JOURNAL OF VIROLOGY, Oct. 1976, p. 133-141

Vol. 20, No. 1
Printed in U.S.A.

Polyoma Genome in Hamster BHK-21-C13 Cells: Integration into Cellular DNA and Induction of the Viral Replicon

WILLIAM R. FOLK* AND JANE E. BANCUK

Department of Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48109

Received for publication 12 May 1976

When grown at 39.5°C, BHK-21-C13 cells transformed by *A* gene mutants of polyoma virus contain viral sequences that are predominantly associated with cellular DNA pelleted in the Hirt lysis procedure. At this temperature, in cells that are inducible for viral DNA replication (Folk, 1973), the majority of the viral genomes are covalently joined with cellular DNAs containing repetitious sequences. Upon a shift to 31°C, free viral genomes appear and are replicated. Coupled with the replication of the free viral genomes at 31°C is an increase in the viral genomes associated with cellular DNA.

Summers and Vogt have observed that polyoma virus can be rescued from BHK-21-C13 cells transformed by the polyoma ts-a mutant (17). To preserve the rescuable property of these transformants, the *A* gene function must be inactivated shortly after transformation is initiated (9, 27). Under conditions that *A* gene function is not expressed (growth at 39.5°C), tsa-transformed BHK-21-C13 cells contain no detectable infectious viral DNA that can be extracted into the supernatant of a Hirt lysate, but contain two to six viral genome equivalents per cell in nuclear DNA, as measured by DNA reassociation kinetics (9). In this report, we provide direct evidence for the covalent integration of the polyoma genome into the cellular DNA of such transformants grown at 39.5°C. In addition, we further characterize the activation of viral DNA replication that occurs in such cells when *A* gene function is expressed at 31°C, in the absence of fusion with permissive mouse cells.

(A preliminary account of these results was given at the 1975 Cold Spring Harbor Tumor Virus Meeting on SV-40, Polyoma and Adenoviruses, 13-17 August 1975, Cold Spring Harbor, N.Y.)

MATERIALS AND METHODS

Cells and media. The BHK-21-C13 cell line and its transformed derivatives have been described previously (9). Additional subclones of the original transformed cell lines are described in the text. Mouse 3T6 cells were provided by B. Griffin, and 3T3 cells were provided by G. DiMayorca. Whole mouse embryo secondary cultures (WME) were prepared from HA-ICR mice obtained from Spartan Animals, Haslett, Mich. The media and sera were obtained from Flow Laboratories and Grand Island Biological Laboratories, respectively, and have been previously described (10).

Virus assays. Assays for infectious virus, infectious viral DNA, and infectious centers were performed on 60-mm petri plates (Lux Plastics) as described earlier (10).

Extraction and purification of DNAs. Labeled viral [^{3}H]DNA and [^{32}P]DNA were purified as previously described from cells cultured in media containing [^{3}H]thymidine or $^{32}P_i$ (10).

Selective extraction of low-molecular-weight DNA by the Hirt procedure (16) was performed as previously described (10), with the following modifications. After addition of the lysis buffer to cells on petri plates (150-mm dishes; Lux Plastics), a 0.2 volume of 5 M NaCl was added and gently mixed with the lysate. The lysate was scraped into centrifuge tubes and placed at 4°C overnight, and then separated into supernatant and pellet fractions (low- and high-molecular-weight DNAs, respectively) by centrifugation at 20,000 × *g* for 60 min. Proteins were then removed by either of two procedures. (i) Those samples of DNA to be analyzed for viral genome content solely by reassociation kinetics were sonicated briefly before extraction with phenol. (The pelleted DNA was first resuspended in 10 volumes of 10 mM Tris-chloride, pH 7.5, containing 1 mM EDTA.) After two extractions with phenol equilibrated with 0.1 M Tris-chloride, pH 7.5, the nucleic acids were precipitated from the aqueous phases of the supernatant and pellet fractions with 2 volumes of ethanol. (ii) Those DNAs that were to be recovered intact were digested with Pronase and then, without sonication, were extracted with phenol. (The pelleted DNA was first resuspended in 10 volumes of 0.1 M Tris-chloride, pH 7.5, containing 1 mM EDTA, and the supernatant was diluted with an equal volume of 0.1 M Tris-chloride, pH 7.5, containing 1 mM EDTA.) Each fraction was then incubated for 4 to 6 h at 41°C with 500 μg of Pronase (self-digested) per ml. Then each fraction was ex-

tracted with an equal volume of phenol equilibrated with 0.1 M Tris-chloride, pH 7.5, and the nucleic acids were collected by precipitation from the aqueous supernatant with ethanol. After removal of proteins, the precipitated nucleic acids were dissolved in a small volume of 10 mM Tris-chloride, pH 7.5, containing 1 mM EDTA and were frozen at −20°C until analyzed for polyoma sequences. The concentration of total DNA in each sample was determined by the diphenylamine assay, with dAMP as a standard (2).

