Analysis of the Molecular Forms of Simian Virus 40 Deoxyribonucleic Acid Synthesized in Cycloheximide-Treated Cell Cultures

SAUL KIT AND K. NAKAJIMA

Division of Biochemical Virology, Baylor College of Medicine, Houston, Texas 77025

Received for publication 31 August 1970

Cycloheximide addition at various times from 24 to 36 hr after virus infection markedly inhibits the rate of simian virus 40 (SV40) deoxyribonucleic acid (DNA) synthesis in monkey kidney (CV-1) cultures. To determine whether superhelical (form I) SV40 DNA was synthesized in the cycloheximide-inhibited cultures, extracts were prepared by the method of Hirt from cultures labeled with ^3H -thymidine (^3H -dT) and were analyzed by cesium chloride-ethidium bromide (CsCl-EtBr) equilibrium centrifugation and by velocity sedimentation in neutral sucrose gradients. When control or cycloheximide-treated cultures were labeled for 2 or 4 hr with ^3H -dT at 36 or 37 hr after infection, 71 to 83% of the radioactivity soluble in 1 M NaCl was detected in closed-circular SV40 DNA (form I). Cycloheximide treatment did not generate an increase of higher multiple circular forms of SV40 DNA. In pulse-chase experiments with or without cycloheximide treatment, radioactivity first appeared in nicked molecular forms sedimenting faster than open-circular SV40 DNA (form II), and then was chased into superhelical form I SV40 DNA. These results suggest that in cycloheximide-treated SV40-infected cultures: (i) polynucleotide ligase concentrations are adequate, and (ii) duplication errors causing formation of circular oligomers of SV40 DNA are not enhanced.

After inhibition of protein synthesis, for example with chloramphenicol or cycloheximide, the rate of deoxyribonucleic acid (DNA) synthesis is markedly reduced in uninfected and virus-infected bacteria and animal cells (1, 10, 12, 16). In *Escherichia coli* treated with chloramphenicol, DNA synthesis proceeds until the completion of the current cycle of duplication and then stops (12). In *Proteus mirabilis*, higher multiple circular DNA forms of the plasmid Col E₁ are generated after chloramphenicol treatment or amino acid starvation (4). In mouse fibroblast mitochondria, DNA dimers and oligomers accumulate after either amino acid starvation or cycloheximide treatment of the cells (14). In mouse cells transformed by a mutant strain of polyoma virus, transfer of cells from a non-permissive to a permissive temperature also leads to an accumulation of closed-circular dimers and trimers of polyoma DNA. The dimers and trimers occur only in small amounts (less than 1%) in normal 3T3 cells lytically infected with either wild-type or the temperature-sensitive mutant (Ts-a) of polyoma virus (3).

Previous studies had demonstrated that cycloheximide inhibits simian virus 40 (SV40) DNA synthesis when the drug is added early or late in infection (10). In extending those studies, two questions were investigated. First we asked whether oligomeric circular forms of SV40 DNA would increase in cycloheximide-inhibited cultures. Second, we asked whether cycloheximide treatment would inhibit the conversion of nicked forms to closed-circular forms, a step postulated by Levine et al. (13) to be rate-limiting in SV40 DNA replication. It had previously been shown that the activities of several enzymes functioning in deoxyribonucleotide interconversions and DNA synthesis were high several hours after addition of cycloheximide to infected cultures (10). However, polynucleotide ligase was not one of the enzymes measured. If cycloheximide treatment enhanced the turnover or reduced the synthesis of polynucleotide ligase, the formation of completed superhelical molecules might be prevented. This could be detected by the accumulation of nicked molecular forms.

MATERIALS AND METHODS

Cell lines and virus strains. CV-1 cells, an established line of African green monkey kidney, were grown in Eagle's minimal essential medium (Auto-Pow MEM, Flow Laboratories, Rockville, Md.) supplemented with 10% calf serum. CV-1 cells were subcultured at weekly intervals. Six- to 7-day-old confluent cultures were used for experiments, and the cells were fed with fresh grown medium 1 day before each experiment.

Parental SV40 clone 307L and SV40tsTNG-1, a temperature-sensitive mutant (11), were grown and assayed in monolayer cultures of CV-1 cells (7). Replication of mutant tsTNG-1 at the nonpermissive temperature (38.7 C) is 3,000-fold less than at the permissive temperature (33.5 C). Parental SV40 clone 307L replicated equally well at 38.7 or 33.5 C.

