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Deoxyribonucleic Acid Replication in Simian Virus 40-Infected Cells

IV. Two Different Requirements for Protein Synthesis During Simian Virus 40 Deoxyribonucleic Acid Replication

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The replication of simian virus 40 (SV40) deoxyribonucleic acid (DNA) was inhibited by 99% 2 hr after the addition of cycloheximide to SV40-infected primary African green monkey kidney cells. The levels of 25*S* (replicating) and 21*S* (mature) SV40 DNA synthesized after cycloheximide treatment were always lower than those observed in an infected untreated control culture. This is consistent with a requirement for a protein(s) or for protein synthesis at the initiation step in SV40 DNA replication. The relative proportion of 25*S* DNA as compared with 21*S* viral DNA increased with increasing time after cycloheximide treatment. Removal of cycloheximide from inhibited cultures allowed the recovery of viral DNA synthesis to normal levels within 3 hr. During the recovery period, the ratio of 25*S* DNA to 21*S* DNA was 10 times higher than that observed after a 30-min pulse with ^{3}H-thymidine with an infected untreated control culture. The accumulation of 25*S* replicating SV40 DNA during cycloheximide inhibition or shortly after its removal is interpreted to mean that a protein(s) or protein synthesis is required to convert the 25*S* replicating DNA to 21*S* mature viral DNA. Further evidence of a requirement for protein synthesis in the 25*S* to 21*S* conversion was obtained by comparing the rate of this conversion in growing and resting cells. The conversion of 25*S* DNA to 21*S* DNA took place at a faster rate in infected growing cells than in infected confluent monolayer cultures. A temperature-sensitive SV40 coat protein mutation (large-plaque SV40) had no effect on the replication of SV40 DNA at the nonpermissive temperature.

Infection of primary African green monkey kidney (AGMK) cells with simian virus 40 (SV40) results in the synthesis of viral deoxyribonucleic acid (DNA) and infectious virus (10, 12). SV40 DNA replication begins at about 15 hr after infection and reaches a maximum rate at about 30 hr. When infected cells were pulse-labeled with ^{3}H-thymidine for short periods of time (less than 10 min), little or no mature viral DNA was detected either by neutral or alkaline sucrose gradients or by cesium chloride-ethidium bromide equilibrium centrifugation. Instead, a viral DNA form that sedimented in neutral sucrose gradients at 25*S* was observed. This DNA was completely denaturable in alkali and banded at a lighter density than closed circular mature viral DNA in ethidium bromide-cesium chloride equilibrium gradients. The ^{3}H-labeled 25*S* DNA could be chased into 21*S* mature viral DNA when unlabeled thymidine was added to the culture medium (12).

Electron micrographs of 25*S* DNA showed SV40 replicating circles (two branch points, three branches, and no visible ends) where 90% of the SV40 molecule had completed its replication. At any one time, most of the replicating molecules observed in the infected cell were of this 25*S* replicating type (75% of the replicating molecules were 90% completed). This led to the suggestion that there was a slow or rate-limiting step late in the replication of an SV40 DNA molecule and that this resulted in an accumulation of 25*S* DNA (12).

In the present study, it is shown that cycloheximide (10 μg/ml) inhibits the replication of SV40 DNA. These data are in agreement with previous studies with SV40 (11) and polyoma virus (5). The results indicate that protein synthesis is required at two different stages in the replication of SV40 DNA: (i) the initiation of DNA synthesis, and (ii) the conversion of the 25*S* replicating form to 21*S* mature viral DNA (finishing step).

MATERIALS AND METHODS

Virus. The SV40 large-plaque mutant (16, 17, 19, 20) and wild-type strain (13) were employed in these studies.

Tissue culture. AGMK cells (Flow Laboratories, Inc.) were cultured in either plastic petri dishes (Falcon, 100 by 20 mm) or plastic flasks (Falcon, 75 ml) in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) supplemented with 10% calf serum.

Infectivity assay. Virus stocks were prepared as described previously (12). Infectious virus was titrated on monolayer cultures of AGMK or BSC-1 cells by use of a plaque assay procedure (6).