Total cellular DNA was extracted from cells by the procedure of Gross-Bellard et al. (15), with the following modifications. After lysis of the cells and digestion with proteinase K (EM Laboratories), the viscous lysate was placed in a flask and gently deproteinized with phenol (equilibrated with 0.1 M Tris-chloride, pH 7.5) by repeated inversions. The phases were separated by centrifugation, and the upper phase was collected (without pipetting) and extracted again with phenol. The aqueous phase containing the viscous nucleic acids was dialyzed against 10 mM Tris-chloride, pH 8.0, containing 10 mM NaCl and 10 mM EDTA until the A_{270} of the dialysate dropped below 0.1. The solution was made 50 mM in Tris-chloride, pH 8.0, and treated with RNase A (20 μg/ml, preheated to 90°C for 15 min) and T1 RNase (0.2 μg/ml, preheated to 90°C for 15 min) for 4 h at 41°C. Then proteinase K (to 50 μg/ml) and sodium dodecyl sulfate (to 0.5%) were added, and the remaining steps in the procedure described by Gross-Bellard et al. (15) were followed. In alkaline sucrose gradients, DNAs prepared in this manner have an average sedimentation coefficient of approximately 60S when centrifuged at 40,000 rpm. Over 60% of the DNA sediments with or ahead of polyoma form I DNA.

In early experiments, in which heat was used to denature DNA for the network fractionations, total cellular DNA was prepared essentially as described by Varmus et al. (30) with self-digested Pronase.

Fractionation of cellular DNA on the basis of repetitive sequence content (network formation) (30). Total cellular DNA purified as described above was fractionated on the basis of its content of repetitious sequences, using either the procedure described by Varmus et al. (30) or a modification of Bellet's procedure (3). The following modifications of the latter procedure were introduced in an attempt to ensure that the single-stranded DNA was preserved intact. After denaturation in alkali and dialysis, the DNA was concentrated against polyethylene glycol 6000 and then dialyzed against 50 mM Tris-chloride, pH 7.5, containing 1 M NaCl. The DNA, at an A_{260} of 1 to 3, was mixed with an equal volume of formamide (Matheson, Coleman & Bell) and renatured at 41°C for 15 to 16 h. Under these reassociation conditions, polyoma DNA has a T_m of 61°C and reassociates approximately 2.5 times faster than in 0.12 M sodium phosphate buffer (pH 6.8) at 65°C. The DNA undergoes less than one chain scission per 5,000 base pairs (our unpublished data). Therefore, under these conditions, renaturation occurs to an equivalent C_0t of 20 to 60 (without correction for chain length).

The networks of DNA formed by the reassociation of the repetitive sequences were pelleted by centrifugation at 26,000 rpm in an SW27 (or SW27.1) rotor for 1 h at 10°C. The pellet was resuspended in 10 mM Tris-chloride pH 7.5, containing 1 mM EDTA, and it and the supernatant were separately sonicated for 30 s. Both fractions were dialyzed extensively against 10 mM Tris-chloride, pH 7.5, containing 0.5 M NaCl and then precipitated by the addition of 2 volumes of ethanol. The precipitates were dissolved in a small volume of 10 mM Tris-chloride, pH 7.5, containing 1 mM EDTA. The concentration of DNA was determined by its A_{260} in 10 mM NaOH or by the diphenylamine assay.

To determine the amount of low-molecular-weight DNA pelleted with the network, φX174 replicative form I [^{32}P]DNA (the generous gift of Paul Johnson) was added to each sample before denaturation, and the amount of ^{32}P in the network and supernatant fractions was measured by counting the Cerenkov radiation.

Equilibrium and rate-zonal centrifugation. The description of the procedures used for isopycnic banding of DNAs in CsCl containing 200 μg of ethidium bromide per ml have been reported (10), and the procedure used for analysis of DNAs by sedimentation through 5 to 20% sucrose gradients is described in the text.

Iodination of viral DNA. High-specific-activity polyoma [^{125}I]DNA was prepared as described previously (1). Often, purified denatured [^{125}I]DNA was carried through an additional reassociation to further eliminate DNAs that do not reassociate readily. In those instances, the denaturable [^{125}I]DNA (1) was recovered by elution from hydroxyapatite and incubated at 65°C for 10 to 20 $C_0t_{1/2}$ and then refractionated over hydroxyapatite. Generally, the specific activity of the purified DNA was calculated to be 0.5×10^8 to 2×10^8 cpm/μg.

DNA reassociation. Nucleic acids that had been ethanol precipitated were dissolved in a small volume of 10 mM Tris-chloride, pH 7.5, containing 1 mM EDTA, and the DNA was denatured by addition of 0.1 volume of 3 M NaOH. Samples not fragmented by sonication were boiled for 25 min in 0.3 M NaOH to reduce the size to 500 to 1,000 nucleotides and to hydrolyze RNA. Samples that were sonicated but not treated with RNase were left at room temperature for 25 min in 0.3 M NaOH to hydrolyze RNA. Then polyoma [^{125}I]DNA was added (usually 2,500 to 5,000 counts) followed by an equal volume of 2 M sodium phosphate buffer, pH 6.8. Five or six aliquots (0.1 ml each) were removed and placed in stoppered tubes at 68°C. Tubes were removed at different times, ranging from 1 to 100 h, chilled on ice, and analyzed for the content of duplex DNA by fractionation on hydroxyapatite columns at 60°C (10).

Often, with low concentrations of polyoma DNA sequences and high-specific-activity probe polyoma DNA, only 1 to 5% reassociation occurs throughout the entire incubation. This, together with the small number of aliquots used, lessens the precision of the assay, but the error that is introduced does not alter the qualitative interpretation of the results. The

sensitivity of the assay is limited only by the amount of cellular DNA mixed with the probe. Generally, 0.5 to 1 mg of DNA (from approximately 10^8 cells) was used in each reassociation experiment, and 0.1 to 0.3 viral genome equivalents per cell could be detected.