Infection of CV-1 cultures and ^3H -thymidine (^3H -dT) labeling of SV40 DNA. Confluent monolayer cultures of CV-1 cells were infected for 2 hr at 36.5 C at an SV40 input multiplicity of 50 to 70 plaque-forming units (PFU)/cell. After removal of unadsorbed virus, the cultures were treated with 1- β -D-arabinofuranosylcytosine (ara-C), 15 $\mu\text{g}/\text{ml}$, for 22 hr to synchronize SV40 DNA replication. At 24 hr postinfection, new media containing deoxycytidine (50 $\mu\text{g}/\text{ml}$) were added to reverse the ara-C block and to initiate DNA replication (10, 11). After further incubation for various periods of time, some of the cultures were treated with cycloheximide (25 $\mu\text{g}/\text{ml}$) to inhibit protein synthesis. To measure DNA synthesis, the cultures were incubated with ^3H -methyl thymidine (New England Nuclear Corp., Boston, Mass.; 19.9 to 23 Ci/mmol).

Extraction and analysis of SV40 ^3H -DNA. SV40 DNA was selectively extracted from the infected cells by the sodium dodecyl sulfate-1 M sodium chloride method of Hirt (5). In some instances, the high-molecular-weight cellular DNA fraction (Hirt pellet) was washed three times with 5% trichloroacetic acid at 4 C, hydrolyzed with 0.5 N perchloric acid at 70 C for 30 min, and assayed for radioactivity in a Packard Tri-Carb liquid scintillation counter.

The supernatant fraction (Hirt extract) was deproteinized with an equal volume of 90% phenol, and dialyzed against SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7) until the dialysate was free from phenol, as determined by absorption measurements at 260 nm.

Closed-circular SV40 ^3H -DNA (heavy peak) was separated from nicked forms of SV40 ^3H -DNA (light peak) by equilibrium centrifugation in cesium chloride (CsCl) solutions (density of about 1.57 g/cm³) containing ethidium bromide (EtBr), 300 $\mu\text{g}/\text{ml}$ (6, 11). The heavy and light DNA fractions obtained by the dye-buoyant density procedure were freed from EtBr as follows. The DNA solutions were extracted two times with an equal volume of isoamyl alcohol and then with ether. The extractions with isoamyl alcohol and with ether were repeated, and the DNA solutions were dialyzed against SSC containing 0.001 M ethylenediaminetetraacetic acid (EDTA) and then against SSC without EDTA (11). The DNA fractions were then analyzed by velocity sedimentation in

neutral sucrose gradients (5 to 30%, w/v). Sucrose solutions were dissolved in 0.1 M sodium chloride, 0.001 M EDTA, 0.01 M tris(hydroxymethyl)amino-methane-hydrochloride buffer, pH 7.2. DNA samples (0.1 ml) were layered on top of the gradients (4.4 ml) in polyallomer tubes (0.5 by 2 inches, 1.3 by 5 cm), covered with 0.6 ml of paraffin oil, and centrifuged at 5 C for 210 min at 35,000 rev/min (100,000 $\times g$), in the SW39 or the SW50 rotor of a Spinco L2 centrifuge (10). Ten-drop fractions were collected from the bottom of the tubes on Whatman GF/A paper. The papers were washed with cold 5% trichloroacetic acid and cold ethyl alcohol, and were assayed for radioactivity by liquid scintillation counting.

Assay of SV40 DNA infectivity. The diethylamino-ethyl-dextran method for the plaque assay of SV40 DNA infectivity on CV-1 monolayers has been described (9).

Protein synthesis. Infected cultures were incubated at 36.5 C for 0.5 to 3 hr with ^{14}C -amino acids (reconstituted protein hydrolysate, Schwarz BioResearch Inc., Orangeburg, N.Y.; 25 $\mu\text{Ci}/\text{culture}$). The labeled cells were scraped from the glass, collected by centrifugation, and washed two times with ice-cold balanced salt solution. The proteins were precipitated with excess 5% trichloroacetic acid, washed three times with 5% trichloroacetic acid, and then heated for 15 min at 90 C with 5% trichloroacetic acid to hydrolyze nucleic acids. The proteins were further washed with 5% trichloroacetic acid and 100% ethyl alcohol, and were heated for 5 min at 65 C with ethyl alcohol-ether (3:1). The dried pellets were dissolved in 1.5 ml of 0.5 N NaOH, and samples were assayed for radioactivity and protein.

