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Induction of Virus Synthesis in Polyoma-Transformed BHK-21 Cells

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BHK-21 cells were transformed with polyoma virus mutants Ts-a and Ts-25 by using a temperature shift from 31 to 39 C at 5 days after infection so that rescuable transformants could be isolated. Clones which yielded virus after fusion with mouse cells were scored and maintained at 39 C in the presence of antipolyoma virus antiserum. Generally, no infectious viral deoxyribonucleic acid (DNA) could be found in Hirt supernatant fractions of these lines when maintained at 39 C, but DNA-DNA reannealing measurements detected two to six viral genomes per diploid cell genome in the nuclear DNA. Fusion with permissive cells was not necessary to induce the synthesis of infectious virus; cell lines shifted to 31 C produce the equivalent of 100 viral genomes per cell after 5 days. In some cell lines up to 1% of the cells formed infectious centers upon a shift to 31 C, and 100% of the subclones of a line were inducible. Growth at 31 C selected for a noninducible population which was still transformed.

Generally, polyoma virus cannot be rescued from BHK-21 hamster cells that have been transformed by wild-type stocks of virus (13, 16). Recently, however, Summers and Vogt observed that BHK-21 cells transformed by the polyoma mutant Ts-a would release virus when fused with permissive mouse cells if the infection were permitted to proceed for 5 days at the permissive temperature, and the cells were propagated thereafter at high temperature (13).

Their isolation of "rescuable" Ts-a transformed BHK-21 cells was repeated, and fusion with permissive mouse cells was found not to be a prerequisite for rescue of the resident Ts-a genome. Virus replication in the transformed cells can be induced simply by shifting to a temperature which permits expression of the Ts-a function. Although induction occurs only in a minority of the population of BHK-21 cells at a given time, subclones of a cell line are all inducible. Virus replication, if permitted to occur, is probably cytopathic and may select against transformants supporting such replication.

Because BHK-21 cells are semipermissive, it may be that cells which ultimately become stably transformed contain defectve viral genomes or that they may have become defective in their capacity to support viral replication.

MATERIALS AND METHODS

Cells and viruses. BHK-21 cells were derived from stocks routinely passaged at the Imperial Cancer Research Fund Laboratories and were grown in Dulbecco's modified Eagle medium containing a fourfold-increased concentration of amino acids (E-4) supplemented with 10% calf serum. Transformed cells were grown in E-4 medium containing, in addition to the calf serum, 0.1% antipolyoma virus antiserum (prepared in rabbits against empty polyoma virions).

The wild-type, large-plaque strain of polyoma virus and the Ts-a polyoma mutant (5a) were obtained from M. Fried, and the Ts-25 mutant was obtained from W. Eckhart. Stocks of each were prepared in secondary, whole mouse embryo (WME) cells at 37 or 31 C, respectively.

Virus assays. Generally, medium and cells were combined and assayed for virus after three cycles of freeze-thawing or after disruption in a sonic-bath. A sample (0.3 ml) of each preparation was added to a confluent monolayer of WME cells in a 50-mm petri dish and incubated (with intermittent agitation) for 90 min at 37 C. Then 10 ml of E-4 medium containing 0.9% agar and 3% horse serum (Flow Laboratories) was added, and the plates were incubated at 31 C for three weeks, or at 37 C for 12 days, before staining with neutral red.

Deoxyribonucleic acid (DNA) infectivity assays were performed by a modification of the method of Warden and Thorne (15). Usually, 0.2 ml of a 1 mg solution of diethylaminoethyl (cellulose)-dextran (Sigma) per ml in tris(hydroxymethyl)aminomethane (Tris)-saline buffer (per liter: 8 g of NaCl, 0.38 g of KCl, 0.1 g of Na_2HPO_4, 1 g of dextrose, 3 g of Tris, 1.5 mg of phenol red, 50,000 units of penicillin, and 0.5 gm of streptomycin, pH 7.4) was added to a washed monolayer of WME cells. After 10 min at room temperature, 0.1 ml of a solution containing DNA (in Tris-saline buffer) was added,

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and the plates were incubated at 37 C for 30 min. Then the cells were washed with 5 ml of Tris-saline buffer, and E-4 medium containing 0.9% agar and 3% horse serum (Flow Laboratories) was added. After 12 days at 37 C, or 21 days at 31 C, the cells were stained with neutral red.