RESULTS

History of the transformed cell lines. The BHK-21-C13 cells transformed by tsa or by ts-25 were described previously (9). In brief, BHK-21-C13 cells were infected with virus at 31°C and placed in soft agar at that temperature for 5 days. Then they were placed at 39.5°C, and clones arising after 1 to 2 weeks of incubation were isolated. After recloning in soft agar containing antipolyoma virus antiserum (at 39.5°C), those transformants that produced virus upon fusion with WME cells at 31°C were selected for further study. Shortly thereafter it was noted that fusion with mouse cells was not prerequisite for the induction of virus synthesis. Virus is produced by the transformants, in the absence of mouse cells, upon a shift to 31°C (the temperature at which the *A* gene product is expressed) (9).

Since their original isolation, cell lines tsa-1b2, tsa-2a2, and ts 25-17 (all independently transformed) have been grown in culture at 39.5°C for over 3 years. On several occasions, cells have been frozen in liquid nitrogen for up to a year and then thawed. The cell lines tsa-1b2 and tsa-2a2 (9) have been recloned in soft agar, and several subclones of each line have been tested for the formation of infectious centers when suspended over a monolayer of WME cells at 31°C. In every case (eight subclones and the two parental cell lines), between 0.05 and 1.5% of the transformed cells formed infectious centers. These are the same levels observed with the original transformants several years before (9). The capacity to produce virus (inducibility), therefore, appears to be stably inherited, although the probability is low that any single colony will produce infectious virus when shifted to 31°C.

Quantitation of polyoma DNA sequences by reassociation kinetics. Initially, the induction of polyoma DNA synthesis in these transformed cells was studied by assaying the number of infectious DNA molecules present in Hirt supernatants of cell lysates. Since this assay measures only those viral DNAs that are infectious, the quantitation of those DNAs rests upon the assumption that the specific infectivity of the DNA isolated from the induced cells is the same as that of purified viral DNA used as a control. To provide an alternative measure of the polyoma DNAs and to enable a more detailed examination of the state of the viral genome in these transformed cells, we have measured polyoma DNA sequences by reassociation kinetics (13).

We have developed a procedure for iodinating polyoma DNA to a specific activity of approximately 10^8 cpm/μg (1). The utility of this polyoma [^{125}I]DNA as a probe for unlabeled polyoma sequences is demonstrated in Fig. 1. When the denatured probe [^{125}I]DNA is incubated with increasing amounts of sonicated polyoma DNA, the initial rate of reassociation of the [^{125}I]DNA increases linearly with the concentration of total polyoma DNA present. This is expected from the known second-order rate of reassociation of DNA (33).

Polyoma DNA in inducible transformants at 39.5°C. At 39.5°C, tsa-transformed BHK-21-C13 cells contain in their nuclei two to six viral genome equivalents. Less than 1% of these genomes, however, can be detected by infectivity assays of the DNA extractable either into a Hirt pellet or into a Hirt supernatant (9; our unpublished observations). Essentially all of the viral genomes in these cells are uninfectious, but either must be associated with the chromosomal DNA that is pelleted in the Hirt lysis procedure or are present in the supernatant in an uninfectious form.

To differentiate between these alternatives,

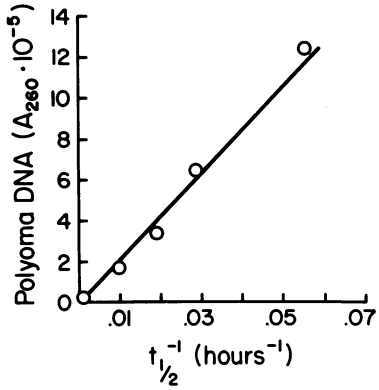

FIG. 1. *Relationship between the concentration of polyoma sequences and the $t_{1/2}$ of reassociation of probe [^{125}I]DNA. The times required for half-reassociation ($t_{1/2}$) of polyoma [^{125}I]DNAs were obtained by measuring the reassociation of a series of mixtures of polyoma DNA containing varying amounts of sonicated, unlabeled DNA and a constant amount of probe [^{125}I]DNA. The probe DNA alone reassociated at a rate such that 50% reassociation would require incubation for 2,000 h. The $t_{1/2}$ is inversely proportional to the initial rate of reassociation. Analogous standard curves were carried out in each experiment in which the quantity of polyoma sequences in unknown DNAs was examined.*

we measured, using reassociation kinetics, the number of viral genomes in the supernatant and pellet fractions of cells lysed and fractionated by the Hirt procedure. In a variety of transformed cell lines grown at 39.5°C, the majority of the viral genomes are found in the Hirt pellet (Table 1). This indicates that they are not free, as are the mature unit-length viral DNAs that are produced during vegetative growth of the virus in mouse cells.

In contrast to the minimum number of polyoma genome equivalents in Hirt supernatants of these cells estimated by infectivity assays (less than 0.01 genome per cell), 0.3 to 0.5 viral genome equivalents per cell were detected by reassociation kinetics. In some experiments, this figure represents the upper limit of the number of sequences that could have been present and may be an overestimate. The lack of infectivity of these sequences suggests they may be derived from randomly sheared cellular DNA associated with the viral genomes in the pellet. To further investigate this notion, we examined the physical properties of the polyoma sequences in the Hirt supernatant of BHK tsa-2a2c1 d at 39.5°C. If the viral sequences are derived from sheared cellular DNA, they will not be covalently closed and can be differentiated from mature viral DNAs by their density in CsCl containing ethidium bromide. Upon examination, over 90% of the viral sequences present in the Hirt supernatant of these transformed cells were not covalently closed, since they banded at the density of linear and nicked circular DNA (see below, Table 3).

