrophoresis. Electrophoresis was carried out in cylindrical gels (6 by 150 mm) consisting of 2.9% acrylamide, 0.15% bisacrylamide, and 0.5% agarose in 0.09 M Tris-borate buffer (pH 8.3), 0.0025 M EDTA, 10% glycerol, and 0.15% SDS (18). After electrophoresis, the gels were fractionated with a Gilson gel slicer and analyzed for radioactivity.

RESULTS

Isolation of cells surviving polyoma infection. Growing 3T6 mouse fibroblast cells were infected with polyoma virus at a multiplicity of about 50 PFU per cell. Two days after infection, most cells had died. However, occasional surviving cells were observed. The cultures were left for a total of 4 days before the medium was changed, to ensure that all susceptible cells were infected. Under these conditions surviving cells grew out, forming colonies. In reconstruction experiments, the frequency of colony formation was about one in 10^4 infected cells. The surviving cells were grown to a mass culture. Clones from single cells were then established. Seven such clones were collected and propagated.

We wanted to study only cells that were not deficient in virus adsorption or penetration. The clones were therefore analyzed for the presence of the virus-induced T-antigen before and after reinfection, because appearance of T-antigen after reinfection shows that the virus is both adsorbed and expressed. One set of cultures was infected at high multiplicity, and another set was left uninfected. Fifty hours after reinfection, the cells were fixed and treated with T-antiserum. With the dilution of antiserum used in this experiment (1:32), the uninfected cells were T-negative. After infection, one clone (clone 4) showed about 20% T-positive cells. Of the other six clones, four showed occasional T-positive cells (<5%). Cells from clone 4 were then recloned, and six colonies from single cells were picked and propagated. Of these clones, only two showed T-positive cells after reinfection with polyoma virus (clones 41 and 43), which indicates either that clone 4 was not homogenous or that the ability of the cells to support T-antigen expression was easily lost. In no case was any cytopathic effect observed after the virus infections. Clone 41 was used for further studies.

Properties of 3T6 clone 41 cells. To investigate whether the resistance to superinfection

with polyoma virus was a stable characteristic of the 3T6 clone 41 cells, the cells were propagated for about 3 months. Again reinfection at about 50 PFU/cell did not result in any detectable cell death. However, reinfection no longer resulted in a stimulation of T-antigen expression. Both before and after reinfection, about 2% of the cells expressed T-antigen. This result indicates that during passage in culture the cells became completely nonsusceptible to reinfection with polyoma virus.

During growth on a plastic surface, the morphology of clone 41 cells was similar to that of the parental 3T6 cells. In medium containing 5% fetal calf serum, both the growth rate and the final cell density were similar for the two kinds of cells (Fig. 1). However, the clone 41 cells were considerably more difficult to maintain in crowded cultures. Many of the cells were loosely attached to the surface and shed off into the medium. In medium containing 0.5% fetal calf serum, the clone 41 cells had a shorter doubling time than 3T6 cells. However, both types of cells grew to a similar final density, which was about five times lower than the saturation density in 5% serum (Fig. 1). In medium with a low serum concentration, the clone 41 cells were easy to maintain in crowded cultures. Under these conditions the cells formed a monolayer, being nonrefractive in the light microscope.

We tested the clone 41 cells for anchorage-independent growth by plating them in soft agar. Uninfected 3T6 cells were used as a negative control in this experiment. Clone 41 cells had a high plating efficiency in soft agar (Table 1). In 12 days about 40% of the cells grew out to

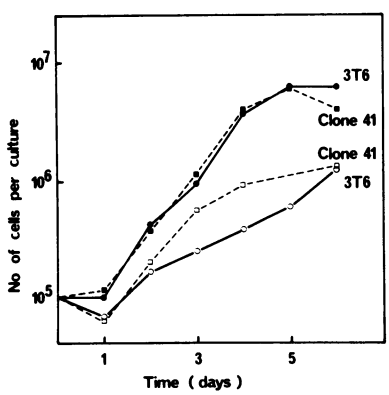

FIG. 1. *Growth curves of 3T6 and clone 41 cells in 5 or 0.5% fetal calf serum. Cells were seeded at a density of 10^5 per 50-mm petri dish. Medium was changed after 3 days. Cells from duplicate cultures were counted in a hemocytometer. Open symbols represent cultures grown in 0.5% serum.*