RESULTS

Effect of cycloheximide on DNA synthesis at various times after infection. DNA synthesis can be partially synchronized by treating SV40-infected CV-1 cultures with ara-C (10, 11). After the addition of deoxycytidine, the ara-C block of DNA synthesis is reversed and a rapid burst of cellular and viral DNA synthesis takes place (10).

In a previous study, the total radioactive DNA was extracted from infected cells and then fractionated by band centrifugation in CsCl gradients or by chromatography of heated DNA on nitrocellulose columns (10). In the present study, most of the cellular DNA was separated from SV40 DNA by the selective extraction method of Hirt (5). However, the Hirt extracts contained some radioactive "low-molecular-weight" cellular DNA. A preliminary experiment was therefore carried out to identify a time interval during active SV40 DNA synthesis when the labeling of low-molecular-weight cellular DNA might be minimal. SV40-infected CV-1 cells were treated with ara-C from 2 to 24 hr postinfection, and then new media containing deoxycytidine

were added to initiate DNA synthesis. The cultures were labeled with ^3H -dT from 24 to 28, 28 to 32, 32 to 36, and 36 to 40 hr after infection. Half of the cultures were also treated with cycloheximide at the same time that ^3H -dT was added. Low-molecular-weight DNA was selectively extracted by the method of Hirt (5) and analyzed by equilibrium centrifugation in CsCl-EtBr density gradients.

Table 1 shows that after the reversal of the ara-C block the rate of DNA synthesis rapidly increased in untreated and in cycloheximide-treated cultures. However, cycloheximide treatment reduced the rate of DNA synthesis 66 to 80% during each of the labeling periods.

When the ^3H -DNA was analyzed by CsCl-EtBr equilibrium centrifugation, it was found that at 24 to 28 hr postinfection about half of the ^3H -DNA had a light buoyant density (nicked circular or linear DNA forms), and about half had a heavy buoyant density (superhelical DNA). At later times, the per cent radioactivity in the light DNA peak decreased, so that by 36 to 40 hr only 14 to 16% of the Hirt extract radioactivity was in the light fraction.

The light and the heavy ^3H -DNA fractions were further analyzed by velocity sedimentation in neutral sucrose gradients. The heavy ^3H -DNA peak obtained from either untreated or cycloheximide-treated cells consisted mostly (70 to 93%) of form I (superhelical) DNA sedimenting at about 21S. The remainder of the ^3H -DNA sedimented more slowly than the main peak and may have arisen through the nicking of the form

I DNA during storage or processing of the samples. About four to seven times as much radioactivity was found in the form I DNA of control cultures as in that of cycloheximide-treated cultures. Rapidly sedimenting circular oligomeric forms of SV40 DNA did not accumulate during the 4-hr cycloheximide treatment period.

When the light ^3H -DNA peaks from CsCl-EtBr centrifugation were analyzed by sucrose gradient centrifugation, three principal regions of radioactivity were detected (Fig. 1): (i) a slow (16 to 18S) region (fractions 24 to 28); (ii) an intermediate (21S) region (fractions 20 to 23); and (iii) a fast-sedimenting region (fractions 10 to 19). The slow 16 to 18S region contains nicked circular SV40 DNA. In previous studies in which SV40-infected cell cultures were labeled with ^3H -dT at 26 to 30 hr after infection, about 73.2% of the total infectivity of the light CsCl-EtBr fraction sedimented in this region (11). The ^3H -DNA sedimenting in the intermediate peak region probably contains some of the replicative intermediates described by Bourgaux et al. (2) and by Levine et al. (13). SV40 infectivity was also detected in the intermediate region (11). In contrast, the rapidly sedimenting fraction (Fig. 1) was not infective, and hence contained mostly cellular DNA (11).