TABLE 1. *Polyoma sequences in Hirt lysates of transformed cells grown at 39.5°C*

BHK cell line[a]	Polyoma DNA[b] (nmol)	Cell DNA[c] (μmol)
tsa-2a2c1 d		
1.[d] Pellet	5.4	3,400
Supernatant	1.2	430
2. Pellet	11.1	4,800
Supernatant	1.2	ND[e]
3. Pellet	12.3	10,300
Supernatant	≤0.85[f]	800
ts25-17E		
1. Pellet	11.5	3,000
Supernatant	2.2	170
2. Pellet	8.1	7,700
Supernatant	5.7	1,000
tsa-1b2(31°C)		
1. Pellet	8.0	7,800
Supernatant	1.9	ND

[a] All cells were grown at 39.5°C. Subgroupings denote separate experiments performed with independent preparations of DNAs.

[b] Quantity of polyoma DNA in each fraction determined by reassociation kinetics with [^{125}I]DNA probe and expressed as nanomoles of nucleotides.

[c] Quantity of cell DNA in each fraction determined by the diphenylamine assay, with dAMP as a standard, and expressed as micromoles nucleotide.

[d] Values in this experiment are averages of two determinations performed at separate times upon the same DNA. Less than 10% variation from the mean was observed.

[e] ND, Not determined.

[f] Upper limit on the amount present.

In summary, these experiments indicate that the majority of the viral sequences in these inducible cells are associated with cell DNA that pellets in the Hirt lysis procedure. The fraction of viral sequences that remain in the supernatant (10 to 20%) is not covalently closed and often is approximately the same as the fraction of total cell DNA that does not pellet (Table 1). It is likely that these sequences are in large part derived from randomly sheared cell DNA. In any event, they do not properly reflect the number of infectious (free) viral genomes.

To further investigate the state of the polyoma genomes in these cells, we determined whether they are covalently joined with cellular DNA. This is most simply done by ascertaining whether the viral sequences are joined to cellular repetitive sequences, which if denatured will reassociate at a very low C_0t value. The renatured repetitive sequences cause the cellular DNA to form large networks (30). These networks can be pelleted at low speeds in the centrifuge, and their content of viral sequences can be determined by reassociation kinetics with polyoma probe DNA.

The cellular DNA from several transformed lines was purified, with care being taken to avoid shear damage. It was denatured, either by heating or by treatment with alkali, and incubated briefly to permit reassociation of repetitive sequences interspersed throughout the genome. The large networks of cellular DNA that formed were pelleted, and their content of polyoma sequences was compared with that of the DNA in the supernatants (which did not form networks).

In every experiment, a large number of the polyoma sequences in the inducible cell lines (tsa-2a2c1 d and ts25-17E) entered into networks with cellular DNA (Table 2). The contrast between the fraction of the total polyoma sequences that are found in the network pellet and the fraction of the phage φX174 [^{32}P]DNA, added to monitor trapping, that is found in the pellet is striking and offers strong evidence for the association of polyoma DNA with repetitive cell DNAs. This association was stable to alkali (0.3 M NaOH) and to boiling.

TABLE 2. *Polyoma sequences in network-fractionated DNAs from cells grown at 39.5°C*

Expt[a]	BHK cell line[b]	Viral DNA (%)		Cell DNA[c] (%)	
		Pellet polyoma[d]	Supernatant polyoma	Pellet	Supernatant
1	tsa-2A2c1 d	71 (2)[e]	29	61	39
	ts25-17E	85 (4)	15	83	17
	tsa-1b2 (31°C)	47 (4)	53	89	11
2	ts-2A2c1 d	55 (3)	45	71	29
	ts25-17E	59 (2)	41	93	7
	tsa-1b2 (31°C)	27 (2)	73	74	26
3	tsa-2A2c1 d	66 (ND)[f]	34	77	23
	ts25-17E	68 (ND)	32	82	18

[a] Each experiment was performed independently, on separate preparations of DNA. Experiments 1 and 2 were performed as described in the text, with alkali denaturation of the DNA (purified as described in the text). Experiment 3 was performed by the procedure of Varmus et al. (30), in which heat was used to denature the DNA.

[b] All cells were grown at 39.5°C.

[c] Quantity of cell DNA in each fraction determined by its A_{260} in 10 mM NaOH.

[d] Quantity of polyoma DNA in each fraction determined by reassociation kinetics with [^{125}I]DNA.

[e] Numbers in parentheses indicate quantity of φX174 [^{32}P]DNA in each fraction determined from Cerenkov radiation immediately after centrifugation.

[f] ND, Not determined.