TABLE 1. *Colony formation of cells in soft agar*

Cell type	No. of cells plated	Colony formation (%)	Colony size
3T6	10^4	0	
3T6	10^5	5.1	Small
Clone 41	10^4	44	Large
Clone 41	10^5	45	Large

form large colonies. The 3T6 cells, in contrast, grew poorly. When the cells were plated at a high density, about 5% formed small colonies. These experiments clearly showed that the clone 41 but not 3T6 cells fulfilled the usual criteria for transformation. We believe that the cells were transformed by polyoma virus and that the low frequency of cells being T-antigen positive only reflected the insensitivity of our polyoma T-antigen assay.

The production of polyoma virus by clone 41 cells was tested before and after reinfection. Permissive 3T6 cells were similarly tested as a control (Table 2). Infected 3T6 cells produced approximately 10^{10} PFU/culture, corresponding to a few thousand PFU/cell. Clone 41 cells produced a constant amount of virus whether they were infected or not. From this experiment it is not clear whether the virus produced by a growing culture of clone 41 cells represented a normal burst produced by just a few cells or whether all cells produced a small amount of virus. Two things are clear, however: (i) superinfection of clone 41 cells with polyoma did not increase the yield of virus, and (ii) clone 41 cells in both situations produced virus giving small plaques on mouse embryo cells. This is noteworthy, since large-plaque virus was used both for the original selection of surviving cells and for the reinfections.

To determine the proportion of clone 41 cells that were producing virus, an infectious-center assay was done. Fifty, 200, or 1,000 cells were plated on mouse embryo indicator cells. Most of the clone 41 cells produced virus or at least could be induced to produce virus when their growth was arrested by plating in 0.9% agar (Table 3). The exact proportion was hard to assess, since the plating of few cells is inexact. All plaques were small and of similar size, indicating that the amount of virus produced by individual clone 41 cells did not vary too much.

Polyoma DNA in clone 41 cells. Clone 41 cells were analyzed for the presence of polyoma DNA by labeling growing cells with [^{3}H]thymidine for 20 h and then extracting low-molecular-weight DNA. This fraction of the DNA, which contained 1.5% of the total radioactivity, was further purified by equilibrium centrifugation in CsCl-PDI. In the gradient,

TABLE 2. *Assay of infectious particles from uninfected and infected 3T6 and clone 41 cells*[a]

Cell type	Virus yield/ culture (PFU)	Plaque morphology
3T6 mock infected	$<2 \times 10^2$	
3T6 infected	1.1×10^{10}	Large
Clone 41 mock infected	3.7×10^7	Small
Clone 41 infected	1.7×10^7	Small

[a] The cell cultures with medium were harvested 4 days after infection, and virus was extracted by three cycles of freezing and thawing.

TABLE 3. *Infectious-center assay of 3T6 and clone 41 cells*

Cell type	No. of cells plated	No. of plaques[a]
3T6	1,000	0, 0, 1
Clone 41	50	20, 35, 37
Clone 41	200	>100
Clone 41	1,000	Semiconfluent lysis

[a] Values represent plaque counts from triplicate assays.

11% of the radioactivity banded in the position of covalently closed circular DNA (form I), whereas the rest of the radioactivity banded in the position of relaxed DNA. The two pools of DNA were collected and analyzed by annealing to polyoma DNA bound to nitrocellulose filters (Table 4). About 40% of the radioactivity in the form I DNA bound to the filters, whereas the corresponding value for the relaxed DNA fraction was much lower. From this experiment it is clear that there was a considerable amount of intracellular polyoma DNA in the form of covalently closed circles. The two fractions of DNA from the CsCl-PDI gradient were then analyzed by sedimentation in neutral sucrose gradients (Fig. 2). The ^{3}H-labeled form I DNA (Fig. 2A) formed a major peak cosedimenting with a polyoma form I DNA marker (20S). A second, smaller peak, probably consisting of mitochondrial DNA, sedimented faster, with an approximate *S* value of 35. The molecules sedimenting at 20S contained about 45% of the total radioactivity in the gradient, which was in good agreement with the proportion of the radioactivity binding to polyoma filters (Table 4). The fraction from the CsCl-PDI gradient containing relaxed DNA had a different sedimentation profile (Fig. 2B). The labeled DNA sedimented as a broad peak extending from the bottom to the top of the gradient. Little DNA was found to sediment at the position of the polyoma DNA marker.