Measurement of the rate of DNA synthesis. The rate of viral DNA synthesis was measured by the addition of ^{3}H-thymidine (14 to 18 Ci/mmole, New England Nuclear Corp.) to infected cell cultures. After an appropriate exposure to the isotope, the cells were washed twice with ice-cold phosphate-buffered saline (PBS, 0.01 M sodium phosphate buffer at *p*H 7.2 and 0.15 M NaCl) and lysed with 0.6% sodium dodecyl sulfate (SDS) in 0.01 M sodium phosphate buffer, *p*H 7.2, 0.15 M NaCl, and 0.01 M disodium ethylenediaminetetraacetic acid (EDTA). Viral DNA was separated from cellular DNA by the Hirt procedure (9). A portion of the 1 M NaCl-SDS soluble fraction (small-molecular-weight DNA) was sedimented through a 5 to 20% linear sucrose gradient for 3 hr at 40,000 rev/min in an SW 50.1 rotor. The ^{3}H-labeled component that sedimented at 21*S* was used to quantitate the levels of mature viral DNA synthesis. The quantity of ^{3}H-labeled DNA that sedimented at 25*S* was employed to determine the amounts of replicating SV40 DNA made during the labeling period (12). The quantity of ^{3}H counts per minute in the 1 M NaCl-SDS precipitable fraction (large-molecular-weight DNA) was employed to determine the rate of cellular DNA synthesis. Only the fraction of the ^{3}H-label that was trichloroacetic acid-precipitable and resistant to alkaline degradation (0.3 M KOH, 37 C for 18 hr) was used to measure the rate of cellular DNA synthesis.

After a 1-hr exposure of SV40-infected cells with ^{3}H-thymidine, over 90% of the trichloroacetic acid-precipitable radioactive label in the 1 M NaCl-SDS soluble fraction is virus-specific (12). Greater than 90% of the ^{3}H-labeled DNA in the 1 M NaCl-SDS precipitable fraction is cellular DNA (12).

Benzoylated-naphoylated diethylaminoethyl cellulose (BNC) chromatography. BNC was synthesized by the procedure of Gillam et al. (7) as modified by Sedat, Lyon, and Sinsheimer (18). BNC columns were prepared and utilized as described previously (12).

Centrifugation techniques and isotope measurements. Sucrose density centrifugation was performed as previously described (12). The details of each sedimentation run are given in the figure legends. Radioactive samples were prepared and counted in a Beckman liquid scintillation counter as described previously (12).

Cycloheximide treatment. Cycloheximide was obtained from Sigma Chemical Co. At the concentration employed (10 μg/ml), cycloheximide inhibited the incorporation of ^{3}H-leucine (30 to 50 Ci/mmole, New England Nuclear Corp.) into hot trichloroacetic acid-precipitable (100 C for 45 min) material by 96% during the first hour and 99% by the second hour of treatment. Infected and uninfected AGMK cells behaved in an identical manner.

RESULTS

Effect of cycloheximide on SV40 DNA replication. To examine the effects of cycloheximide on the replication of SV40 DNA, the following experiment was performed. Six monolayer cultures of AGMK cells were infected with SV40 large-plaque mutant, and 30 hr later five of these cultures were treated with 10 μg of cycloheximide per ml. One cycloheximide-treated culture and one untreated control culture were then labeled with ^{3}H-thymidine (1 μCi/ml) for 1 hr at 36 C. A second cycloheximide-treated infected culture was labeled from 1 to 2 hr after the addition of the antibiotic (31 to 32 hr after infection). Each additional culture was labeled in an identical manner during the 3rd, 4th, or 5th hr after exposure to cycloheximide. At the end of the 1-hr pulse-labeling period, each culture was washed two times with ice-cold PBS, and the cells were lysed with 0.6% SDS as described in the preceding section. A 0.2-ml sample of the 1 M NaCl-SDS soluble fraction was sedimented through a 5 to 20% linear sucrose gradient for 3 hr at 40,000 rev/min. The results of this experiment are presented in Fig. 1.

The addition of cycloheximide to virus-infected AGMK cells results in the cessation of SV40 DNA replication. The synthesis of SV40-specific DNA was inhibited by 99% after 2 hr of exposure to this antibiotic. Cycloheximide inhibited the production of both 25*S* (replicating) DNA and 21*S* (mature) SV40 DNA. However, after an initial 1-hr delay, the ratio of 25*S* to 21*S* DNA was observed to increase from 0.3 to 1.0. This can be seen in the sucrose gradient profile presented in Fig. 1 (compare 1A with 1E or 1F).