The polyoma sequences that do not form networks (and therefore are found in the supernatant fraction) may derive from either of two sources. They may be free, mature genomes unassociated with cell DNA, or they may be polyoma sequences associated with cell DNA that has not entered into networks. In some cases, as many as 40% of the total polyoma sequences in the inducible cell lines are in the supernatant fraction. This is substantially more than that amount appearing in the supernatant fraction after lysis of the cells by the Hirt procedure (Table 1). Furthermore, in view of the upper estimate of the total polyoma sequences that are covalently closed (Table 3), it is clear that the polyoma sequences in the network supernatant fraction are not mature viral genomes. We favor the interpretation that they are genomes integrated into cellular DNA that is sparse in repetititve sequences, but at present have no other data to support this notion.

Polyoma DNA in the uninducible tsa-1b2 (31°C) cell line. One line of cells, tsa-1b2(31°C), is no longer inducible for virus replication. Shortly after the isolation of tsa-1b2 (an inducible cell line), a culture of cells was placed at 31°C and passaged continuously at that temperature, with antipolyoma virus antiserum added to the medium to inactivate free virus. After 6 months at 31°C, no free virus could be detected in the culture fluid, nor could virus be rescued from the cells after they were fused with mouse cells. This cell line does not produce infectious centers when plated over WME cells, nor can infectious viral DNA be detected in a Hirt supernatant of the cells. They are therefore analogous to cells transformed by wild-type virus, from which polyoma cannot be rescued or induced (20, 32).

These cells contain polyoma sequences in their DNA. To learn more about the state of these sequences, we performed experiments similar to those described in the preceding section. As in the inducible cell lines, the majority of the polyoma sequences are found in the pellet fraction of cells lysed by the Hirt procedure (Table 1). Furthermore, the significant quantities of polyoma sequences, which appear in the pellet of network-fractionated cell DNAs (Table 2), provide strong evidence that some of the viral sequences are integrated. However, there is an apparent difference in the distribution of polyoma sequences between the pellet and supernatant fractions of network-fractionated tsa-1b2 (31°C) cell DNA and the DNAs of the inducible cell lines. This difference may reflect the content of repetitive sequence surrounding the sites the polyoma genome occupies.

Induction of the polyoma replicon at 31°C. When the inducible ts25- or tsa-transformed BHK-21-C13 cells are shifted to 31°C, infectious viral DNAs appear in the Hirt supernatant and increase exponentially throughout 72 h (9). The number of infectious genomes doubles every 10 h. Some of the genomes are encapsidated, since

TABLE 3. *Viral genomes in BHK tsa-2a2c1 d cells after a shift to 31°C*

Hours after shift to 31°C	Hirt supernatant		Hirt pellet (viral genome equivalents/cell)[a]
	Viral genome[a] equivalents/cell	Percent covalently closed[b]	
0	0.34	<10%	3.0
12	1.2	37%	3.5
24	1.9	72%	4.9
48	9.7	82%	6.4
72	22	ND[c]	13

[a] Calculated as described by Gelb et al. (13). For both calculations, the quantity of polyoma sequences (in genome equivalents, assuming the molecular weight of polyoma DNA to be 3×10^6) determined by reassociation kinetics was divided by the quantity of cell DNA in the Hirt pellet (in genome equivalents, assuming the molecular weight of the haploid mammalian genome to be 2×10^{12}).

[b] Fraction of the total found to band with form I simian virus 40 or φX174 DNA in CsCl-ethidium bromide gradients.

[c] ND, Not determined.

the cells form infectious centers, and infectious virus can be recovered (9; our unpublished observations).

We have confirmed several of these earlier observations, using reassociation kinetics rather than DNA infectivity assays to measure the quantity of viral sequences. Upon a temperature shift to 31°C, the number of viral genome equivalents per diploid cell genome appearing in the Hirt supernatant of tsa-2a2cl d cells increased from 0.3 to 30 over a period of 72 h (Fig. 2, Table 3). The rate of increase and the final number of genome equivalents reached after 72 h were very similar when measured either by DNA infectivity or by reassociation kinetics.

To more fully examine the structures of the viral DNAs produced after the temperature shift, samples of nucleic acid in the Hirt supernatants from each time point were banded in CsCl-ethidium bromide. The DNAs banding at the density of covalently closed DNA (form I) or at the density of linear or nicked circular DNA (forms II and III) were collected and analyzed for their content of polyoma sequences (Table 3). Before the shift to 31°C, all detectable viral sequences in the Hirt supernatant were covalently open, probably being derived from sheared cellular DNA. After the shift to 31°C, viral sequences were detected at the density of covalently closed DNAs. After 48 h, the majority of the genomes were covalently closed, as expected of mature viral DNA.

To determine whether there are significant quantities of oligomeric viral DNAs produced in these cells (6), a sample of DNA from the Hirt supernatant of cells shifted to 31°C for 72 h was sedimented through a neutral sucrose gradient. The gradient was fractionated into four parts, and the content of polyoma sequences in each fraction was analyzed by reassociation kinetics. The predominant DNAs containing viral sequences had the same size as mature polyoma DNA (Fig. 3). Less than 10% of the