These experiments show that the clone 41 cells contained a relatively large number of "free" polyoma DNA molecules. A rough calculation of the average number of copies per cell was obtained by relating the radioactivity in polyoma DNA to the total radioactivity in cellular DNA. Thus it appears that about 0.12% of the total labeled DNA was polyoma specific. This assumes that the DNA recovered from the Hirt pellet did not contain large amounts of polyoma DNA, which was confirmed in a separate experiment. Knowing the approximate

TABLE 4. *Annealing of labeled DNA to polyoma DNA bound to nitrocellulose filters*[a]

Origin of radioactive DNA (cpm)	Input radioactivity (cpm)	Input bound to filters (cpm)	Input bound to filters (%)	
			Uncorrected	Corrected
Authentic polyoma	9,827	6,295	64.1	100
Closed circular[b]	7,770	1,980	25.4	39.6
Relaxed[b]	11,720	360	3.0	4.7

[a] Nitrocellulose filters containing 1 μg of polyoma DNA were incubated with radioactively labeled sonically sheared DNA in 4× SSC and 0.1% SDS at 65°C for 24 h as described before (24).

[b] DNA fractions from a CsCl-PDI gradient.

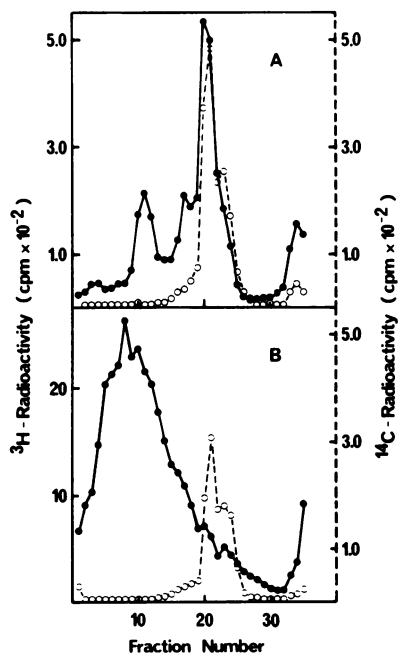

FIG. 2. *Sedimentation of DNA in neutral sucrose gradients. ^{3}H-labeled low-molecular-weight DNA from clone 41 cells was fractionated in a CsCl-PDI gradient into a convalently closed (A) and a relaxed (B) fraction and mixed with ^{14}C-labeled polyoma form I marker DNA. The DNA was then sedimented in neutral 5 to 20% sucrose gradients. Direction of sedimentation was from right to left. Symbols:* (●) *^{3}H;* (○) *^{14}C.*

molecular weight of a diploid mammalian genome (4×10^{12}) (7) and polyoma DNA (3×10^6), 0.12% of the total DNA mass corresponds to 1,600 copies of polyoma DNA.

Characterization of polyoma DNA from clone 41 cells. Polyoma form I DNA labeled with [^{3}H]thymidine was isolated from clone 41 cells and then purified by equilibrium centrifugation in CsCl-PDI and sedimentation in neutral sucrose gradients. The size of the closed circular DNA was determined by electrophoresis in agarose gels before and after digestion with endo R·*Eco*RI or endo R·*Bam*HI. In all cases the clone 41 DNA migrated as a symmetric peak with a slightly lower mobility than that of authentic polyoma DNA (data not shown). The experiments show that the clone 41 DNA was slightly larger than polyoma DNA and, like authentic polyoma DNA, had one cleavage site for each of the two restriction endonucleases.

To localize the addition in the clone 41 DNA, the DNA was cleaved with a mixture of endo R·*Bam*HI and endo R·*Hha* I. By cleaving polyoma DNA with these two enzymes, four fragments were generated. The size of the fragments was measured by gel electrophoresis (Fig. 3). It is clear that fragments 1, 2, and 4 were of identical size in clone 41 and wild-type polyoma DNA. However, fragment 3 of clone 41 DNA was significantly larger than the corresponding wild-type fragment. It is also clear that the clone 41 DNA did not contain any wild-type DNA molecules, since no ^{3}H label was present in the position of wild-type fragment 3.