The per cent radioactivity in the three regions varied at different times after infection. For example, at 24 to 28 hr after infection, about 56% of the radioactivity in control cultures was found in the rapidly sedimenting fractions, 11% in the intermediate region, and 16% in the slow region

TABLE 1. Effect of cycloheximide (CH) on the synthesis of SV40 DNA at various times after infection of ara-C-treated CV-1 cells with SV40tsTNG-1^a

Time postinfection incubated with ^3H -dT	Counts per min per culture in Hirt extract		Radioactivity recovered in fractions after CsCl-EtBr centrifugation (%)			
	No CH	With CH	No CH		With CH	
			Heavy ^b	Light	Heavy ^b	Light
<i>hr</i>						
24-28	226 $\times 10^3$	63 $\times 10^3$	47.5	51.4	54.8	46.6
28-32	871 $\times 10^3$	173 $\times 10^3$	76.6	21.8	70.5	27.1
32-36	1,232 $\times 10^3$	428 $\times 10^3$	80.0	17.3	79.1	18.4
36-40	1,781 $\times 10^3$	615 $\times 10^3$	82.6	13.9	81.9	15.8

^a Confluent monolayer cultures of CV-1 cells (9×10^6 cells/culture) were infected with SV40tsTNG-1 (50 PFU/cell) for 2 hr, and then incubated at 38.7 C (nonpermissive temperature) until 24 hr postinfection with ara-C (15 $\mu\text{g}/\text{ml}$). The ara-C media were removed and new media with deoxycytidine (50 $\mu\text{g}/\text{ml}$) were added. Cultures were labeled with ^3H -thymidine (5 μCi and 0.5 $\mu\text{g}/\text{ml}$) for the times indicated. Cycloheximide (25 $\mu\text{g}/\text{ml}$) was also added at the time of ^3H -thymidine addition to experimental cultures and was present until the cells were harvested.

^b The heavy ^3H -DNA fractions consisted of 70 to 93% form I (21S) DNA, as determined by sucrose gradient analysis. The remainder of the ^3H -DNA sedimented more slowly than form I DNA. The radioactive peaks sedimented similarly in the sucrose gradients to those shown for form I DNA in Fig. 3a and c.

FIG. 1. Velocity sedimentation in neutral sucrose gradients (5 to 30%, w/v) of SV40tsTNG-1 DNA from light peak fractions obtained after equilibrium centrifugation in CsCl-EtBr density gradients. (See Table 1 for experimental details.)

(Fig. 1a). At later times, the per cent radioactivity in the rapidly sedimenting region declined and the per cent radioactivity in the intermediate and slow regions increased (Fig. 1b to 1d). For example, at 36 to 40 hr after infection, 12, 28, and 46% of the radioactivity was detected in the fast, intermediate, and slow regions of the control cultures; 23, 27, and 37% of the radioactivity was detected in the corresponding fast, intermediate, and slow regions of the cycloheximide-treated cultures.

The experiments shown in Fig. 1d and previous studies of ara-C-treated cultures (11) suggest that by 36 to 40 hr after infection there are reduced amounts of radioactivity in the cellular DNA of Hirt extracts. Also, the rate of SV40 DNA synthesis is maximal at about this time (8). The time span between 36 and 40 hr was therefore chosen for further study.

Inhibition of protein synthesis by cycloheximide.

It was essential to verify definitely that cycloheximide treatment shut off protein synthesis under the conditions of our experiments. Therefore, SV40-infected cultures that had been partially synchronized with ara-C were treated at 36 hr after infection with cycloheximide. At 37 hr after infection, ^{14}C -amino acids were added and the cultures were further incubated for 30 to 180 min. Figure 2 shows that protein synthesis was in-

hibited in cycloheximide-treated cultures by 98% or more.

Effect of cycloheximide pretreatment on the formation of SV40 DNA molecular forms. In the experiments of Table 1, cycloheximide and ^3H -dT were added to ara-C-treated cultures at the same time, and the cultures were incubated for 4 hr. There is probably a lag of several minutes before cycloheximide inhibits protein synthesis, during which time labeling of SV40 DNA might be taking place. To avoid this complication, cycloheximide was added to SV40-infected cultures at 36 hr after infection for 1 hr prior to the addition of ^3H -dT. The cultures were then incubated for an additional 2 hr in the presence of both cycloheximide and ^3H -dT. As in the previous experiment, cultures were partially synchronized by ara-C treatment 2 to 24 hr after infection (Table 2; Fig. 3).

