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Polyoma and Cell DNA Synthesis in Mouse L Cells Temperature Sensitive for the Replication of Cell DNA

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Polyoma (Py) virus multiplies, at 34 and 38.5 C, in wild-type (WT-4) and in *ts* A1S9 mouse L cells, which are temperature sensitive for growth and for DNA replication (R. Sheinin, 1976; L. H. Thompson et al., 1970). De novo synthesis of double-stranded, fully covalently closed Py DNA has been shown to proceed by semiconservative replication in WT-4 and *ts* A1S9 cells at the permissive and nonpermissive temperatures. Cell DNA is made late during infection, by both cell types and at both temperatures. Semiconservative replication of cell DNA proceeds in Py-infected WT-4 cells incubated at 34 or at 38.5 C and in Py-infected *ts* A1S9 cells incubated at 34 C. In virus-infected *ts* A1S9 cells incubated at 38.5 C, cell DNA synthesis appears to proceed almost entirely by a process analogous to repair replication. The inability of *ts* A1S9 cells to produce large-molecular-weight chromosomal DNA strands, at 38.5 C, by the normal mechanism is not overcome by Py infection.

The transforming capacity of polyoma (Py) virus derives from the interaction of viral DNA synthesis with the specific DNA metabolism of host cells, the final result being integration of viral into chromosomal DNA (14, 33). This interaction can take one of several pathways, depending upon the physiology of infection (7, 15, 37, 38, 40). Of interest in the present context are those events which proceed in productively infected cells. Early during infection, the synthesis of apparently normal cell DNA is stimulated or derepressed (7, 13, 15, 37, 38). In association with the onset of Py DNA replication, cell DNA synthesis becomes aberrant (37, 40) such that incomplete, single-stranded DNA accumulates (10-12). Available evidence suggests that this phenomenon results from multiple initiation events, which ultimately tax the normal mechanism of ligation.

Stimulation of cell DNA synthesis in Py-infected cells probably results from the expression of an early Py gene (17, 20, 31). It seems likely that most proteins which participate in Py and in cell DNA synthesis in virus-infected cells are encoded in the cell genome. Py DNA appears to carry little if any information in excess of that required to code for the virion polypeptides (cf. 17, 21, 33), the protein responsible for the temperature-sensitive *ts* a function (19, 20, 31) and the Py-specific T antigen (22, 31).

Little definitive information is available regarding either the proteins that participate in the replication of Py DNA or the mechanism by which cell DNA synthesis is induced. One approach to this problem is to study Py infection of mutants of permissive mammalian cells defective in DNA synthesis. The present work describes such experiments with a line of mouse L cells that is *ts* for the replication of nuclear DNA (39). These *ts* A1S9 cells cannot convert newly made, small single-stranded DNA segments to large-molecular-weight chromosomal DNA at the nonpermissive temperature. However, they do support the multiplication of Py virus and normal Py DNA replication. Cell DNA synthesis occurs, but it does not follow the pattern seen in wild-type (WT-4) cells or in *ts* A1S9 cells infected at the permissive temperature.

MATERIALS AND METHODS

Cells. WT-4 and *ts* A1S9 mouse L cells, the properties of which are described elsewhere (39, 43), were grown at 34 C, either in suspension or on a solid substratum, a α-minimum essential medium (42) lacking nucleosides but supplemented with 7.5% (vol/vol) fetal calf serum (Reheis Chemical Co.). Suspension cultures (40-ml volumes in 15- by 2.5-cm tubes) were incubated in New Brunswick Rollotherm cabinets. They were inoculated at 2×10^4 to 5×10^4 cells/ml and grown to late logarithmic phase prior to subculture. In the case of cells grown on glass, confluent cultures were subcultured as follows. The medium was decanted, and the cells were washed twice with phosphate-buffered saline (16) and then incubated at 34 C for 5 min with a solution of

hyaluronidase and collagenase (50 μg/ml each; Worthington Biochemicals) in a volume just adequate to cover the culture surface. This solution was replaced with 0.1% trypsin (Difco) in citrate saline (34) for an additional 5 min. The cells were then suspended in medium and plated at concentrations of about 5×10^4 to 5×10^5 cells/ml (in volumes adequate to cover the growing surface of culture bottles).

Virus. Py TSP1 (41) was prepared by the procedure of Winocour (47). A single stock having a titer of 6.8×10^{10} PFU/ml was used throughout. Plaque assays were performed on mouse embryo cultures (34).

Infection procedures. To infect cells grown on glass, medium from subconfluent cultures was removed; virus (in a volume adequate to form a film over the cells) was added and allowed to adsorb for 1 h. Medium containing 1% (vol/vol) fetal calf serum was added, and the cultures were incubated at the appropriate temperature. In the case of suspension cultures, cells were collected by centrifugation (10 min, $800 \times g$) and incubated with intermittent mixing for 30 min in 0.5 ml of virus suspension before medium was added and incubation at the desired temperature was continued. For mock infection, virus was replaced with an equal volume of phosphate-buffered saline.

Antibody to Py virus. Antiserum to Py virus, with a neutralization coefficient of $K = 23\ \text{min}^{-1}$ (1), was raised in rabbits (36). In some experiments this antiserum was used directly. In others, the antiviral gamma globulin coupled with fluorescein was used for immunofluorescent staining of mock- or Py-infected cells grown on cover slips (40, 46).

Experimental regimen for studying DNA synthesis. Unless otherwise noted, the following procedure was used for the study of DNA synthesis. Cells were grown at 34 C through three to four generations to midlogarithmic phase in medium supplemented with 0.01 μCi of [^{14}C]thymidine (dThd) per ml (approximate specific activity, 60 mCi/mmol; approximate concentration, 0.2 μM). This medium was then replaced by nonradioactive medium, after which the cells were incubated at either 34 or 38.5 C for 16 to 24 h, a period adequate to permit full expression of the *ts* lesion of *ts* A1S9 cells (39, 43). [*methyl*-^{3}H]dThd (approximate specific activity, 20 mCi/mmol; approximate concentration, 0.05 μM) was then added, as noted in individual experiments, to label DNA newly made under test conditions.

Assessment of DNA synthesis. [^{14}C]- and [^{3}H]DNA was examined by velocity sedimentation and by equilibrium centrifugation by procedures previously described. For velocity sedimentation studies in neutral sucrose density gradients (6, 8), approximately 10^7 cells, lysed in 2 ml of a 1% solution of sodium dodecyl sulfate in SSC, were layered onto a 15 to 30% (wt/wt) linear sucrose density gradient (29 ml, in 0.01 M Tris [pH 7.4]-0.1 NaCl-0.001 M EDTA-0.5% sodium dodecyl sulfate) formed over a 5-ml cushion of 70% sucrose in SSC and centrifuged for 16 h at 20 C in a Beckman SW27 rotor at 22,000 rpm. One-milliliter fractions were collected with an Isco density gradient fractionator and monitored for optical density at 260 nm, which permitted identification of the internal sedimentation markers, 18*S* and 28*S* rRNA.