To get a more precise location of the addition in the clone 41 DNA, the DNA was cleaved with endo R·*Hpa* II. Digestion of wild-type polyoma DNA with this enzyme resulted in the formation of eight fragments (Fig. 4). The previous experiment indicated that the addition in clone 41 DNA was located in *Hpa* II fragment 3 or 5. After *Hpa* II cleavage, all restriction fragments formed from wild-type DNA were also formed from clone 41 DNA. However, cleavage of clone 41 DNA generated an extra fragment with a slightly higher mobility than that of fragment 7. In the figure only the first seven fragments of wild-type DNA are shown. Fragment 8 was run off the end of the gel to improve the separation of the other fragments. In separate experiments, it was shown that clone 41 DNA contained normal amounts of *Hpa* II fragment 8 and no other small fragments. In Fig. 5, the logarithm of the radioactivity of each *Hpa* II fragment has been plotted versus the mobility of the respective fragment. The result shows that cleavage of both wild-type and clone 41

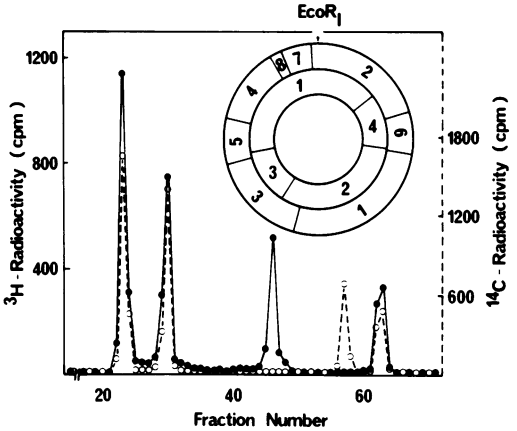

FIG. 3. *Digestion of closed circular polyoma clone 41 DNA with a mixture of endo R·BamHI and endo R·Hha I. ^{3}H-labeled polyoma clone 41 DNA was mixed with ^{14}C-labeled authentic polyoma DNA and digested with the restriction endonucleases. The generated fragments were separated by gel electrophoresis as described in Materials and Methods at 80 V for 14 h. Symbols: (●) ^{3}H; (○) ^{14}C. The inset shows the physical map of polyoma DNA, as determined by Griffin et al. (8, 9), with the cleavage sites for endo R·BamHI, Hha I, and Hpa II oriented relative to the single endo R·EcoRI site. The numbers in the outer circle represent the Hpa II fragments, and the numbers in the inner circle represent the fragments generated by cleavage with both endo R·BamHI and Hha I.*

DNA with endo R·*Hpa* II resulted in molar yields of all fragments.

The restriction endonuclease digestions demonstrate that the addition in clone 41 DNA was located between *Hpa* II fragments 3 and 5. The extra *Hpa* II fragment in clone 41 DNA was about 4.5% of the genome, corresponding to about 230 base pairs.

Infectivity of clone 41 polyoma DNA. The infectivity of polyoma DNA purified from clone 41 cells was tested on secondary mouse embryo cells. Similarly purified wild-type polyoma DNA was used as a control. The cells were transfected by the DEAE-dextran technique. The specific infectivity of clone 41 DNA was 4×10^4 PFU per μg of DNA, whereas the corresponding value for wild-type DNA was 3×10^5 PFU per μg of DNA. Clone 41 DNA produced small plaques as opposed to the large-plaque morphology of wild-type DNA. To exclude that the infectivity observed with clone 41 DNA was due to the presence of wild-type DNA in the clone 41 DNA preparations, individual plaques were picked. The virus of these plaques was then propagated by two cycles of infection on 3T6 cells. During the second cycle of infection,

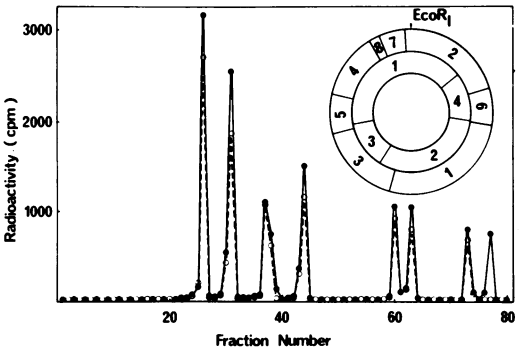

FIG. 4. *Endo R·Hpa II cleavage of closed circular polyoma clone 41 DNA. ^3H-labeled polyoma clone 41 DNA mixed with ^{14}C-labeled authentic polyoma DNA was digested with endo R·Hpa II and the generated fragments were separated by gel electrophoresis at 100 V for 8 h. Symbols: (●) ^3H; (○) ^{14}C. The inset is the same as in Fig. 3.*