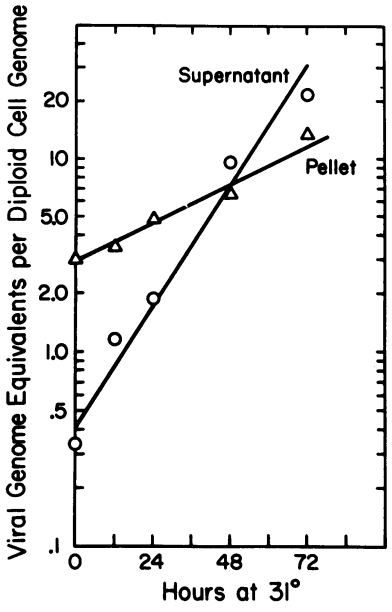

FIG. 2. *Induction of polyoma replication in BHK tsa-2a2cl d at 31°C. Semiconfluent cultures of BHK tsa-2a2cl d cells growing at 39.5°C were shifted to 31°C. Samples (5 to 10 plates each) were lysed by the Hirt procedure and fractionated into pellet and supernatant fractions, and the nucleic acids in each fraction were purified as described in the text. The content of polyoma sequences in the pellet was normalized to the quantity of cell DNA (determined by diphenylamine), and the polyoma genome equivalents per diploid cell genome were calculated as described by Gelb et al. (13). The quantity of viral sequences in the supernatant was normalized to the quantity of cell DNA in the pellet of that sample. Results are from a single experiment, but similar values have been obtained from other induction experiments with BHK ts25-17 as well as with BHK tsa-2a2cl d, in which fewer samples were taken. Symbols: (○) Hirt supernatant viral genome equivalents; (△) Hirt pellet viral genome equivalents.*

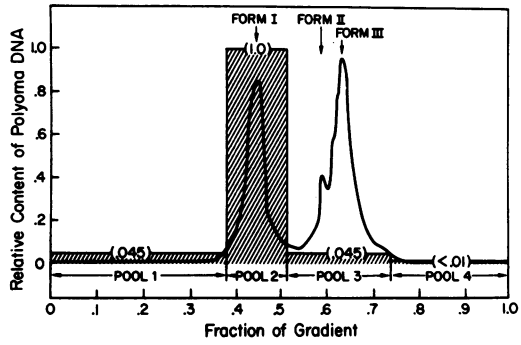

FIG. 3. *Sedimentation of DNAs through a neutral sucrose gradient: 0.5 ml of a Hirt supernatant fraction (deproteinized and ethanol precipitated as described in the text) was layered on a 5 to 20% sucrose gradient containing 10 mM Tris-Chloride and 1 mM EDTA, pH 7.5. On an identical gradient, a mixture of polyoma [^{32}P]DNA (form I and form II) and [^{3}H]-DNA (EcoRI linears) in 0.5 ml was layered as sedimentation marker. The gradients were centrifuged in an SW27.1 rotor at 24,000 rpm for 15 h at 20°C and collected from the bottom of the tubes. After analyzing the fractions containing the labeled polyoma DNAs (smooth curve in the figure), pools 1 to 4 were made of the fractions from the gradient containing the Hirt supernatant DNAs. A 100-μg amount of salmon sperm DNA was added to each pool, and the nucleic acids were ethanol precipitated and analyzed for polyoma DNA content as described in the text. The labeled areas indicate the relative content of polyoma sequences in each pool.*

polyoma sequences sedimented faster or slower than form I DNA, and there were no sequences detected sedimenting more slowly than form II DNA. A more detailed examination of the polyoma DNAs induced or rescued from these cells is the subject of a subsequent paper (D. M. Anderson and W. R. Folk, manuscript in preparation).

The previous experiments confirm that the replication of free viral DNAs was induced in these cells by a shift to 31°C. In addition, viral genomes that were associated with the chromatin in the Hirt pellet were selectively increased (Table 3; Fig. 2). Over a period of 72 h, the number of viral genomes per diploid cell genome recovered in the Hirt pellet increased from 3 to approximately 13. It is unlikely that this increase was an artifact due to trapping of free viral genomes in the pellet: reconstruction experiments indicated that over 90% of the viral DNA added to cells immediately before lysis was recovered in the Hirt supernatant. There would need to be approximately 100 genomes per cell for the amount detected in the pelleted DNA to be due to trapping. Although it is not likely that reconstruction experiments of this sort adequately mimic the viral DNA in the cell nucleus, it seems improbable that the increased quantity of viral genomes in the pellet at early times before there are equivalent numbers of "free" genomes in the supernatant fraction (Fig. 2) is due to trapping. Furthermore, at no time (either at 39.5°C or after a shift to 31°C) were there significant quantities of infectious viral DNA present in the pellet (our unpublished observations).

To provide further support for the notion that the viral genomes in the pelleted DNA are not trapped mature viral genomes, a sample of the pelleted DNA at 72 h after a temperature shift was banded in CsCl-ethidium bromide. The distribution of polyoma sequences between covalently open and covalently closed DNAs was measured, using reassociation kinetics. Over 75% of the polyoma sequences banded as covalently open, indicating they are not form I DNAs.

In summary, the temperature shift to 31°C induces the replication of free viral genomes as well as an increase in genomes that are associated with cell DNAs in the Hirt pellet. Further studies are being carried out to determine the nature of the genomes associated with the cellular DNA and to determine whether there is a precursor-product relationship between the genomes isolated in the pellet and the supernatant fractions.

DISCUSSION

Other workers have observed that the viral genome in papovavirus-transformed cells can be induced to replicate. To get activation of the viral genome in transformants that are nonpermissive, the transformed cells must be fused with permissive cells (20, 32). Semipermissive cells, such as transformed hamster or rat embryo fibroblasts, can be stimulated to produce virus by a variety of physical or chemical treatments (8, 17). Permissive cells, which support the vegetative growth of the virus, are rarely transformed, as they are usually killed by the virus. Only if virus replication is interfered with are permissive cells viably transformed. In both mouse 3T3 cells and BHK-21-C13 cells, manipulation of the ability of polyoma virus to replicate can be achieved by transforming with a mutant of the A complementation group. Shortly after transformation is initiated, the *A* gene function can be inactivated sufficiently to prevent killing of the cells by this virus while permitting expression of the transformed phenotype (9, 27, 31).