For analysis of alkaline-denatured DNA, approximately 5×10^5 to 5×10^6 cells (in 0.2 ml) were added to 2 ml of 0.2 M NaOH-0.01 M EDTA layered on a 5 to 20% sucrose density gradient (30 ml) in 0.9 M NaCl-0.3 M NaOH-0.001 M EDTA established over a 4-ml cushion of 70% sucrose (8). This was left at 2 C for at least 12 h before being centrifuged at 22,000 rpm at 2 C for 9 h in a Beckman SW27 rotor. Purified ^{14}C-labeled Py form I DNA (4) was run in parallel gradients to serve as marker. Fractions were collected as noted above. All fractions were analyzed for the presence of trichloroacetic acid-precipitable radioactively labeled material. (Radioactive dThd was purchased from Amersham-Searle Corp.). The amount of any given component was calculated by expressing the counts per minute in the corresponding peak as a percentage of the total acid-precipitable counts per minute recovered from the gradient.

Equilibrium centrifugations were performed at 2 C for 48 h at 48,000 rpm in a Beckman SW50.1 rotor. For neutral gradient analysis (27), DNA samples were diluted to 5 ml with CsCl in 0.01 M Tris-0.01 M EDTA, pH 7.4 (final density, 1.70 g/cm^3). Poly(dA:dT) and poly(dC:rG) (Miles Laboratories) were added as optical density markers. To prepare DNA for alkaline gradient analysis, samples were heated for 30 min at 50 C with one-tenth volumes of 1 M NaOH (26) and then diluted to 5 ml with saturated CsCl solution in 0.01 M Tris, pH 8.0, to a final density of 1.76 g/cm^3. Poly(dA·dT) was used as optical density marker. Fractions were collected from the bottom of each tube and monitored for optical density at 260 nm, trichloroacetic acid-insoluble material, and refractive index. Calculations of density were made from the latter measurements.

DNA-DNA hybridization. To establish the nature of the 20*S* Py DNA made in infected L cells, DNA-DNA hybridization studies were performed as described previously (6, 8), using cellulose nitrate filters carrying either 0.1 μg of purified form I Py DNA or 10 μg of L-cell DNA, isolated by established procedures (37). Prior to use, labeled DNA was sheared by sonic vibration, boiled at 100 C, and quick-cooled in ice.

RESULTS

Growth of Py virus in WT-4 and ts A1S9 cells. Several years ago it was reported that certain strains of Py virus grow in some lines of mouse L cells (2, 3, 23, 28). Initially, therefore, experiments were performed to determine whether WT-4 and *ts* A1S9 cells would support the growth of Py TsP1. As may be seen from the data presented in Table 1, virus multiplication does proceed in these cells, at both 34 and 38.5 C, even in the presence of antiviral antiserum added to inactive unadsorbed virus. This antiserum had little effect on virus formation of WT-4 cells or in *ts* A1S9 cells incubated at 34 C, but it did reduce the yield of Py from *ts* A1S9

TABLE 1. *Multiplication of Py TSP1 in WT-4 and ts A1S9 mouse L cells*[a]

Cells	Temp (C)	Antiserum added	Adsorbed MOI (PFU/cell)	Plaque titer (PFU/ml)		
				2 h p.i.	24 h p.i.	48 h p.i.
WT-4	34.0	–	0.04	1.4×10^4	3.0×10^5	6.2×10^6
		+	0.04		4.0×10^3	2.1×10^5
	38.5	–	0.03	8.0×10^3	3.1×10^5	1.0×10^6
		+	0.03		8.5×10^3	1.1×10^6
ts A1S9	34.0	–	0.01	1.6×10^4	1.5×10^4	1.4×10^5
		+	0.01		9.5×10^3	1.5×10^5
	38.5	–	0.01	8.0×10^3	3.4×10^4	1.2×10^6
		+	0.01		4.6×10^4	3.0×10^5

[a] Replicate cultures (in 2-oz [ca. 0.06-liter] Brockway bottles) of the L cells, in mid-logarithmic phase of growth, were incubated for 24 h at 34 or 38.5 C. They were then infected with 0.2 ml of Py TSP1 (10 to 20 PFU/cell). After the adsorption period, one-half of the cultures were treated for 2 h at the appropriate temperature with antiserum to virus, sufficient to inactivate unadsorbed virus. At the intervals noted the cultures were washed, incubated in medium, and plaque assayed for virus on mouse embryo cells. L-cell cultures (including medium) were subjected to three cycles of freeze-thawing prior to titration by plaque assay. No virus was detected in control or mock-infected cultures. The absorbed multiplicity of infection (MOI) was calculated as the amount of infectious virus associated with the L cells, released from the glass, and counted after the 2-h incubation with or without antiserum.

cells incubated at 38.5 C. This may be related to the increased fragility of the *ts* cells after prolonged incubation at this temperature.

The preceding growth experiments were conducted at low multiplicities of infection. The biochemical experiments to be described below are performed at high multiplicities of infection to obtain totally infected cell populations. Evidence that this objective was achieved was obtained from two kinds of experiments. Data from one experiment (Table 2) indicated that all WT-4 and *ts* A1S9 cells infected at either low or high temperature with Py TSP1 at a multiplicity of infection of $\cong 2 \times 10^3$ PFU/cell plated as infectious centers. Further evidence that all cells incubated under such conditions were infected was obtained from studies of immunofluorescent staining. WT-4 and *ts* A1S9 cells were grown at 34 C to about two-thirds confluence in cultures containing cover slips (40). Replicate cultures were infected at an estimated multiplicity of 2,000 PFU/cell and incubated at either 34 or 38.5 C. After 24 and 48 h the cover slips were removed and stained with fluorescein-conjugated antipolyoma gamma globulin (40, 46). Almost all nuclei of infected cultures (but not of mock-infected cells) exhibited weak fluorescence at 48 h postinfection (p.i.), indicating the presence of newly made virion proteins (40, 46).

DNA synthesis in Py-infected L cells. Previous studies (39, 43) have shown that *ts* A1S9 cells are defective in DNA synthesis at 38.5 C. After incubation at this temperature for 16 to 24 h, the incorporation of dThd into DNA falls to a level about 1 to 5% of that observed either with WT-4 cells incubated at 38.5 C or with both WT-4 and *ts* A1S9 cells incubated at 34 C. Such incorporation increases if *ts* A1S9 cells are incubated further at 38.5 C and can approach 10% of control levels 48 to 72 h later.

TABLE 2. *Infection of WT-4 and ts A1S9 cells by Py virus*[a]

Cells	Temp (C)	Infectious centers ($\times 10^{-4}$)	
		Expected	Observed
WT-4	34.0	2.7	2.4
	38.5	1.2	2.9
ts A1S9	34.0	0.7	1.6
	38.5	1.6	1.3

[a] Replicate cultures (in 4-oz [ca. 0.12-liter] Brockway bottles) in late logarithmic phase were incubated for 24 h at 34 and 38.5 C. Individual cultures were then either infected with Py TSP1 (input multiplicity of infection, $\simeq 2 \times 10^3$ PFU/cell) or mock infected. They were incubated at the appropriate temperature with medium containing antibody to inactivate unadsorbed virus. At 16 h p.i., cells were washed, harvested, counted, and plated on indicator mouse embryo fibroblasts (cf. 34). The observed infectious centers were measured by this plaque assay. The expected values are calculated from the actual cell numbers counted prior to plating.