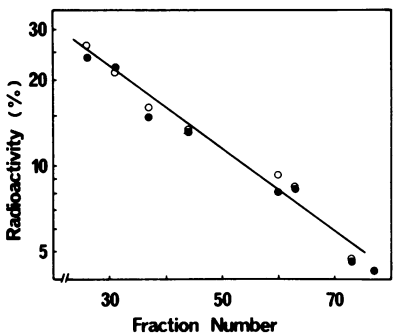

FIG. 5. *Diagrammatic representation of the logarithm of the radioactivity of the restriction fragments from Hpa II cleavage (Fig. 4) plotted versus the electrophoretic mobility of respective fragment. Symbols: (●) Clone 41 polyoma DNA; (○) authentic polyoma DNA.*

the DNA was labeled with [^{3}H]thymidine and viral DNA was purified. Cleavage of the DNA with endo R·*Hpa* II showed that the viral DNA indeed had the same structure as the DNA isolated from clone 41 cells. Thus the clone 41 polyoma DNA was infectious, but the specific infectivity was about one-tenth that observed for wild-type polyoma DNA.

DISCUSSION

The properties that make a cell permissive for polyoma virus infection are not well understood. A basic requirement for permissiveness is that the virions be adsorbed to the cell surface, penetrate into the cell, and be uncoated. These requirements are fulfilled in both permissive mouse cells and nonpermissive hamster or human cells (2, 13). In infected mouse cells, viral early functions are first expressed, followed by replication of viral DNA and expression of late genes. In hamster or rat cells, the polyoma infection is abortive. Early viral genes are expressed and remain continuously expressed in the fraction of the cells that becomes transformed (11). Normally there is no replication of "free" viral DNA or expression of late genes in transformed cells. However, also in nonpermissive cells progeny virus can be obtained. BHK cells transformed with a temperature-sensitive mutant of polyoma (ts-a) did not produce virus when maintained at 39°C, but were induced to produce progeny virus after a shift-down of temperature (5). Clones of polyoma-transformed rat cells were spontaneously producing virus at a low frequency and could by certain manipulations be induced to produce virus at a high frequency (4). These findings indicate that cells which normally are nonpermissive for polyoma can, under certain conditions, support a normal lytic growth cycle.

Furthermore, there are reports of abortive polyoma and simian virus 40 infection of permissive cells. In most of these cases the transforming virus was defective in productive infection (21). There might, however, be mouse cells present in a culture that transiently or permanently are unable to support the full expression of viral functions required for lytic growth and therefore survive the acute infection of a nondefective virus and become transformed.

Here we studied 3T6 cells surviving polyoma infection. After infection of growing cells at high input multiplicity, the cultures were left in medium containing high titers of virus for several days to allow for infection of cells that might have been transiently resistant to polyoma at the initial infection. Clones from single surviving cells were isolated, and one clone was further studied (clone 41). This clone seemed completely resistant to reinfection with polyoma. However, the reinfecting virus could at least be partially expressed in the cells, as judged by an increase in the number of T-antigen-positive cells.

The majority of the clone 41 cells produced polyoma virus at low levels (Tables 2 and 3). In spite of this, the growth of the cells did not seem to be affected. The difficulty of maintaining clone 41 cultures at high cell densities (Fig. 1) might, however, be explained by the presence of virus in the cells. The polyoma virus from clone 41 cells had a small-plaque morphology, as opposed to the large-plaque virus used for the selection of the cells. A similar phenomenon was reported by Vogt and Dulbecco (23). It is presently not clear whether the virus was a

"normal" small-plaque variant with a change in the major viral capsid protein (16) or whether the small plaques were a result of an abnormal infection cycle. We favor the latter alternative for reasons discussed below.