The polyoma *A* gene product is very likely T antigen or a component thereof (24). One of its functions is to modulate the initiation of new grounds of replication of free polyoma genomes in productively infected cells (11). Furthermore, *A* gene function is necessary for the initiation of transformation of BHK-21-C13 (12). The analogous simian virus 40 *A* gene product modulates the initiation of new rounds of DNA replication (28) and is believed to control the phenotype of transformed cells (4, 19, 22, 23, 29). Evidence of a similar role for the polyoma *A* gene product in the maintenance of the transformed phenotype is inconclusive (12, 18). Models in which the *A* gene product modulates the initiation of DNA replication of both the virus and host genomes in transformed cells have been proposed (4, 5, 22, 28), but no direct tests of these models have yet been made.

We can detect no infectious polyoma genomes in Hirt supernatants of these inducible transformed BHK-21 cells at 39.5°C, a temperature at which the *A* gene product is inactive. Polyoma sequences are present in the supernatants, as detected by DNA reassociation kinetics, but they are covalently open and are probably derived from sheared cellular DNA. They are present in less than one viral genome equivalent per cell.

Many of the viral sequences that are present in the inducible cells at 39.5°C are associated with cellular repetitive DNAs by bonds that are resistant to boiling and alkali. The viral sequences not associated with repetitive DNAs may be free, or they may be associated with cellular DNAs that are not highly repeated. The uninducible tsa-1b2(31°C) cell line con-

tains viral sequences that are predominantly not associated with repetitive cell sequences. The covalent integration of simian virus 40 DNA into the genomes of mouse cells and of polyoma DNA into the genome of rat cells has previously been established (14, 21, 26).

The constant number of viral genomes in these inducible cell lines when grown at 39.5°C, and in the uninducible cell line, argues strongly for a tight coupling of replication of the viral genome and the cellular genome. In several years of continuous passage, we have detected no change in the number of viral genome equivalents per diploid cell genome. Every subclone we have examined contains, within experimental error, the same number of genomes as the parental cell line (our unpublished observations), and all are inducible for viral DNA replication. These observations suggest to us either of two possibilities: (i) all the viral sequences in these cells are integrated, but their sites of integration differ with respect to the quantity of nearby repetitive sequences; or (ii) these cell lines contain both integrated and free viral genomes (the latter being uninfectious), both of whose replication is tightly coupled with that of the cell. We are biased toward the first possibility, because it is simpler and because it is not apparent how the free genomes would replicate in the absence of active *A* gene product or why they would be uninfectious. Furthermore, there is evidence that the sites of integration of the simian virus 40 genome in transformed cells differ from one cell line to another (14).

An important question that is yet to be resolved is that of the origin of the infectious viral genomes that appear in the Hirt supernatant after a temperature shift to 31°C. Their absence from Hirt supernatants of cells grown at 39.5°C and the uniform inducibility of subclones indicate that they must derive from sequences associated with cellular DNA in the Hirt pellet. The *A* gene product may or may not have a direct role in the early formation of these free viral genomes.

A very interesting feature of the induction of the viral replicon in these cells at 31°C is the increase in the viral genomes associated with the cellular DNA in the Hirt pellet. Since the *A* gene product modulates the initiation of rounds of replication of free genomes, one might expect it to have a similar effect upon integrated genomes. Cellular DNA replication might be activated by the *A* gene function as well, but preferential replication of the viral genome would result if the *A* gene product exhibited a higher affinity for the viral origin of replication (4, 5, 21) than for cellular origins of replication. Selective replication of gene *N* mutants of λ while integrated into the *Escherichia coli* chromosome, and of mutants of phage P22 while integrated into the *Salmonella typhimurium* chromosome, provide prokaryotic models for this speculation (7; S. Weaver and M. Levine, personal communication).

Clearly, considerably more information about the site occupied by the viral genome(s) in these cells is required before we can understand the events that take place when the viral *A* gene product is activated. Does selective amplification of the integrated viral genome provide a source of viral DNA that can be excised and enter into rounds of vegetative growth? This and other questions about the biology of these cells are currently being studied.

ACKNOWLEDGMENTS

We thank Barbara Fishel and Mary Wolf for providing help with some of the early experiments.

This work was supported by grant NP 172 from the American Cancer Society and in part by Public Health Service grant 5-R01-CA13978 from the National Cancer Institute.