Direct studies on the pattern of DNA replication indicate that this is not due to restoration of any capacity for normal semiconservative DNA synthesis, but results from repair replication (cf. 39; R. Sheinin, manuscript in preparation; and below).

Because the replication of Py DNA is known to be closely linked to cell DNA synthesis (cf. 6, 7, 17, 38), it was of interest to study the pattern of formation of both Py and cell DNA in *ts* A1S9 cells incubated at permissive and nonpermissive temperatures.

The first approach was to examine total Py and cell DNA synthesis by velocity sedimentation analysis in neutral sucrose density gradients. *ts* A1S9 cells were grown to midlogarithmic phase and then incubated at 34 or 38.5 C for 24 h. The cultures were then infected with Py virus and incubated further at the appropriate temperature. Twenty-four hours p.i. (i.e., late during the infectious cycle) cultures were treated for 6 h with [^{3}H]dThd to label newly made DNA, which was analyzed by velocity sedimentation in neutral sucrose density gradients as described in the legend to Fig. 1. Two major components were obtained, one of which sedimented with the high-molecular-weight cell DNA of mock-infected cultures (data not shown) and was collected onto the 70% sucrose cushion at the bottom of the gradient (e.g., fractions 30–38 in Fig. 1a and 39–42 in Fig. 1b). The second component was found in the same position as marker (20*S*) [^{14}C]Py DNA isolated from purified virus particles.

In alkaline sucrose density gradients (discussed below) the ^{3}H-labeled 20*S* DNA isolated for Py-infected *ts* A1S9 cells cosedimented with the [^{14}C]Py DNA marker at 53*S* (8). Similar data were obtained with WT-4 cells. Essentially identical results were obtained with cells grown in suspension and on glass, labeled at either 24 or 48 h p.i. In all instances the sedimentation profiles obtained resembled those observed with other productively infected cells.

The finding that the ^{3}H-labeled 20*S* DNA of Py-infected WT-4 or *ts* A1S9 cells cosedimented with ^{14}C-marker Py DNA in neutral and alkaline sucrose density gradients suggested that the former is double-stranded, covalently closed form I Py DNA (14, 45). To further establish its identity, the following experiment was done. Cells were grown, infected, and labeled as

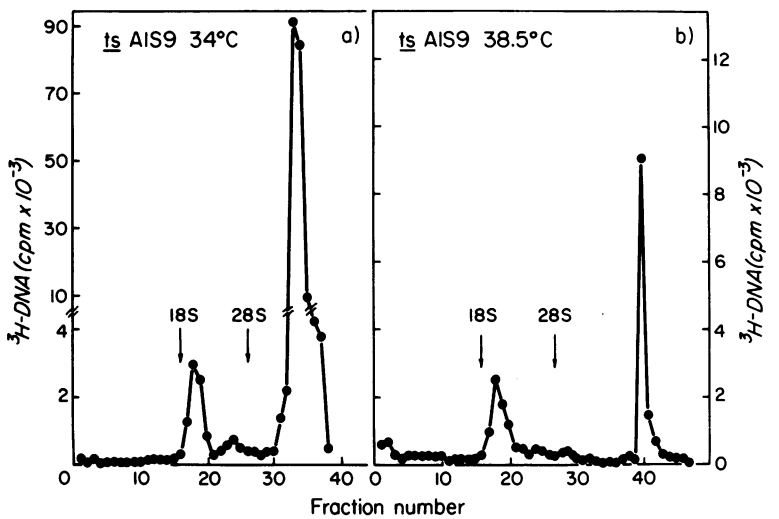

FIG. 1. *Velocity sedimentation analysis in neutral sucrose density gradients of DNA synthesized in Py-infected ts A1S9 cells. Replicate suspension cultures were grown at 34 C to mid-logarithmic phase. One-half of the cultures was then shifted to 38.5 C, and incubation was continued for 16 h. All subsequent manipulations of cells were carried out at the temperature of incubation. The cells were centrifuged at 800 × g for 10 min, infected with 0.5 ml of a Py suspension (multiplicity of infection, ≅2,000 PFU/cell), resuspended to their original concentration in medium lacking nucleosides, and re-incubated. At 0, 24, and 48 h p.i., the cultures were sampled for plaque assay. At 24 h p.i., [^{3}H]dThd (1 μCi/ml) was added to each culture, and 6 h later approximately 2 × 10^7 cells were harvested by centrifugation and washed once with SSC. The [^{3}H]DNA was analyzed by velocity sedimentation in neutral sucrose density gradients as described in Materials and Methods. The arrows mark the position of 18S and 28S rRNA. The plaque titers at time zero were about 4 × 10^6 PFU/ml for all cultures. After 48 h they were 2.5 × 10^7 and 3.0 × 10^7 PFU/ml for ts A1S9 cells incubated at 34 and 38.5 C, respectively.*

described in the legend to Fig. 1. The [^{3}H]DNA was separated in neutral sucrose density gradients. The material sedimenting at 20*S* (e.g., fractions 13–21 of Fig. 1a) was isolated and tested for its capacity to hybridize with Py and mouse cell DNA. The ^{3}H-labeled 20*S* DNA isolated from WT-4 and *ts* A1S9 cultures infected and labeled at either 34 or 38.5 C hybridized with purified form I Py DNA with an efficiency equivalent to that exhibited by [^{3}H]Py DNA extracted from infected mouse embryo cells (Table 3). Little or no hybridization was detected between ^{3}H-labeled 20*S* DNA and cell DNA isolated from uninfected mouse L cells (WT-4 or *ts* A1S9). It was therefore concluded that the ^{3}H-labeled 20*S* DNA is in fact Py DNA.

In some instances [^{3}H]DNA sedimenting between 21 to 28*S* (e.g., in fractions 21–26 of Fig. 1a) was observed in Py-infected cultures. On the basis of our own studies (P. E. Branton and R. Sheinin, manuscript in preparation) and those of others (30), it has been assumed that this material is [^{3}H]Py DNA in the replicating form.

These experiments indicate that *ts* A1S9 cells, in which normal cellular DNA replication is blocked at 38.5 C, are able to synthesize apparently normal Py DNA in amounts comparable to those produced by infected WT-4 cells (at 34 and 38.5 C) and at 34 C by infected *ts* A1S9 cells.