Vogt and Dulbecco (23) reported that cultures of polyoma virus-transformed mouse embryo cells produced infectious virus. The continued production of virus was dependent on cross-infection of cells in culture and could be prevented by cloning cells in medium with polyoma antiserum. The maintenance of the transformed state of the cells did not seem to be related to the production of infectious virus. It seems unlikely that the persistent infection of the clone 41 cells was maintained by a similar carrier state infection for the following reasons. (i) The clone 41 cells appeared totally resistant to reinfection with wild-type virus. (ii) There was no increase in virus yield (Table 2), and no cytopathic effect was observed after reinfection. (iii) Furthermore, measurements of polyoma RNA and DNA synthesis showed no increase in the rate of viral nucleic acid synthesis after reinfection. (iv) Finally, the infectious-center assay (Table 3) showed that most cells in a clone 41 culture produced virus when plated in agar. The clone 41 cells contained in average about 1,500 copies of "free" polyoma DNA per cell. Whether there were additional copies integrated in the host genome is not known, but analysis of the high-molecular-weight DNA fraction from the cells showed that integrated copies of DNA could not represent more than a fraction of the total intracellular viral DNA.

The viral DNA from the clone 41 cells had the normal physical characteristics of polyoma DNA, i.e., a covalently closed circular structure with an *S* value of about 20 in neutral sucrose gradients (Fig. 2A). However, electrophoresis of linearized molecules showed that the clone 41 DNA had a slightly larger size than wild-type polyoma DNA. The addition to the viral genome was located by restriction endonuclease mapping (Fig. 3 and 4). It appeared to be an insertion between *Hpa* II fragments 3 and 5, with a size of about 4.5% of the genome. The additional *Hpa* II fragment, in fact, represented a duplication of the DNA sequences around the junction between *Hpa* II fragments 3 and 5 (Magnusson and Nilsson, manuscript in preparation). The origin of DNA replication is located in this region of the DNA (9), and consequently the clone 41 DNA probably contained two DNA replication origins. The large number of viral DNA copies with a duplication of the replication origin that were present in the cells might explain the resistance to reinfection of the clone 41 cells. It seems likely that the intracellular viral DNA would interfere with the replication of new viral genomes introduced into the cells after reinfection, similar to the interference with wild-type polyoma DNA replication caused by defective genomes generated by high-multiplicity infection (6). Such defective interfering particles were shown to contain DNA with multiple replication origins (8). However, these defective genomes usually had gross deletions of other DNA sequences, and therefore needed a nondefective helper for virus multiplication. One difference between the polyoma clone 41 DNA and the DNA of most naturally occurring defective papovaviruses is that the former was larger whereas the latter generally were smaller than wild-type DNA.

Why the clone 41 cells survived the initial infection remains to be explained. The most likely explanation is that the original cultures contained cells that had a decreased susceptibility to polyoma virus. These cells would conceivably only support a limited replication of viral DNA, resulting in an equilibrium between virus production and cell multiplication. Such an equilibrium could possibly not be established with wild-type virus. Experiments supporting this hypothesis showed that polyoma virus from clone 41 cells had a strong cytocidal effect on normal mouse cells. A large amount of viral DNA, but only little progeny virus, was produced (manuscript in preparation). The low yield of virus after infection of normal cells and the reduced infectivity of the viral DNA from clone 41 cells was probably caused by the addition to the DNA. This addition might simply prevent packaging of viral DNA, or it might interfere with the expression of viral genes. The 5′ ends of mRNA from both early and late genes map close to the juncture between *Hpa* II fragments 3 and 5 (12), which is close to the duplicated region of the polyoma clone 41 DNA. The altered structure of the DNA might result in a reduced amount of functional mRNA molecules being formed.

The clone 41 cells were transformed, as judged by their ability to grow in medium with a low serum concentration (Fig. 1) and their high plating efficiency in soft agar (Table 1). It is possible that the transformed character of these cells was caused by the "free" viral DNA molecules in the cells and not by viral genomes integrated in the host DNA. A somewhat similar system of polyoma-transformed cells carrying multiple copies of "free" viral DNA was recently reported by Prasad et al. (19). In those polyoma-transformed rat cells, 20 to 50 copies of free viral DNA were found. This DNA, like the

polyoma clone 41 DNA, had a reduced infectivity as measured by transfection of mouse cells. However, the rat transformants, being nonpermissive for polyoma, did not produce any infectious virus.

ACKNOWLEDGMENTS

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