For infectious-center assays, a confluent monolayer of WME cells in a 50-mm plate was covered with 1.5 ml of E-4 medium containing 0.9% agar and 3% horse serum. When the agar had solidified, an additional 1.5 ml of E-4 medium containing 0.9% agar, 3% horse serum, and varying numbers of transformed cells was added over the first layer. When the agar in the second overlay had solidified, an additional 7 ml of E-4 medium containing 0.9% agar and 3% horse serum was added, and the plates were incubated and stained as described above.

Cell fusion. Generally, 5×10^5 washed BHK-21 cells were mixed with 1 to 2×10^6 washed WME cells and centrifuged briefly. Then they were suspended in 0.2 ml of phosphate-buffered saline (PBS) containing per liter: 10 g of NaCl, 0.25 g of KCl, 1.44 g of Na_2HPO_4, and 0.25 g of KH_2PO_4), and then they were mixed with 0.1 ml of β-propiolactone-inactivated Sendai virus (4,000 hemagglutinating units per ml). After 10 min on ice, the cells were placed at 37 C for 15 min ,and then were gently pipetted into a 90-mm petri dish containing E-4 medium supplemented with 5% horse serum. After 24 h, the medium was changed, and the plates were incubated for 5 more days at 31 C. The cells were scraped from the dish, frozen, and thawed with the medium and assayed for virus.

Immunofluorescent staining. Cover slips containing cells were washed in PBS and fixed in a solution of acetone:methanol (3:1) for T antigen or 100% acetone for V antigen. The T-antigen staining was kindly performed by V. Defendi with an antiserum prepared from rabbits containing tumors induced by polyoma virus (10). The antiserum contained no detectable cross-reactivity with intact polyoma virus. Polyoma virus V-antigen antiserum was obtained from rabbits inoculated with polyoma virus empty capsids and conjugated with fluorescein.

In each case the cells were examined by fluorescence microscopy immediately after staining. A negative control (untransformed BHK-21 cells) was included in the T-antigen experiments, and both negative and positive controls (uninfected and infected WME cells) were included in the V-antigen experiments.

Extraction of DNA. Selective extraction of viral DNA was performed by the method described by Hirt (8). Cells on a 90-mm plastic dish were lysed by the addition of 1 ml of 0.6% sodium dodecyl sulfate (SDS) and 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4. After several minutes, the lysate was scraped into a centrifuge tube and made 1.0 M in NaCl. After precipitation of the host cell DNA at 4 C overnight, the extract was centrifuged at 20,000 $\times$ g for 30 min. The supernatant fluid was removed and extracted with an equal volume of phenol equilibrated with 10 mM Tris-chloride, pH 8.0, containing 1 mM EDTA and centrifuged for 10 min at 10,000 $\times$ g to separate the phases. The phenol phase and interface were reextracted with an equal volume of 10 mM Tris-chloride, pH 8.0, containing 50 mM NaCl and 1 mM EDTA and recentrifuged. The two aqueous phases were combined, and the nucleic acid was precipitated by the addition of two volumes of ethanol. After 2 to 24 h at -20 C, the precipitate was collected by centrifugation and dissolved in a small volume of Tris-saline buffer.

Extraction of cell nuclear DNA was performed by a method suggested by P. Berg (personal communication). Cells were detached from burler bottles by treatment with trypsin-EDTA and were harvested by centrifugation. They were washed and frozen as a compact pellet at -20 C. Generally 5 to 7 g (wet weight) of frozen cells were thawed in 30 ml of buffer containing 0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 2.5 mM Tris, pH 7.4, and centrifuged at 13,000 $\times$ g for 10 min. The pellet was resuspended in 30 ml of 0.25 M sucrose in 10 mM Tris-chloride, pH 8.1, containing 0.3% Triton X-100. The cells were disrupted with four strokes of a hand-held homogenizer, and the nuclei were pelleted by centrifugation at 4,500 $\times$ g for 10 min. The pellet was resuspended in 90 ml of 0.15 M NaCl containing 0.1 M EDTA, pH 7.0, homogenized briefly, and lysed by the slow addition of 10 ml of 10% SDS at room temperature. In order to obtain complete lysis, the suspension occasionally had to be heated to 60 C for several minutes.