Cell DNA synthesis in Py-infected WT-4 and ts A1S9 cells: size analysis by velocity sedimentation in alkaline sucrose density gradients. It has been demonstrated that, in cells productively infected with Py virus, cellular DNA synthesis is stimulated several hours prior to the onset of viral DNA replication (13, 15, 37). In addition, it is known that the induced cell DNA synthesis becomes increasingly aberrant after viral DNA replication is initiated (6, 7, 10–12, 37). The abnormality is readily demonstrable by velocity sedimentation analysis in alkaline sucrose density gradients. Since DNA replication in *ts* A1S9 cells is defective at high temperature, it was of interest to compare the patterns of cell DNA synthesis in virus-infected WT-4 and *ts* A1S9 cells at the permissive and nonpermissive temperatures.

Cells were grown at 34 C with [^{14}C]dThd to label cell DNA and were then incubated at 38.5 C for 24 h. They were then infected with Py virus (or were mock infected) and labeled with [^{3}H]dThd from 24 to 30 h p.i. The newly made [^{3}H]DNA was analyzed by velocity sedimentation in alkaline sucrose density gradients (Fig. 2).

The data shown in Fig. 2a were obtained with WT-4 cells. The mock-infected culture gave a sedimentation profile expected for normal cells (10, 11). The majority (81.8%) of the [^{3}H]DNA synthesized at 38.5 C was recovered along with marker [^{14}C]DNA preformed at 34 C (data not shown) on the 70% sucrose cushion (fractions 31–36). In the case of Py-infected WT-4 cells, the sedimentation profile exhibited three components. One (52.6% of the total [^{3}H]DNA) was recovered in the position of high-molecular-weight cell DNA at the bottom of the gradient. A second (12.3% of the total) sedimented in the position of the marker (53*S*) [^{14}C]Py DNA. The third (19.9% of the total) sedimented heterogeneously between these two components (fractions 24–30). On the basis of the close qualitative and quantitative analogy between these data and those obtained from studies of Py-infected mouse embryo cells (10–12) and 3T3 mouse fibroblasts (unpublished data), we have assumed that the heterogeneously sedimenting [^{3}H]DNA results from aberrant cell DNA synthesis, i.e., from the inhibition of chain elongation which occurs late during Py virus growth.

The results obtained with *ts* A1S9 cells incubated at 38.5 C are shown in Fig. 2b. They are, in general, similar to those obtained with WT-4 and other mouse cell systems. However, with both mock- and virus-infected cultures there is more [^{3}H]DNA that sediments heterogeneously throughout the gradient. The centrifugation profiles of both mock- and virus-infected cultures exhibited [^{3}H]DNA (21.3 and 21.0% of the total, respectively), which sedimented as high-molecular-weight DNA to the bottom of the

TABLE 3. *Hybridization of ^{3}H-labeled 20S DNA synthesized in Py-infected WT-4 and ts A1S9 cells, with virus and cell DNA*[a]

^{3}H-labeled 20*S* test DNA derived from:		Input counts/min	% DNA bound to filters carrying:	
Cells	Temp (C)		L-cell DNA	Py I DNA
Mouse embryo	37.0	7,690	ND	34.2
WT-4	34.0	1,830	1.4	27.3
	38.5	2,876	0.9	40.2
ts A1S9	34.0	1,500	ND	26.3
	38.5	2,204	0.05	36.9

[a] ^{3}H-labeled 20*S* DNA was obtained from neutral sucrose density gradient fractions by precipitation with alcohol as described elsewhere (8). L-cell DNA and Py I DNA were purified using established procedures (4, 37). ND, Not detectable.

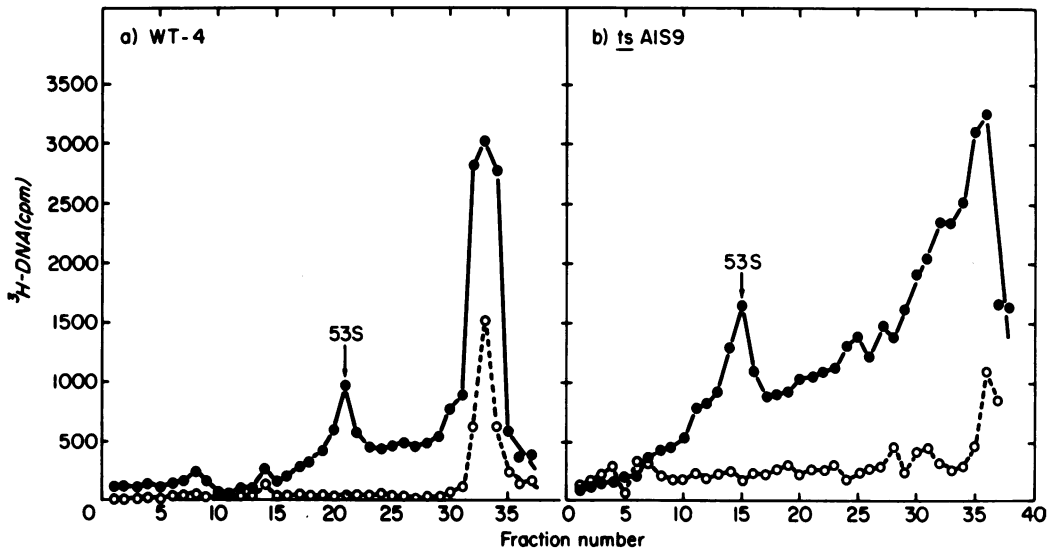

FIG. 2. *Velocity sedimentation analysis in alkaline sucrose density gradients of DNA synthesized in control and Py-infected mouse L cells at 38.5 C. WT-4 and ts A1S9 cells were grown to mid-logarithmic phase at 34 C in Brockway bottles in medium containing [^{14}C]dThd. The cells were washed free of radioactive medium and incubated for 24 h at 38.5 C. They were then infected with Py virus (≃2,000 PFU/cell) labeled with [^{3}H]dThd from 24 to 30 h p.i., after which they were washed and lysed in a 1% solution of sodium dodecyl sulfate in SSC and the labeled DNA was analyzed by velocity sedimentation in alkaline sucrose density gradients as described in Materials and Methods. The arrow marks the position of marker 53S [^{14}C]Py DNA run in parallel gradients. In all instances the ^{14}C-cell DNA preformed at 37 C was recovered on the 70% sucrose cushion (e.g., fractions 31–36 in [a]). Only the data for ^{3}H-labeled DNA are shown. Symbols: ○, mock-infected cells; ●, Py-infected cells.*

gradient. In the case of the Py-infected cells, a very prominent ^{3}H-labeled 53*S* viral DNA peak (12.8% of the total) was detected. The heterogeneously sedimenting cell DNA (fractions 18–34) made up 56.8% of the total.

Data obtained from analyses of DNA synthesized between 24 to 30 h p.i. in mock- and Py-infected WT-4 and *ts* A1S9 cells at 34 C were essentially identical to those illustrated in Fig. 2a.

Cell DNA synthesis: pulse-chase studies. In our earlier work it was found that *ts* A1S9 cells are temperature sensitive in a step involved in the conversion of newly synthesized, short, single-stranded segments into large chromosomal DNA. The experiments described in the foregoing section suggested that Py infection might circumvent this *ts* lesion. It was of interest, therefore, to examine further the process of Py-induced cell DNA synthesis in these cells under permissive and nonpermissive conditions.