Levine et al. (12) demonstrated that 25*S* viral DNA is a precursor of 21*S* mature SV40 DNA. The increased levels of 25*S* DNA relative to 21*S* DNA observed after cycloheximide treatment suggested the possibility that cycloheximide may act by blocking the conversion of 25*S* to 21*S* DNA (as well as inhibiting the initiation of SV40 DNA synthesis). Because of difficulties in quantitating the relative amounts of 25*S* and 21*S* DNA when sucrose gradient centrifugation was used, a second procedure was also employed to separate these two forms of SV40 DNA. BNC chromatography has been shown to fractionate replicating (25*S*) and mature SV40 DNA (12). The experi-

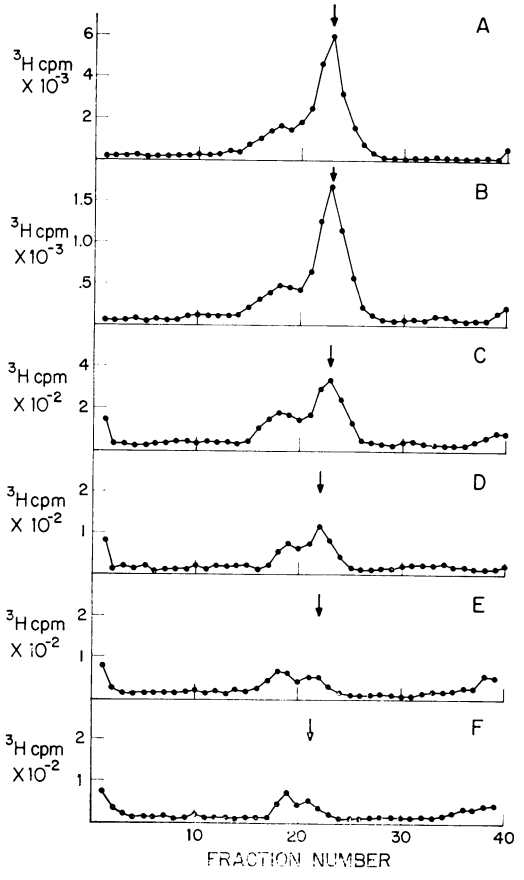

FIG. 1. *Sedimentation of SV40 DNA synthesized after cycloheximide inhibition. Six monolayer cultures of AGMK cells were infected with SV40, and 30 hr later five of the cultures received 10 μg of cycloheximide per ml. One cycloheximide-treated culture (B) and one untreated culture (A) were labeled with ^{3}H-thymidine (1 μCi/ml) for 1 hr. The other cultures were labeled (C) from 1 to 2 hr, (D) from 2 to 3 hr, (E) from 3 to 4 hr, and (F) from 4 to 5 hr after addition of the drug. A sample of the 1 M NaCl-SDS soluble fraction was sedimented for 3 hr at 40,000 rev/min in an SW 50.1 rotor. (●) ^{3}H counts per minute; the arrow represents the peak of a 21S ^{32}P-labeled SV40 DNA sedimentation marker.*

periment presented in Fig. 1 was repeated except that the 1 M NaCl-SDS soluble fraction was phenol-extracted and chromatographed on BNC columns. Mature viral DNA (21S) eluted from the BNC resin with 1 M NaCl. The 25S DNA remained bound to the column under these conditions and was eluted at a later time with 2% caffeine in 1 M NaCl. This procedure permits an excellent separation of replicating and mature viral DNA (12).

TABLE 1. *Inhibition of SV40 DNA synthesis by cycloheximide*

Cycloheximide concn	Labeling time	Sucrose gradient		BNC[b]	
		Per cent of control	Per cent 25S DNA[a]	Per cent of control	Per cent 25S DNA[a]
μg/ml	hr				
0	0–1	100[c]	24	100[c]	27
10	0–1	24	22	31	23
10	1–2	7.5	37	9.3	33
10	2–3	1.0	43	1.8	41
10	3–4	0.9	50	1.0	48
10	4–5	0.6	54	1.7	53

[a] Percentage of total DNA synthesized during the time interval indicated that sedimented at 25S or was eluted with 2% caffeine from a BNC column.