After stirring for at least 10 min, 5.5 g of NaCl was added, and stirring was continued for an additional 10 min. The viscous solution of DNA was centrifuged for 1 h at 20,000 $\times$ g at 4 C, and the supernatant fluid was collected. Then, 100 ml of ethanol were layered over the solution, and the DNA was spooled out.

The DNA was dissolved in 20 ml of 15 mM NaCl plus 1.5 mM sodium citrate (0.1$\times$SSC, pH 7.0) and treated with 1 mg of pancreatic ribonuclease preheated to 90 C for 15 min (Sigma). Then, self-digested Pronase was added (100 μg/ml), and the solution was stirred overnight at room temperature. The next day, 20 ml of 0.1 $\times$ SSC was added, the solution was made 1 M in sodium perchlorate and extracted twice with equal volumes of isoamyl alcohol-chloroform (1:24), and each time the phases were separated by centrifugation at 10,000 $\times$ g for 10 min. Two volumes of ethanol were added to the combined aqueous phases, and the DNA was wound up on a glass rod and dissolved in 10 mM Tris-chloride, pH 8.0, containing 1 mM EDTA. Generally, the yield of DNA was 175 to 250 A_{260} (absorbance at 260 nm).

DNA reannealing. The analysis of polyoma DNA sequences in transformed-cell DNA was carried out by measuring the effect of cellular DNA on the reannealing kinetics of sheared, denatured, ^{32}P-polyoma DNA as described by Gelb, Kohne, and Martin (7) and modified by Dieckmann, Devine, and Berg (personal communication). According to the modification

developed by Dieckmann et al., the ^{32}P-polyoma DNA reagent was synthesized from randomly nicked, unlabeled polyoma DNA and DNA polymerase I of E. coli by using as substrates α-^{32}P-deoxynucleoside triphosphates (prepared by a procedure of R. H. Symons; personal communication). The resulting fragments of ^{32}P DNA were approximately 500 nucleotides in length. This DNA was mixed with 100 A_{260} of unlabeled cellular DNA in a volume of 1.4 ml, denatured by boiling for 2 min, and cooled to 68 C. Then, 0.6 ml of 5 M NaCl was added and, at intervals, 0.1-ml samples were removed and placed at −20 C. After taking samples for 96 h, the single-stranded DNA in the samples (present at the start of the reaction) was distinguished from the double-stranded DNA formed by reannealing at 68 C by digestion with the single-stranded specific nuclease S_1 (1).

The zero time points always contained less than 5% double-stranded DNA. In the calculation of the number of genome copies in transformed cell DNA, it is assumed that the viral DNA used as a probe was uniformly labeled.

RESULTS

Isolation of transformants. BHK-21 cells were infected with Ts-a or Ts-25 (a mutant of the same complementation group as Ts-a Walter Eckhart, personal communication) at an input multiplicity of 10 to 20 plaque-forming units (PFU) per cell and plated in agar at 31 C (11). After 5 days at 31 C, the plates were shifted to 39 C, a temperature at which the Ts-a function is not expressed. After 2 weeks, clones were picked from the agar and grown in medium containing 0.1% antipolyoma virus antiserum at 39 C, and then were recloned at 39 C in agar containing antipolyoma virus antiserum. After the second cloning, the transformed cells were tested for virus production by fusing them with secondary WME cells by using β-propiolactone-inactivated Sendai virus to promote cell fusion. Two out of five different Ts-a transformed clones and two out of 14 Ts-25 transformed clones produced virus at 31 C. From a separate transformation experiment performed by M. Fried with Ts-a, four out of eight different clones produced virus after fusion.