WT-4 and *ts* A1S9 cultures, prelabeled with [^{14}C]dThd, were incubated at 38.5 C for 16 h and then were either mock infected or infected with Py virus. Some cultures of each set were pulse-labeled for 5 min with [^{3}H]dThd at 24 h p.i. Others were pulse-labeled and then incubated for an additional 1 or 6 h in dThd-containing, nonradioactive medium. The [^{14}C]- and [^{3}H]DNA of each culture were analyzed by velocity sedimentation analysis in alkaline sucrose density gradients.

Figure 3a and b presents results from experiments with mock- or Py-infected WT-4 cells incubated at 38.5 C. Similar data were obtained in analyses of virus-infected WT-4 and *ts* A1S9 cells incubated at 34 C. In all instances the ^{14}C-marker cell DNA was recovered primarily as high-molecular-weight material, at the bottom of the gradients. The [^{3}H]DNA synthesized at 38.5 C during a 5-min pulse period by virus- or mock-infected WT-4 cells sedimented heterogeneously throughout the whole gradient. The smallest components were about 10*S*; the largest cosedimented with the ^{14}C-marker DNA (i.e., with *S* values of >300 [29]). In the Py-infected cultures no clear 53*S* component was detectable. After a chase of 1 h, the pattern of sedimentation of the [^{3}H]DNA was altered greatly. In mock-infected cultures (Fig. 3c), the pulse-labeled DNA was recovered as high-molecular-weight material at the bottom of the gradient, with the [^{14}C]cell DNA preformed at

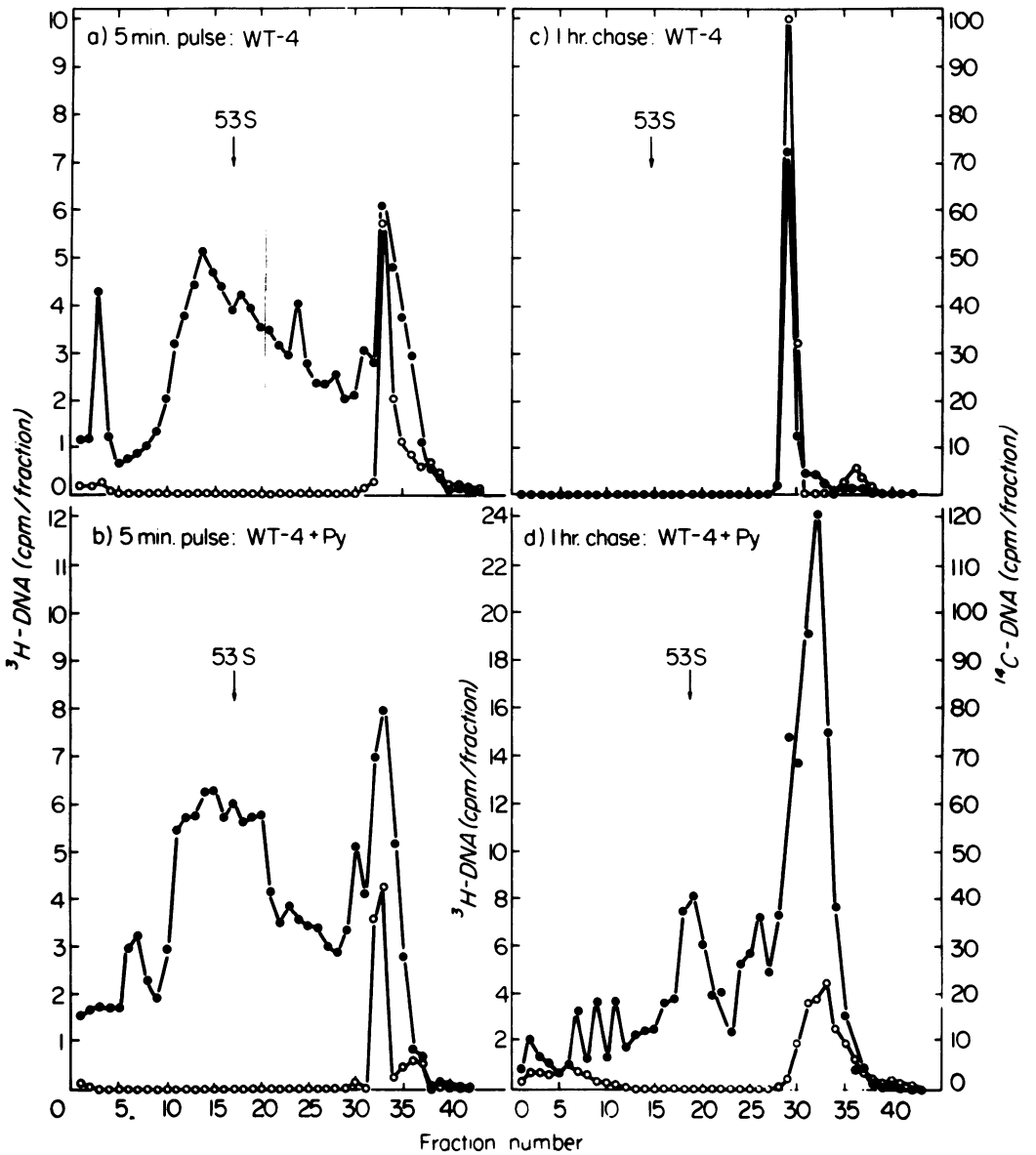

FIG. 3. *Velocity sedimentation analysis in alkaline sucrose density gradients of DNA synthesized during pulse and chase intervals by Py-infected WT-4 cells incubated at 38.5 C. Replicate suspension cultures of WT-4 cells were grown to mid-logarithmic phase at 34 C in medium containing [^{14}C]dThd (0.1 μCi/ml). The cells were resuspended in fresh nonradioactive medium and incubated at 38.5 C for 16 h. They were then infected with Py virus, or mock infected, as described in the legend to Fig. 1. After 24 h the cultures received [^{3}H]dThd (25 μCi/ml) for 5 min. The labeled DNA of one mock- and one virus-infected culture was analyzed immediately by velocity sedimentation in an alkaline sucrose density gradient. The other cultures received nonradioactive dThd (0.05 mM) and were incubated further for 1 h prior to analysis. Symbols: ●, [^{3}H]DNA; ○, [^{14}C]DNA preformed at 34 C. The arrows mark the position of ^{14}C-labeled 53S Py DNA, run in parallel gradients.*

34 C. In the Py-infected WT-4 cells (Fig. 3d), the sedimentation profile of the [^{3}H]DNA resembled that shown in Fig. 2a. Material (15.4% of the total) sedimenting at 53*S*, in the position of Py DNA, was detected, as was a major component (58.5% of the total) cosedimenting with high-molecular-weight preformed [^{14}C]DNA. In addition, some of the [^{3}H]DNA (28.2%) sedimented as heterogeneous material between these two peaks. In some experiments

the chase interval was extended to 6 h. The sedimentation profiles observed after a 6-h chase resembled those shown in Fig. 3 and 4 for 1-h chase period.