Cycloheximide pretreatment inhibited the incorporation of ^3H -dT into the DNA of the Hirt extract and Hirt pellet (high-molecular-weight cellular fraction) by about 77 to 79% (Table 2). Analysis of the Hirt extracts by CsCl-EtBr centrifugation indicated, however, that 74 to 77% of the radioactivity was in the heavy fraction (superhelical DNA) in both control and cycloheximide-treated cultures. Thus, the modified procedure in which cycloheximide was present

FIG. 2. Effect of cycloheximide on the incorporation of ^{14}C -amino acids into proteins of SV40-infected CV-1 cell cultures. Confluent monolayer cultures (7×10^6 cells/culture) were infected with SV40 and treated from 2 to 24 hr postinfection with ara-C (15 $\mu\text{g}/\text{ml}$). At 24 hr, the ara-C-containing media were removed; fresh medium containing deoxycytidine (50 $\mu\text{g}/\text{ml}$) was added; and the cultures were further incubated until 36 hr after infection. The media were again removed and prewarmed media consisting of 10% calf serum, balanced salt solution, and 2 mM glutamine were added. Cycloheximide (25 $\mu\text{g}/\text{ml}$) was also added to experimental cultures. At 37 hr, ^{14}C -amino acids were added. The cultures were harvested at various times after addition of the ^{14}C -amino acids, as indicated in the figure.

1 hr before the ^3H -dT labeling period did not appreciably change the relative amount of radioactivity in superhelical SV40 DNA (Table 2).

The samples were next analyzed by velocity centrifugation in neutral sucrose gradients. Sucrose sedimentation patterns from a typical experiment are illustrated in Fig. 3. Most of the radioactivity from the heavy CsCl—EtBr fractions sedimented at about 21S in the sucrose gradient (Fig. 3a and c). In cycloheximide-treated samples, there was only a small increase in radioactivity of the leading front (fractions 15 to 19, Fig. 3a and c) of the 21S fractions (Table 2). In the case of the light CsCl—EtBr fractions, 49 to 61% of the radioactivity was found in the 18S region. The remaining radioactivity was about equally distributed in the 21S and fast-sedimenting regions (Fig. 3b and d), and presumably consisted of a mixture of cellular DNA and replicative intermediates.

Pulse-chase experiments on cycloheximide-treated cultures. When polyoma virus- or SV40-

infected cultures were subjected to short pulses of ^3H -dT, most of the radioactive DNA of Hirt extracts was found in the light CsCl—EtBr fraction. This DNA sedimented more rapidly than nicked circular viral DNA (2, 13). The radioactivity of pulse-labeled DNA could be chased into superhelical viral DNA, indicating that it consisted of intermediates in viral DNA replication. The experiments of Table 3 were carried out to determine whether the replicative intermediates could be chased into form I SV40 DNA in the presence of cycloheximide.

Cycloheximide was added to experimental cultures at 36 hr after infection. At 36.75 hr, 5-fluorodeoxyuridine and uridine were added to deplete the endogenous thymidine pools. At 37 hr, control and cycloheximide-treated cultures were

TABLE 2. Effect of cycloheximide on the synthesis of SV40 DNA after infection of ara-C pre-treated CV-1 cells with SV40 cl 307L^a

Analysis of DNA fractions labeled with ^3H -dT at 37 to 39 hr	No cycloheximide	With cycloheximide, 36 to 39 hr
Counts per min per culture in		
Hirt pellet.....	439×10^3	101×10^3
Hirt extract.....	331×10^3	71×10^3
Per cent radioactivity in fractions after CsCl—EtBr centrifugation of Hirt extract		
Heavy peak.....	76.6	74.1
Light peak.....	21.6	22.0
Per cent radioactivity recovered in heavy CsCl—EtBr fraction after sucrose sedimentation		
Fast.....	8.0	6.3
21S.....	62.0	70.2
18S.....	24.1	22.4
Per cent radioactivity recovered in light CsCl—EtBr fraction after sucrose sedimentation		
Fast.....	21.0	22.3
21S.....	25.4	24.7
18S.....	55.6	61.1

^a Confluent monolayer cultures of CV-1 cells (6.3×10^6 cells/culture) were infected with parental SV40 cl 307L (50 PFU/cell) for 2 hr and then incubated at 36.5 C until 24 hr postinfection with ara-C (15 $\mu\text{g}/\text{ml}$). At 24 hr postinfection, new media containing deoxycytidine (50 $\mu\text{g}/\text{ml}$) were added. Cultures were pulse-labeled with ^3H -thymidine (^3H -dT, 0.5 μCi and 5 $\mu\text{g}/\text{ml}$) for the times indicated. Experimental cultures were also treated with cycloheximide (25 $\mu\text{g}/\text{ml}$) for 1 hr before ^3H -dT was added. Cycloheximide was present throughout the period of labeling.