These cloned lines generally produced 10^5 to 10^6 PFU of virus after 1 week at 31 C when 5×10^5 transformed hamster cells were fused with 1 to 2×10^6 WME cells in the presence of 400 hemagglutinating units of Sendai virus.

Synthesis of infectious viral DNA. The rescuability of polyoma virus from these transformed cells must depend upon the viral DNA persisting in such a state that it can be converted into a template for viral DNA replication under permissive conditions. Conceivably, the viral DNA is maintained in these transformed cells as a plasmid. To examine this possibility, DNA was extracted from cells by a procedure which separates low-molecular-weight DNA (up to a size several times that of polyoma) from that of the large chromosomal DNA (8). The fraction containing the low-molecular-weight DNA was assayed for viral DNA infectivity on WME cells by using diethylaminoethyl (cellulose)-dextran. In a reconstruction experiment the sensitivity of this assay was 2×10^6 PFU per 1 μg of purified polyoma form I DNA, or approximately one PFU per 10^5 molecules of polyoma DNA.

When the transformed cell lines were grown at 39 C, no infectious viral DNA was detected. If each cell in the 3×10^7 cells from which the DNA was extracted had contained one molecule of infectious viral DNA, then 300 PFU would have been detected. Generally, no PFU were detected in 0.2 vol of such an extract, so a maximum estimate is 5 per 300 PFU, or 0.02 molecules of free, infectious, viral DNA per cell.

However, when the cells were shifted to 31 C (without fusing them to WME cells) infectious, viral DNA appeared and gradually increased to as much as 10^4 PFU per 10^4 cells (Fig. 1 and Table 1). In several experiments, the amount of infectious, viral DNA varied by as much as a factor of 10. The reason for this variability is not yet understood, but it might reflect differences in

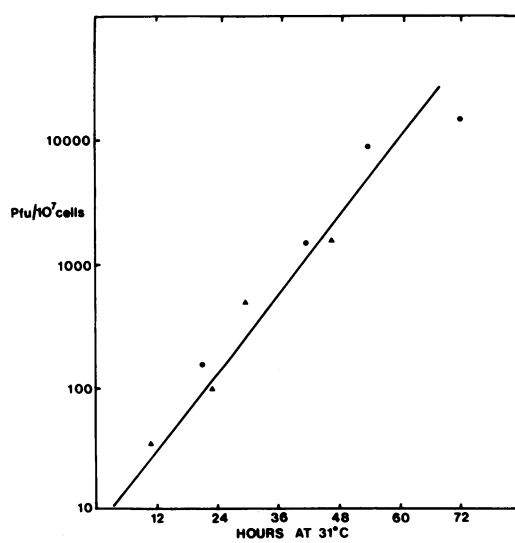

FIG. 1. *Time course of synthesis of infectious DNA. Plates containing approximately 10^7 BHK-Ts-a/1b2 cells in reinforced Eagle medium containing 10% calf serum and 0.1% antipolyoma virus antiserum were shifted to 31 C. At each stated time, viral DNA was selectively extracted from one plate. Results are from two independent experiments.*

the number of cells in a population in which viral DNA synthesis was activated. The infectivity was sensitive to deoxyribonuclease, insensitive to ribonuclease, (Table 2) and banded at a light density in $CsSO_4$ (less than 1.45 g/cm³).

Virus synthesis at 31 C. If the BHK-21 transformed cells can support viral-DNA replication, can they also support the synthesis of the viral proteins required for virion assembly? To answer this question, disrupted cells were combined with their growth media (containing no antiserum) and assayed for infectious virus.

When the cells were grown at 31 C, infectious virus was produced (Table 3). Cells which had been passaged continuously for over 6 months at 39 C in the presence of antipolyoma virus antiserum continued to be inducible at 31 C. Because the cells divided every 15 to 16 h, this represented nearly 300 generations, a sufficient number to have removed by dilution any virus remaining from the initial infection. Usually no virus PFU could be detected in extracts made of cells kept at 39 C. In two instances, a few PFU were detected in the most concentrated extracts of cells kept at 39 C, but these might have been due to a small amount of virus induction caused by the incubator temperature dropping below 39 C.