Data obtained in pulse-chase studies with mock- and Py-infected *ts* A1S9 cells incubated at 38.5 C are shown in Fig. 4. In all instances the [^{14}C]DNA preformed at 34 C was recovered as large-molecular-weight material on the 70% sucrose cushion. In mock-infected *ts* A1S9 cells (Fig. 4a) the pulse-labeled [^{3}H]DNA sedimented heterogeneously, with a majority (58.5%) being recovered as low-molecular-weight material at the top of the gradient. After a 1-h chase interval (Fig. 4c), there was a redistribution of ^{3}H-labeled material, with only 29.9% being recovered in this low-molecular-weight range. These observations, made 48 h after *ts* A1S9 cells were shifted to 38.5 C, are in accord with those made earlier (39) with cells incubated for only 16 to 24 h at the nonpermissive temperature. The *ts* A1S9 cells differ from the WT-4 cells in that they are *ts* in the process of conversion of newly made small segments of DNA to chromosomal DNA strands (cf. 39).

The sedimentation profile of the [^{3}H]DNA of *ts* A1S9 cells infected at 38.5 C and pulse-labeled for 5 min 24 h p.i. (Fig. 4b) closely resembled those obtained with material from mock-infected cultures. No 53*S* viral DNA was detected. However, after a 1-h chase interval such [^{3}H]Py DNA was clearly visible (Fig. 4d).

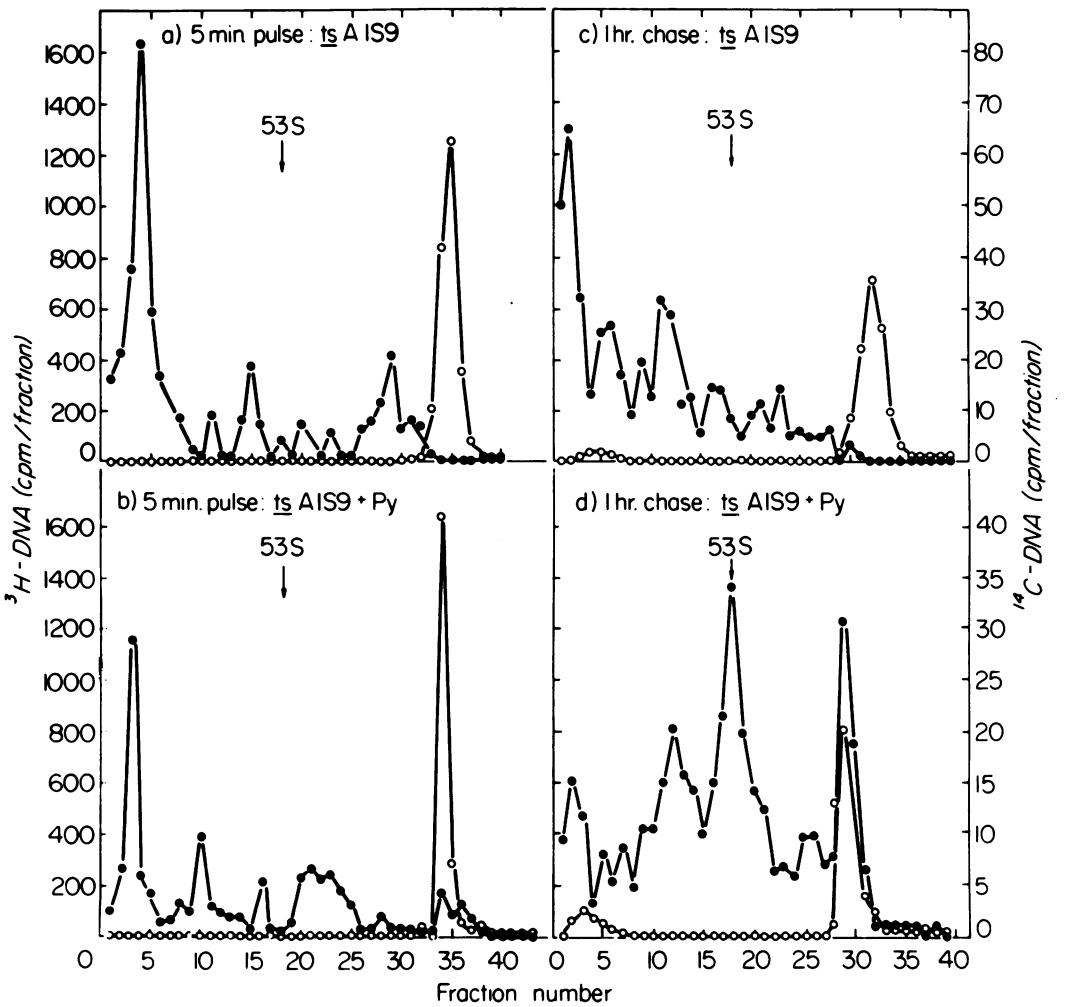

FIG. 4. *Velocity sedimentation analysis in alkaline sucrose density gradients of DNA synthesized during pulse and chase intervals in Py-infected ts A1S9 cells incubated at 38.5 C. Replicate cultures of ts A1S9 were processed as described in Fig. 3. Symbols: ●, [^{3}H]DNA synthesized at 38.5 C 24 h p.i.; ○, [^{14}C]DNA preformed in uninfected cells at 34 C.*

It made up 36.2% of the total DNA, of which about 20.6% cosedimented with preformed ^{14}C-labeled chromosomal DNA. Approximately 11.5% of the [^{3}H]DNA sedimented between these components, whereas the remainder was recovered as heterogeneously sedimenting material of molecular size smaller than 53*S*.

Essentially quantitative recovery of both preformed [^{14}C]DNA and newly synthesized [^{3}H]DNA was achieved in such studies. No evidence suggesting degradation and reutilization of labeled DNA was obtained. The experiments indicate that *ts* A1S9 cells incubated at 38.5 C for 48 h are seriously impaired in their capacity to synthesize chromosomal DNA. In contrast, Py-infected *ts* A1S9 appear able to make such large-molecular-weight DNA.

Cell DNA synthesis studied by BUdR labeling. Under the experimental conditions employed, i.e., incubation of *ts* A1S9 cells for 48 to 54 h post-temperature shift, incorporation of [^{3}H]dThd into cell DNA in uninfected cells was about 10 to 15% of that observed in WT-4 cells and about 8 to 10% of that detected in either *ts* A1S9 or WT-4 cells incubated continuously at 34 C. As a result of Py infection, such incorporation was stimulated from 1.5- to 3.5-fold in *ts* A1S9 cells at the high temperature. In addition, the Py-infected cells synthesized some DNA which cosedimented in alkaline sucrose density gradients with ^{14}C-marker chromosomal DNA, suggesting that the virus may have effected reversal of the *ts* lesion. The following experiment was performed to determine whether Py infection of *ts* A1S9 cells resulted in the restoration of normal semiconservative DNA replication.