FIG. 3. Velocity sedimentation in neutral sucrose gradients (5 to 30%, w/v) of parental SV40 cl 307L DNA from heavy peak fractions (a, c) and light peak fractions (b, d) obtained after equilibrium centrifugation in CsCl-EtBr gradients. Cycloheximide (25 μ g/ml) was added to experimental cultures at 36 hr postinfection and cultures were pulse-labeled with ^3H -dT at 36.5 to 38.5 hr postinfection.

pulse-labeled for 7.5 min with ^3H -dT. The cultures of group 1 were harvested at this time. The media were removed from the cultures of group 2, and these cultures were washed with prewarmed media containing thymidine and deoxycytidine. The wash media of the cycloheximide-pretreated cultures (group 2b) also contained cycloheximide. New media with thymidine and deoxycytidine, or thymidine, deoxycytidine, and cycloheximide, were added to the control and cycloheximide-pretreated cultures, respectively, and the cultures were further incubated for 1 hr.

Table 3 shows that cycloheximide treatment inhibited the incorporation of ^3H -dT into the DNA of Hirt extract and Hirt pellet fractions by 83 to 89%. About two to three times as much radioactivity was found in the Hirt pellet fraction (high-molecular-weight cellular DNA) as in the Hirt extracts. During the 60-min chase period, the presence of excess nonradioactive thymidine did not completely prevent incorporation of ^3H -dT into DNA of the Hirt pellet fraction. However, the total radioactivity of the Hirt extracts did not increase significantly during the chase period.

Analyses of the Hirt extracts by CsCl-EtBr equilibrium centrifugation demonstrated that 75

to 86% of the total radioactivity was in the light fraction after the 7.5-min pulse (Table 3). However, after the 60-min chase, only 7 to 14% of the total radioactivity was found in the light fraction, and 86 to 91% of the label was found in the heavy fraction.

Sucrose sedimentation analyses of the heavy (groups 2a and 2b) and the light (group 1a) CsCl-EtBr fractions of Table 3 are shown in Fig. 4. The remaining samples did not have sufficient radioactivity for accurate analysis by sucrose gradient centrifugation. Figure 4b shows that the light CsCl-EtBr fraction from the 7.5-min ^3H -dT pulse consisted mostly of DNA sedimenting more rapidly than form II SV40 DNA. Thus, the Hirt extracts of group 1a, Table 3, contained mostly nicked forms of DNA sedimenting faster than the nicked circular form of SV40 DNA. Since the radioactivity was chased into form I DNA, it is apparent that much of this radioactivity was in the form of replicative intermediates. The heavy CsCl-EtBr fractions obtained after the chase period, with or without cycloheximide, sedimented in the position of form I SV40 DNA (21S). These experiments demonstrate that radioactivity can be chased from replicative interme-

TABLE 3. Effect of cycloheximide on the pulse-labeling and chase of SV40 DNA at 37 hr after infection of ara-C pretreated CV-1 cell cultures^a

Group	Time post-infection cycloheximide added	Duration of chase	Counts per min per culture ($\times 10^{-3}$)		Radioactivity in fractions after CsCl-EtBr centrifugation of Hirt extract (%)	
			Hirt pellet	Hirt extract	Heavy	Light
1a	None	None	73.4	47.5	8.8	85.5
1b	36	None	10.2	6.5	21.9	74.9
2a	None	1	145.5	48.4	85.7	14.0
2b	36	1	16.1	8.0	91.0	6.6