The virus that was produced at 31 C was insensitive to deoxyribonuclease and was sensitive to the antipolyoma virus antiserum added to the medium (Table 4). It was still thermosensitive for plaque formation and banded in CsCl between 1.33 and 1.367 g/cm³, the density of polyoma virus.

Every cell line which gave virus upon fusion with WME cells could be induced by a temperature shift. Cell lines which did not produce virus upon fusion were not inducible. Between 0.05 and 1% of the cells of several different cell

TABLE 1. *Synthesis of infectious DNA*

Cell line	DNA PFU per 3 × 10^7 cells	
	(39 C)	(31 C)[a]
Ts-a/1b2	<5	7×10^3
Ts-a/1b2, cl 6	<5	4×10^3
Ts-a/1b2, cl 7	<5	3×10^3
Ts-a/2a2	<5	2×10^3
Ts-a/3b2	<5	7×10^3
Ts-25/17E	<5	1×10^3
Ts-a/1b2 (31 C)	<5	<5

[a] After 144 h at 31 C.

TABLE 2. *Characteristics of infectious DNA*[a]

DNA source	Untreated	DNAse treated[b]	RNAse treated[c]
Polyoma DNA	3×10^5	<5	3×10^5
Ts-a/1b2	7×10^3	<5	3×10^3
Ts-25/17E	3×10^3	<5	3×10^3

[a] Values are expressed as plaque-forming units per milliliter.

[b] Incubated in Tris-saline buffer containing 10 mM $MgCl_2$ and 10 μg of pancreatic DNAse (Sigma, Electrophoretically purified) per ml for 30 min at 37 C.

[c] Incubated in Tris-saline buffer containing 100 μg of pancreatic RNAse (Sigma, preheated to 90° for 15 min) per ml for 30 min at 37 C.

TABLE 3. *Synthesis of infectious virus*

Cell line	Virus PFU per 10^7 cells	
	(39 C)	(31 C)[a]
Expt 1		
Ts-a/1b2	<15	400
Ts-a/2a2	<15	1000
Ts-a/3a2	<15	250
Expt 2		
Ts-a/1b2	<15	1.5×10^4[b]
Ts-a/1b2 cl 6	60	1×10^5
Ts-25/17E	<15	3×10^4

[a] Cells were shifted to 31 C for 96 h before harvesting.

[b] Cells were shifted to 31 C for 144 h before harvesting.

TABLE 4. *Properties of induced virus*

Virus stock	Virus titer[a]		
	Untreated	DNAse treated[b]	Antiserum treated[c]
Wild-type polyoma	2,000	2,000	180
Ts-a/2a2	1,200	1,600	18
Ts-a/1b2	1,500	1,450	ND[d]
Ts-25/17E	1,800	1,500	ND

[a] Values are expressed as plaque-forming units per milliliter.

[b] Incubated with 100 μg of pancreatic DNAse (electrophoretically purified) per ml for 30 min at 37 C.

[c] Mixed with an equal volume of medium containing 0.1% antipolyoma virus antiserum and incubated for 30 min at 37 C. Titer is corrected for dilution.

[d] Not done.

lines formed infectious centers when plated in agar over WME cells (Table 5). Adding the transformed cells directly to the WME cells (under agar) did not significiently increase the number of infectious centers. Although this was only a small fraction of the population, all (17 out of 17) of the subclones of one cell line (BHK-Ts-a-/1b2) yielded virus after fusion, and all of those subclones tested (three) were inducible. It is not understood why only a small fraction of the population is induced at any given time, but a similar inefficiency has been observed in the rescue of virus from SV40 transformed cells after fusion with permissive cells (9).