Cultures of *ts* A1S9 cells, prelabeled with [^{14}C]dThd at 34 C, were incubated at 34 or 38.5 C for 16 h, after which they were either infected with Py or mock infected. Twenty-four hours p.i., the cultures were treated with bromodeoxyuridine (BUdR), 5'-fluorodeoxyuridine, and [^{3}H]dThd to mark the newly made DNA with both a density and radioactive label. After 6 h the cell DNA was separated by velocity sedimentation in neutral sucrose density gradients and then analyzed by equilibrium centrifugation in neutral CsCl density gradients as described in the legend to Fig. 5.

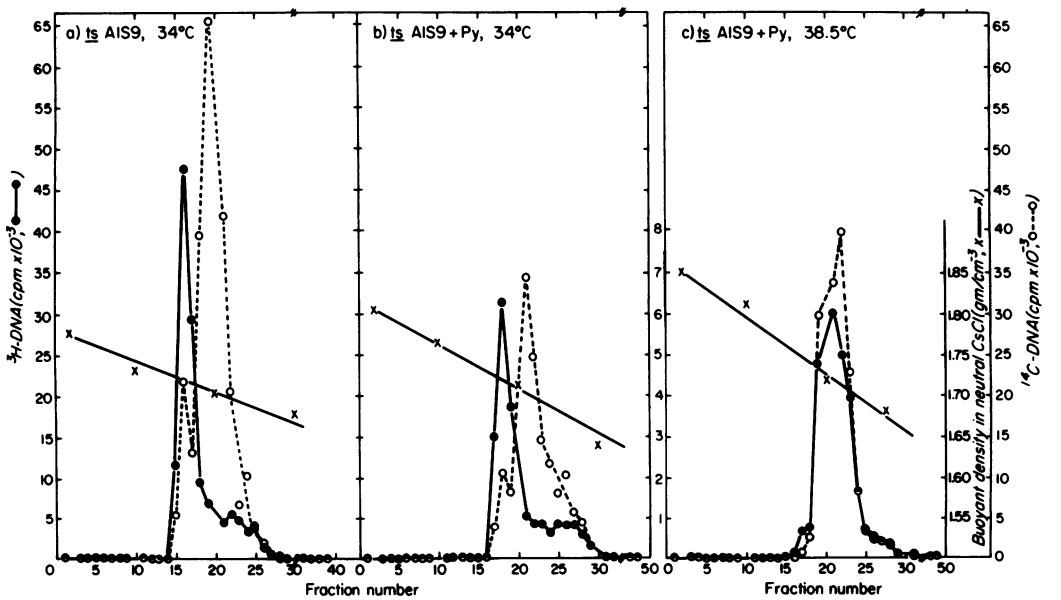

FIG. 5. *Equilibrium centrifugation analysis of CsCl of cell DNA synthesized in ts A1S9 cells. Suspension cultures of ts A1S9 cells were grown to mid-logarithmic phase at 34 C with [^{14}C]dThd, incubated in nonradioactive medium at 34 or 38.5 C for 16 h, and then either mock infected or infected with Py virus as described in the legend to Fig. 1. After 24 h BUdR (16 μM), 5'-fluorodeoxyuridine (FUdR) (1 μM), and [^{3}H]dThd (1 μCi/ml) were added to each culture. Six hours later, the cells were harvested, washed in SSC, and lysed in SDS, and the Py and cell DNA were separated by velocity sedimentation in neutral sucrose density gradients (see Fig. 1). The cell DNA fraction with maximum radioactive label (e.g., fractions 33 or 34, Fig. 1a) was further analyzed by equilibrium centrifugation in neutral CsCl gradients as described in Materials and Methods. Symbols: O, [^{14}C]DNA preformed at 34 C; ●, [^{3}H]DNA made in the presence of BUdR and FUdR; X, buoyant density. (a) Mock infected, 34 C; (b) Py-infected cells, 34 C; (c) Py-infected cells, 38.5 C.*

The [^{14}C]DNA preformed in light medium by uninfected *ts* A1S9 cells was recovered as two components after the 6-h period of incubation at 34 C in BUdR-containing medium (Fig. 5a). One had a buoyant density equal to that of normal mouse DNA (27), 1.706 g/cm^3. The other had a density of 1.720 g/cm^3. The data suggested that approximately 26.9% of the [^{14}C]DNA had undergone semiconservative replication in the presence of BUdR in the experiment depicted. Approximately 70.7% of the [^{3}H]DNA co-banded with this [^{14}C]DNA.

In *ts* A1S9 cells infected with Py (Fig. 5b) at 34 C, about 15.1% of the preformed [^{14}C]DNA was recovered at a heavier than normal density. About 58.5% of the [^{3}H]DNA co-banded with it. Similar observations were made with WT-4 cells infected at 34 or 38.5 C.

Results obtained with cell DNA from Py- and mock-infected *ts* A1S9 cells incubated at 38.5 C differed from those seen with WT-4 cells. Data from virus-infected cells are shown in Fig. 5c. The centrifugation profiles of both [^{14}C]- and [^{3}H]DNA were broad, but exhibited a mean buoyant density characteristic of unreplicated DNA, i.e., $\cong$1.71 g/cm^3. A very small fraction (0.06%) of the [^{3}H]DNA was found at a higher density, 1.745 g/cm^3.

Mode of Py replication in WT-4 and ts A1S9 cells. The experiments described in earlier sections suggested that Py DNA replication proceeds normally in *ts* A1S9 cells incubated at the nonpermissive temperature. However, the results of the study outlined in the preceding section indicate that the cell DNA synthesis that proceeds in either uninfected or Py-infected *ts* A1S9 cells at 38.5 C is almost entirely abnormal. The mechanism of Py DNA replication was therefore re-examined using BUdR as density label.

Once again WT-4 and *ts* A1S9 cells were prelabeled with [^{14}C]dThd at 34 C and incubated at either 34 or 38.5 C for 16 h. They were then infected with Py virus at the appropriate temperature. They were density labeled with BUdR and radioactively labeled with [^{3}H]dThd at 24 h p.i. After 6 h, the ^{3}H-labeled 20*S* Py DNA was isolated from neutral sucrose density gradients and analyzed in neutral CsCl density gradients, as described in the legend to Fig. 6.

The ^{3}H-labeled 20*S* Py DNA produced by WT-4 and *ts* A1S9 cells, at both 34 and 38.5 C, in the presence of BUdR had a buoyant density heavier than that of the light-infecting virus DNA (1.709 g/cm^3 [24]). The centrifugation profiles were heterogeneous, suggesting that the fractions examined contained Py DNA molecules with varying degrees of BUdR substitution. Two major components appeared to be resolved in each case. Those from cells incubated at 34 C had buoyant densities of about 1.75 and 1.73 g/cm^3; those from cells incubated at 38.5 C banded at approximately 1.79 and 1.75 g/cm^3.