LITERATURE CITED

1. **Anderson, D. M., and W. R. Folk.** 1976. Iodination of DNA. Studies of the reaction and iodination of papovavirus DNA. Biochemistry 15:1022–1030.
2. **Ashwell, F.** 1957. Colorimetric analysis of sugars, p. 99–102. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. III. Academic Press Inc., New York.
3. **Bellet, A. J. D.** 1975. Covalent integration of viral DNA into cell DNA in hamster cells transformed by an avian adenovirus. Virology 65:427–435.
4. **Butel, J. S., J. S. Brugge, and C. A. Noonan.** 1974. Transformation of primate and rodent cells by temperature-sensitive mutants of SV-40. Cold Spring Harbor Symp. Quant. Biol. 39:25–36.
5. **Cheevers, W. P., and J. B. Hiscock.** 1973. DNA synthesis in polyoma virus infection II. Relationship between viral DNA replication and initiation of cellular DNA replicons. J. Mol. Biol. 78:237–245.
6. **Cuzin, F., M. Dieckmann, M. Vogt, and P. Berg.** 1970. Induction of virus multiplication in 3T3 cells transformed by a thermosensitive mutant of polyoma virus. J. Mol. Biol. 47:317–333.
7. **Eisen, H., L. Pereira da Silva, and F. Jacob.** 1968. The regulation and mechanism of DNA synthesis in bacteriophage λ. Cold Spring Harbor Symp. Quant. Biol. 33:755–764.
8. **Fogel, M.** 1972. Induction of virus synthesis in polyoma transformed cells by DNA antimetabolites and by irradiation after treatment with 5-bromodeoxyuridine. Virology 49:12–22.
9. **Folk, W. R.** 1973. Induction of virus synthesis in polyoma-transformed BHK-21 cells. J. Virol. 11:424–431.
10. **Folk, W. R., and H. E. Wang.** 1974. Closed circular DNAs with tandem repeats of a sequence from polyoma virus. Virology 61:140–155.
11. **Francke, B., and W. Eckhart.** 1973. Polyoma gene function required for viral DNA synthesis. Virology 55:127–135.

12. **Fried, M.** 1965. Cell transforming ability of a temperature sensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. 53:486–491.
13. **Gelb, L. D., D. E. Kohne, and M. Martin.** 1971. Quantitation of SV-40 sequences in African green monkey, mouse and virus transformed cell genomes. J. Mol. Biol. 57:129–145.
14. **Gelb, L. D., and M. A. Martin.** 1973. SV-40 integration within the genome of virus-transformed mammalian cells. Virology 51:351–357.
15. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1973. Isolation of high molecular weight DNA from mammalian cells. Eur. J. Biochem. 36:32–38.
16. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365–367.
17. **Kaplan, J. C., S. Wilbert, and P. Black.** 1972. Analysis of simian virus 40-induced transformation of hamster kidney tissue in vitro. VIII. Induction of infectious simian virus 40 from virogenic transformed hamster cells by amino acid deprivation or cycloheximide treatment. J. Virol. 9:448–453.
18. **Kimura, G.** 1975. Temperature-sensitive growth of cells transformed by ts-a mutant of polyoma virus. Nature (London) 253:639–641.
19. **Kimura, G., and A. Itagaki.** 1975. Initiation and maintenance of cell transformation by SV-40: a viral genetic property. Proc. Natl. Acad. Sci. U.S.A. 72:673–677.
20. **Koprowski, H., F. C. Jensen, and Z. Steplewski.** 1967. Activation of production of infectious tumor virus SV-40 in heterokaryon cultures. Proc. Natl. Acad. Sci. U.S.A. 58:127–133.
21. **Manor, H., M. Fogel, and J. Sachs.** 1973. Integration of viral into chromosomal DNA in an inducible line of polyoma-transformed cells. Virology 53:174–185.
22. **Martin, R. G., J. Y. Chou, J. Avila, and R. Saral.** 1974. The semiautonomous replicon: a molecular model for the oncogenicity of SV-40. Cold Spring Harbor Symp. Quant. Biol. 39:17–24.
23. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene *A* function and maintenance of transformation. J. Virol. 15:636–644.
24. **Paulin, D., and F. Cuzin.** 1975. Polyoma virus T antigen: synthesis of modified heat-labile T antigen in cells transformed with the ts-a mutant. J. Virol. 15:393–397.
25. **Reed, S. I., J. Ferguson, R. W. Davis, and G. R. Stark.** 1975. T Antigen binds to SV-40 DNA at the origin of replication. Proc. Natl. Acad. Sci. U.S.A. 72:1605–1609.
26. **Sambrook, J., H. Westphal, P. R. Srinivasan, and R. Dulbecco.** 1968. The integrated state of viral DNA in transformed cells. Proc. Natl. Acad. Sci. U.S.A. 60:1288–1295.
27. **Summers, J., and M. Vogt.** 1970. Recovery of virus from polyoma transformed BHK-21, p. 306–311. *In* L. G. Silvestri (ed.), Second Lepetit Symposium: The biology of oncogenic viruses. North Holland Publishing Co., Amsterdam.
28. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. 10:591–598.
29. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene *A* in transforming infection. J. Virol. 15:613–618.
30. **Varmus, H. E., P. K. Vogt, and J. M. Bishop.** 1973. Integration of DNA specific for Rous sarcoma virus after infection of permissive and nonpermissive hosts. Proc. Natl. Acad. Sci. U.S.A. 70:3067–3071.
31. **Vogt, M.** 1970. Induction of virus multiplication in 3T3 cells transformed by a thermosensitive mutant of polyoma virus I. J. Mol. Biol. 47:307–316.
32. **Watkins, J. F., and R. Dulbecco.** 1967. Production of SV-40 virus in heterokaryons of transformed susceptible cells. Proc. Natl. Acad. Sci. U.S.A. 58:1396–1403.
33. **Wetmur, J., and N. Davidson.** 1968. Kinetics of renaturation of DNA. J. Mol. Biol. 31:349–370.