Estimate of the viral genome copies per diploid cell genome at 39 C. To obtain an estimate of the number of viral genomes present in each cell at 39 C, the kinetics of reannealing of denatured polyoma DNA in the presence of transformed cell DNA were examined. Denatured, sonicated, ^{32}P-polyoma DNA reannealed with a $Cot_{\frac{1}{2}}$ (initial concentration $\times$ time for 50% reannealing) of 3.1×10^4 mol-s per liter at 68 C in 1.5 M NaCl with 50 A_{260} salmon sperm DNA added to normalize viscosity (Fig. 2 and Table 6). Substitution of untransformed BHK-21 DNA for the salmon sperm DNA did not affect the rate of reannealing. When transformed BHK-21 cell DNA was added in place of the salmon sperm DNA, the rate of reannealing was increased; a reconstruction experiment in which the effect of unlabeled polyoma DNA upon the rate of reannealing of the ^{32}P-polyoma DNA was measured provided a means of judging the amount of polyoma DNA present in the transformed cell DNA (Fig. 2 and Table 6). Three different inducible lines were examined, and the number of polyoma genomes per diploid cell genome was found to vary between two and six (Fig. 3 and Table 6). Because 5 to 10% of the cells in these transformed lines were multinucleate (unpublished data) it was not possible to get an accurate figure for the number of polyoma genomes per cell.

TABLE 5. *Formation of infectious centers*

Cell line	Infectious centers (%)
Ts-a/1b2	0.2
Ts-a/1b2, cl 6	0.3
Ts-a/1b2, cl 7	0.06
Ts-a/1b2, cl 8	0.1
Ts-a/2a2	0.9
Ts-a/3b2	1.0
Ts-25/17E	0.1
Ts-a/1b2 (31 C)	<0.001

Synthesis of polyoma-specific antigens. By using a fluorescent antiserum specific for polyoma virus T antigen, several inducible BHK-21 transformed lines were examined for T-antigen production after growth at 39 C and after 48 h at 31 C. All of the cell lines tested exhibited some nuclear fluorescence at both

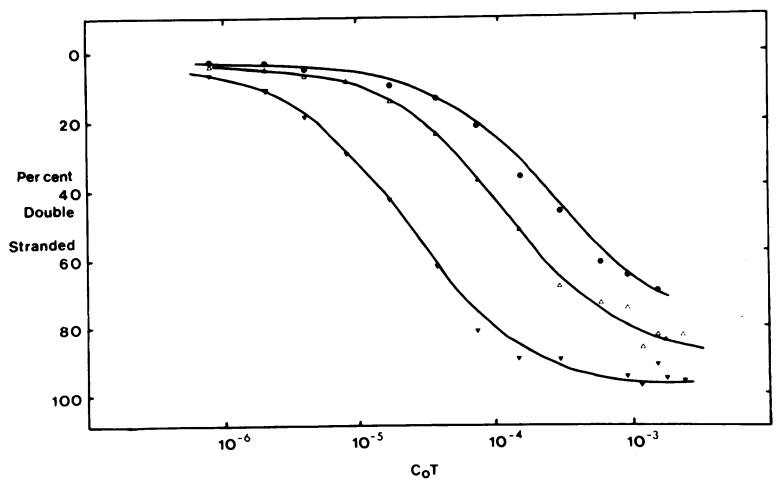

FIG. 2. *Reconstruction of polyoma DNA reannealing. The amount of ^{32}P-DNA resistant to S_1 nuclease (and therefore double stranded) is plotted against the product of the initial concentration $\times$ the time of reannealing (hours) at 68 C. Curves are from computer plots of second-order functions which best fit the experimental points.* (●) *^{32}P-polyoma DNA with salmon sperm DNA;* (△) *^{32}P-polyoma DNA with 6.3×10^{-5}, A_{260} nonradioactive polyoma DNA and salmon sperm DNA.* (▼) *^{32}P-polyoma DNA with 6.3×10^{-4}, A_{260} nonradioactive polyoma DNA and salmon sperm DNA.*