^a Confluent monolayer cultures of CV-1 cells (11.6×10^6 cells/culture) were infected with parental SV40 cl 307L and then incubated at 36.5 C from 2 until 24 hr postinfection with ara-C. At 24 hr, new media containing deoxycytidine were added. Experimental cultures (1b and 2b) were treated with cycloheximide at 36 hr postinfection. At 36.75 hr postinfection, all cultures were treated with 5-fluorodeoxyuridine (25 μ g/ml) and uridine (12.2 μ g/ml) to deplete endogenous thymidine (dT) pools. At 37 hr postinfection, all cultures were pulse-labeled with ³H-dT (25 μ Ci/ml) for 7.5 min, and groups 1a and 1b were harvested. The media of groups 2a and 2b were removed and the cultures were washed with prewarmed media containing dT (100 μ g/ml) and deoxycytidine (10 μ g/ml). The wash of group 2b also contained cycloheximide (25 μ g/ml). New media containing dT and deoxycytidine (groups 2a) or dT, deoxycytidine, and cycloheximide (group 2b) were then added, and the cultures were further incubated for 1 hr at 36.5 C.

diates to superhelical SV40 DNA in the presence or in the absence of cycloheximide.

DISCUSSION

Goebel and Helinski (4) observed a striking increase in higher multiple circular DNA forms of the bacterial plasmid Col E₁ in *P. mirabilis* after chloramphenicol treatment or amino acid starvation. They proposed that the generation of multiple circular forms may result from an imbalance in the formation or concentration of the enzymes responsible for Col E₁ duplication. This might also be true for mouse fibroblast mitochondria (14). In the case of Ts-a-3T3 cells, Cuzin et al. (3) suggested that one possible mechanism for polyoma DNA oligomer formation might entail mistakes in replication of an autonomous non-integrated structure during growth at the non-permissive temperature.

In the present experiments, SV40-infected CV-1 cell cultures were treated with cycloheximide in an

effort to induce the accumulation of multiple circular forms of SV40 DNA. In some experiments, cycloheximide was added with ³H-dT and the SV40 DNA molecular forms were studied after a 4-hr labeling period. Studies have also been carried out in which SV40 DNA was first labeled with ³H-dT and then the fate of the labeled DNA was investigated after a 5-hr chase in the presence of cycloheximide (11). It was postulated that monomers might combine to form dimers under these conditions. In addition, infected cultures have been pretreated with cycloheximide for 1 hr and then incubated with ³H-dT and cycloheximide for 2 hr. None of these procedures induced an accumulation of multiple circular forms. These results suggest that errors in SV40 DNA replication, similar to those which take place during duplication of Col E₁ DNA in *P. mirabilis* and of mitochondrial DNA in mouse fibroblast cells, are not increased after cycloheximide treatment of SV40-infected CV-1 cells.

The results presented in this study also indicate that polynucleotide ligase activities are adequate

FIG. 4. Velocity sedimentation in neutral sucrose gradients (5 to 30%, w/v) of heavy and light CsCl-EtBr fractions from Table 3.

in cycloheximide-treated and SV40-infected cultures. Pulse-chase experiments have provided direct evidence that SV40 DNA synthesis can proceed in the presence of cycloheximide until the completion of the current cycle of duplication. Thus, replicative intermediates generated in the presence of cycloheximide were converted to superhelical SV40 DNA, despite the continued presence of the drug (Table 3, Fig. 4). Furthermore, most of the SV40 ³H-DNA formed in cycloheximide-inhibited cultures during longer labeling periods (2 to 4 hr) was superhelical (Tables 1 and 2). Form I SV40 ³H-DNA was generated in cycloheximide-inhibited cultures shortly after SV40 DNA synthesis started (24 to 28 hr) and also at later times (28 to 40 hr, Table 1).

Previous studies of polynucleotide ligase activity in SV40-infected cultures are consistent with the present findings. Sambrook and Shatkin (15) observed that polynucleotide ligase activity is enhanced in SV40-infected cultures and is maximal at about 36 to 40 hr after infection. Most likely, ligase activity is not reduced during cycloheximide treatment below the level needed to complete the formation of superhelical SV40 DNA. Previous studies have also shown that cycloheximide addition to SV40-infected cultures at 36 hr did not significantly reduce the activities of thymidine kinase, deoxycytidylate deaminase, thymidylate kinase, and DNA polymerase at 42 hr (10).

During the interval after infection investigated in this study (24 to 40 hr), the rate of SV40 DNA synthesis increased rapidly in the presence or absence of cycloheximide (8–10; Table 1). However, at any time between 24 and 40 hr, cycloheximide treatment markedly inhibited the formation of ³H-SV40 DNA. It would appear that enzymes catalyzing nucleotide interconversions, DNA polymerase, polynucleotide ligase, and endonucleases, are all available. This suggests that a cycloheximide-sensitive protein different from the aforementioned proteins is essential for maximal rates of SV40 DNA synthesis. The function of the essential protein is unknown, but three possibilities may be suggested. The cycloheximide-sensitive protein may be: (i) an initiator of new rounds of SV40 DNA replication; (ii) a component of newly generated SV40 DNA replication sites; or (iii) a maturation protein which might accelerate the removal of the completed SV40 DNA superhelices from active replication sites.