The ^{3}H-labeled material banding at 1.75 g/cm^3 likely corresponds to Py DNA, in which only one strand is fully substituted with the density label, whereas that at 1.79 g/cm^3 is probably Py DNA, which carries BUdR in both strands (24, 25). The material recovered at 1.73 g/cm^3 likely represents viral DNA molecules in which only partial substitution has occurred. A very small proportion of the ^{3}H-labeled 20*S* Py DNA obtained from WT-4 cells incubated at 34 or 38.5 C, and from *ts* A1S9 cells incubated at 34 C, cosedimented at a buoyant density of about 1.704 g/cm^3 with [^{14}C]DNA preformed in light medium at 34 C. This is probably pseudovirion DNA (44), comprising cellular DNA molecules.

This study clearly establishes that Py DNA replicates primarily in a semiconservative mode in WT-4 and in *ts* A1S9 cells at both 34 and 38.5 C. There is little evidence of repair replication.

DISCUSSION

The experiments described here reveal that Py DNA is replicated by mouse cells, under conditions nonpermissive for the duplication of their own chromosomal DNA (see also 39). Such DNA synthesis normally proceeds by the formation of small, single-stranded DNA segments that are joined to produce intermediates of increasing molecular size, giving rise ultimately to large-molecular-weight chromosomal DNA (cf. 26). The evidence already obtained (39; Sheinin, manuscript in preparation) indicates that *ts* A1S9 cells can effect limited DNA synthesis and perform those ligating events involved in making the small-molecular-weight intermediates (up to $\cong 5 \times 10^6$ daltons) and in repair replication. They are *ts* in that process that results in the joining, or conversion, of such intermediates to chromosomal DNA strands. Clearly, the protein required for this latter cellular process is not essential for the replication of Py DNA form II and its maturation to the covalently closed form I DNA. These studies leave unresolved the question of whether ring closure of Py DNA, a process that requires continual protein synthesis, is dependent upon a cellular protein or one which is virus specified (5–9; P. E. Branton, Ph.D thesis, Univ. of Toronto, Toronto, Ont., 1972; Branton and Sheinin, manuscript in preparation).

The multiplication of Py in mouse L cells is associated with a stimulation of cell DNA

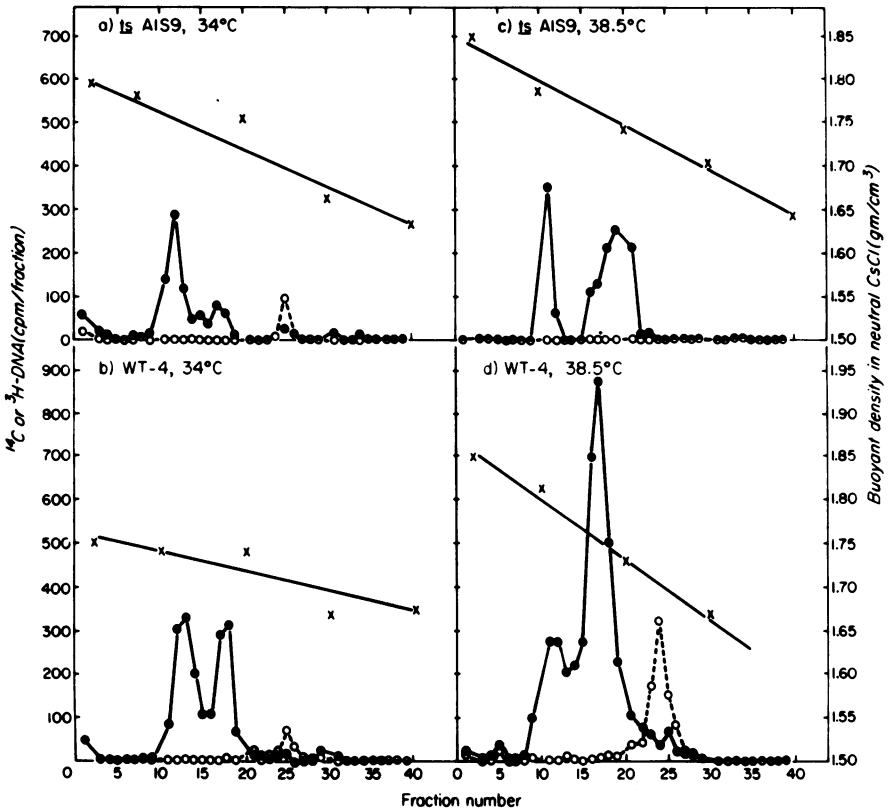

FIG. 6. *Equilibrium centrifugation analysis of Py DNA synthesized in the presence of BUdR in Py-infected WT-4 and ts A1S9 cells at 38.5 C. Replicate cultures of WT-4 and ts A1S9 cells were grown in [^{14}C]dThd at 34 C and then grown for 16 h at 38.5 C and infected with Py virus (or mock infected) as described in the legend to Fig. 1. Twenty-four hours p.i., the cultures were incubated with BUdR, 5'-fluorodeoxyuridine and [^{3}H]dThd as noted in Fig. 4. Six hours later, the labeled 20S DNA fraction was isolated from neutral sucrose density gradients and analyzed by equilibrium centrifugation in neutral CsCl density gradients. [^{14}C]Py DNA, analyzed in parallel gradients, was seen to have a buoyant density of 1.709 g/cm^{3}. Symbols: ●, [^{3}H]DNA synthesized in the presence of BUdR; ○, [^{14}C]DNA, preformed at 34 C in light medium; X, buoyant density.*

synthesis. In this respect it resembles other permissive systems. In Py-infected mouse embryo cells (10–12) or mouse 3T3 cells (unpublished data), the stimulated cell DNA synthesis occurs by the stepwise elongation process, discussed above. As shown here, cell DNA synthesis in Py- or mock-infected WT-4 cells incubated at 38.5 C occurs by the semiconservative mechanism (26). Under similar incubation conditions, the cell DNA made in virus- or mock-infected *ts* A1S9 cells appears to result almost entirely from a process akin to repair replication (18, 32; Sheinin, manuscript in preparation). These observations suggest that the Py genome does not carry information for a protein that repairs the major *ts* defect of *ts* A1S9 cells.

Cell DNA synthesis, which occurs late during Py infection of WT-4 and *ts* A1S9 cells, is similar to that observed in Py-infected mouse embryo cells (10–12). It is characterized by the accumulation of single-stranded segments of molecular weight ranging from about 10^{6} to 10^{8}. This process, which results from premature or multiple initiation, is accentuated in *ts* A1S9 cells infected at 38.5 C. The data indicate that, in *ts* A1S9 cells in which the temperature-sensitive lesion is well established, this process proceeds primarily by repair replication. If so, it would suggest that the abnormal DNA synthesis that occurs late in Py-infected *ts* A1S9 cells is initiated by endonucleolytic cleavage at chromosomal DNA loci other than normal sites of origin. Such abnormal initiation may also explain the aberrant pattern of cell DNA synthesis observed late during productive infection by Py virus in all permissive cells.

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