TABLE 6. *Viral genomes in cell nuclear DNA*

Unlabeled DNA	^{32}P-polyoma DNA Cot½	Viral genomes per diploid cell genome (calculated)[a]
Salmon sperm	3.1×10^{-4}[b]	0
Salmon sperm	1.27×10^{-4}	3.6
+6.3×10^{-5}, A_{260} polyoma DNA		
Salmon sperm	0.20×10^{-4}	32.2
+6.3×10^{-4}, A_{260} polyoma DNA		
BHK-21	3.1×10^{-4}	0
BHK-Ts-a/1b2	0.84×10^{-4}[c]	6.0
	0.84×10^{-4}[c]	6.0
BHK-Ts-a/2a2	1.51×10^{-4}	2.34
BHK-Ts-25/17E	1.09×10^{-4}	4.1

[a] Based upon 4 pg of DNA per diploid mammalian cell genome and a mass of 3×10^6 daltons for the polyoma genome.

[b] Moles × seconds per liter.

[c] DNA from two separate batches of cells.

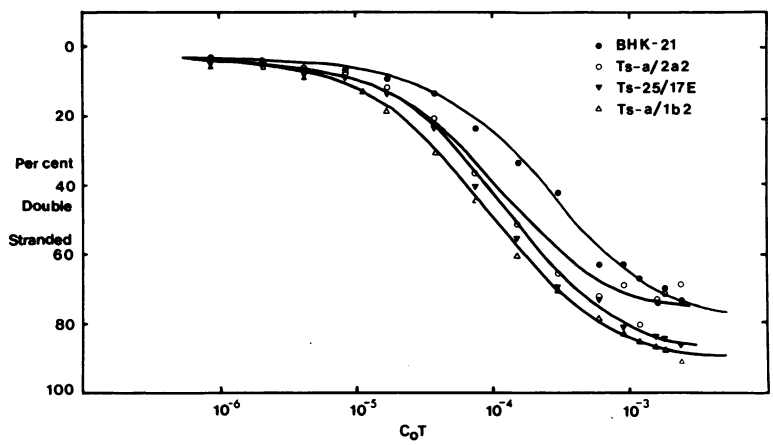

FIG. 3. *Polyoma DNA reannealing with transformed cell DNA. The amount of ^{32}P DNA resistant to S_1 nuclease (and therefore double stranded) is plotted against the product of the initial concentration × the time of reannealing (hours) at 68 C. Curves are from computer plots of second-order functions which best fit the experimental points. (●) ^{32}P-polyoma DNA with BHK-21 DNA. (△) ^{32}P-polyoma DNA with BHK-Ts-a/1b2 DNA. (▼) ^{32}P-polyoma DNA with BHK-Ts-25/17 DNA. (○) ^{32}P-polyoma DNA with BHK-Ts-a/2a2 DNA.*

temperatures (compared with an untransformed BHK-21 control); in several instances, the fluorescence intensity was increased somewhat by the shift to 31 C.

Also, cells grown at 39 C and shifted to 31 C for up to 72 h were stained with a fluorescent antiserum prepared against polyoma virus capsids. Out of several thousand cells examined, from several different cell lines, no significant fluorescence was observed. Although these cells produced some virus, either too few cells were induced to synthesize virus by this time or the threshold concentration of antigen required to fix a detectable amount of antibody was not reached.

Growth at 31 C selects for a noninducible population. A culture from one inducible line (BHK-Ts-a/1b2; 31 C) was shifted to 31 C soon after its isolation and passaged for 6 months at 31 C in the presence of antipolyoma virus antiserum. It became noninducible (Tables 1 and 5), nor could virus be rescued from the cells after fusion with WME cells. It was still transformed by the criterion that it had a high-plating efficiency in agar (10%), and it still had polyoma T-antigen, although it grew in a less random manner than the parental line passaged at 39 C.