ACKNOWLEDGMENTS

This investigation was aided by a grant from the Robert A. Welch Foundation (Q-163), and by Public Health Service grants CA-06656-08 and 1-K6-AI-2352 from the National Cancer Institute and the National Institute of Allergy and Infectious Diseases.

We thank Carolyn Smith and Marjorie Johnson for able technical assistance.

LITERATURE CITED

- Amati, P. 1970. Chloramphenicol: Effect on DNA synthesis during phage development in *Escherichia coli*. *Science* **168**: 1226–1228.
- Bourgaux, P., D. Bourgaux-Ramoisy, and R. Dulbecco. 1969. The replication of the ring-shaped DNA of polyoma virus. I. Identification of the replicative intermediate. *Proc. Nat. Acad. Sci. U.S.A.* **64**:701–708.
- Cuzin, F., M. Vogt, M. Dieckmann, and P. Berg. 1970. Induction of virus multiplication in 3T3 cells transformed by a thermosensitive mutant of polyoma virus. II. Formation of oligomeric polyoma DNA molecules. *J. Mol. Biol.* **47**:317–333.
- Goebel, W., and D. R. Helinski. 1968. Generation of higher multiple circular DNA forms in bacteria. *Proc. Nat. Acad. Sci. U.S.A.* **61**:1406–1413.
- Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365–369.
- Hudson, B., W. B. Upholt, J. Devanny, and J. Vinograd. 1969. The use of an ethidium analogue in the dye-buoyant density procedure for the isolation of closed-circular DNA: The variation of the superhelix density of mitochondrial DNA. *Proc. Nat. Acad. Sci. U.S.A.* **62**:813–820.
- Kit, S., D. R. Dubbs, P. M. Frearson, and J. L. Melnick. 1966. Enzyme induction in SV40-infected green monkey kidney cultures. *Virology* **29**:69–83.
- Kit, S., T. Kurimura, and D. R. Dubbs. 1969. Properties of simian virus 40 rescued from cell lines transformed by ultraviolet-irradiated simian virus 40. *J. Virol.* **4**:585–595.
- Kit, S., T. Kurimura, M. L. Salvi, and D. R. Dubbs. 1968. Activation of infectious SV40 DNA synthesis in transformed cells. *Proc. Nat. Acad. Sci. U.S.A.* **60**:1239–1246.
- Kit, S., T. Kurimura, R. A. de Torres, and D. R. Dubbs. 1969. Simian virus 40 deoxyribonucleic acid replication. I. Effect of cycloheximide on the replication of SV40 deoxyribonucleic acid in monkey kidney cells and in heterokaryons of SV-40-transformed and susceptible cells. *J. Virol.* **3**:25–32.
- Kit, S., S. Tokuno, K. Nakajima, D. Trkula, and D. R. Dubbs. 1970. Temperature-sensitive simian virus 40 mutant defective in a late function. *J. Virol.* **6**:286–294.
- Lark, K. G. 1966. Regulation of chromosome replication and segregation in bacteria. *Bacteriol. Rev.* **30**:3–32.
- Levine, A. J., H. S. Kang, and F. E. Billheimer. 1970. DNA replication in SV40-infected cells. I. Analysis of replicating SV40 DNA. *J. Mol. Biol.* **50**:549–568.
- Nass, M. M. K. 1969. Reversible generation of circular dimer and higher multiple forms of mitochondrial DNA. *Nature (London)* **223**:1124–1129.
- Sambrook, J., and A. J. Shatkin. 1969. Polynucleotide ligase activity in cells infected with simian virus 40, polyoma virus, or vaccinia virus. *J. Virol.* **4**:719–726.
- Ward, C. B., and D. A. Glaser. 1969. Analysis of the chloramphenicol-sensitive and chloramphenicol-resistant steps in the initiation of DNA synthesis in *E. coli* B/r. *Proc. Nat. Acad. Sci. U.S.A.* **64**:905–912.