DISCUSSION

Reports in the past have conflicted as to whether BHK-21 cells support replication of polyoma virus. Fraser and Gharpure (5) observed

a small number of fluorescent nuclei in an infected population of BHK-21 cells stained with fluorescent antibody prepared against polyoma virus, but it is not clear whether this was due to synthesis of new antigen or differential accumulation of antigen from the input virus. Bourgaux (3) showed that infected BHK-21 cells incorporated P^{32}-orthophosphate into material which cosedimented in sucrose and banded in rubidium chloride with polyoma virus, but Basilico et al. (2) were unable to detect any incorporation of ^{3}H-thymidine into viral DNA after infection of BHK-21 cells. In all these experiments, the most sensitive technique available for detecting virus, the plaque assay, could not be used because the massive amount of input virus obscured the small amount of replication which might have occurred. This problem is not encountered here, for these transformed cells can be induced to synthesize infectious virus simply by shifting to a temperature permissive for virus replication.

Initially, these "rescuable" Ts-a transformed cell lines were isolated so that the transcription of the polyoma genome within transformed cells could be studied in cells known to contain the entire viral genome. Because these BHK-21 cells have been found to be capable of producing virus, albeit only a small amount, it is clear that an absolute transcriptional block is not imposed at all times by the cell. Fusion with mouse cells is not essential for any aspect of virus replication, although it may increase the yield of virus from a population of cells.

After a temperature shift to 31, between 0.05 and 1% of a population of BHK-Ts-a cells is induced to form infectious centers. In 72 h, as many as 10^8 to 10^9 infectious DNA molecules are made by a population of 10^7 cells. It is not known how these are distributed among the population, but if only those cells which form infectious centers synthesize viral DNA, then each induced cell may contain 1,000 to 10,000 viral DNA molecules. Why only a fraction of a BHK-Ts-a population is induced to synthesize virus at any given time is an interesting and unanswered question. The limiting factor may be the accessibility of the viral DNA template or a transient restriction of viral DNA synthesis, transcription, or translation.

The inducibility of these BHK-Ts-a cell lines is very similar to that observed with the Ts-a transformed mouse cell line, 3T3-Ts-a (4, 14). When 3T3-Ts-a is shifted to 31 C, a much larger yield of virus is observed, and a greater fraction of the population is induced. However, questions regarding the state and function of the viral genome in the 3T3-Ts-a cell lines are difficult to approach, because there is a small amount of virus synthesis occurring even at a nonpermissive temperature (14).

When the inducible BHK-Ts-a cell lines are grown at 39 C, no infectious viral DNA molecules can be detected in Hirt extracts (less than 0.02 per cell), but DNA reannealing experiments indicate that there are usually two to six viral equivalents per diploid cell genome. At present it is not known if this polyoma DNA is integrated within the cellular DNA, but its lack of infectivity suggests only that is in a configuration different from the free, closed, circular DNA found in infected mouse cells or in virions. If it is integrated, then the manner by which it becomes available to act as a template for further viral DNA synthesis during induction at 31 C is of considerable interest.

A related topic of great interest is the role of the Ts-a gene product in the inducibility of these transformed cells. Summers and Vogt (13) initially observed that BHK-21 cells transformed by Ts-a were unlike most other polyoma-transformed BHK-21 cells in that the resident virus genome could be rescued. They also observed that "rescuability" was dependent upon the Ts-a function being inactivated during outgrowth of transformants. Earlier experiments of Fried (6) demonstrated that the Ts-a function was absolutely required for the establishment of permanent transformation, but that it was not required for the subsequent maintenance of the transformed state. One attractive suggestion is that the Ts-a gene product is required for the integration and excision of viral DNA from the host DNA, as well as for replication of the viral DNA (4). Although the polyoma genome in transformed cells has not yet been shown to be covalently linked to host DNA, as is the case for the SV40 genome in transformed 3T3 cells (12), it seems probable that it is. The continued expression of the function of the Ts-a gene product (as in cells transformed by wild-type virus or by Ts-a at low temperature) may lead to an instability of an integrated viral genome, and thereby be disadvantageous for the maintenance of the transformed state, or it may permit sufficient synthesis of viral DNA to be harmful to the growth of the cell. Thus, inactivation of the Ts-a gene product may be essential in transformed cells, but it is likely that the manner in which this is accomplished may vary: in some cells the resident viral genome may mutate and and become nonexcisable, and in other cells the host may become absolutely nonpermissive.

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