[b] Benzoylated-naphoylated diethylaminoethyl cellulose.

[c] The 100% value for the ^{3}H counts per minute as measured by sucrose gradients is 29,000 counts/min and by BNC is 215,000 counts/min.

Table 1 presents the results of experiments designed to investigate the effects of cycloheximide on SV40 DNA replication. Both the rate of viral DNA synthesis and the relative proportions of 25S and 21S SV40 DNA were measured by use of BNC chromatography and sucrose gradient analysis. Within the first hour after the addition of cycloheximide, viral DNA synthesis decreased to 25 to 30% of the level observed in the untreated control culture. During this time, the relative amounts of 25S and 21S DNA remained the same in the antibiotic-treated and untreated cultures. After a 1-hr exposure to this drug, the rate of viral DNA synthesis continued to fall (from 10 to 1%), but an increasing proportion of the SV40 DNA remained in the replicating form (25S or caffeine-eluted DNA). The percentage of 25S DNA synthesized in a 1-hr pulse-labeling period increased from 25% in the absence of cycloheximide to 50% by 3 to 4 hr after the addition of this drug. The same results were obtained with either sucrose gradient analysis or BNC chromatography.

Recovery of SV40 DNA synthesis after the removal of cycloheximide. The experiments described in the preceding section suggest that cycloheximide might be inhibiting both the initiation of viral DNA synthesis and the conversion of 25S DNA to mature viral 21S DNA. It is difficult however, to make any conclusions about the significance of the relative proportions of 25S and 21S DNA when the rate of viral DNA synthesis is only 1% of the normal levels. For this reason, it was necessary to investigate whether

viral DNA replication could fully recover from this cycloheximide treatment and to determine what happened during this recovery period to the conversion of 25S to 21S DNA.

Cycloheximide (10 μg/ml) was added to five AGMK monolayer cultures at 36 hr after infection. After 3 hr, the cycloheximide was removed from four of these cultures by washing the cells four times with 10 ml of warm (37 C) medium. Viral DNA synthesis was measured by pulse-labeling these cultures with ^{3}H-thymidine (1 μCi/ml) for 30 min at different time intervals after the removal of cycloheximide. The pulse-labeling periods were stopped by washing the cultures with ice-cold PBS and the cells were lysed with 0.6% SDS. A sample of the 1 M NaCl-SDS soluble fraction was analyzed by sucrose gradient centrifugation as previously described. The radioactive sedimentation profiles from these sucrose gradients are presented in Fig. 2. Figure 2A was obtained from an infected culture where cycloheximide was not removed. Figures 2B, C, D, and E were obtained by pulse-labeling with ^{3}H-thymidine at 0 to 0.5 hr, 0.5 to 1 hr, 1.5 to 2 hr, and 3 to 3.5 hr after the removal of cycloheximide. Figure 2F was obtained from a control culture that never received cycloheximide. It can be seen that removal of this drug allows a full recovery of the rate of viral DNA synthesis. The viral DNA synthesized shortly after the removal of cycloheximide (0 to 0.5 hr and 0.5 to 1 hr) was mostly 25S replicating DNA. With increasing time after reversal of cycloheximide inhibition, the ratio of 25S to 21S DNA changed from 3.0 to 0.3.

Table 2 presents the results of experiments that demonstrate the reversal of cycloheximide inhibition of viral DNA synthesis. Both BNC chromatography and sucrose gradient analysis were employed to investigate the rate of viral DNA replication and the relative proportions of 25S and 21S viral DNA. After 3 hr of exposure of infected cells to cycloheximide, the rate of viral DNA synthesis was 1% of that in an uninfected control culture. By 3.5 hr after the removal of this drug, the rate of viral DNA synthesis was normal (87 to 100% of the control value), and the relative amounts of 25S and 21S DNA were identical to those in the untreated infected control culture (30 to 35% of 25S DNA). Between 0 and 2 hr after removal of this drug, the rate of SV40 DNA synthesis recovered from 20% to 70% of the untreated control. At that time, the levels of 25S DNA declined from 75% to 39% of the total viral DNA synthesized during the 30-min pulse-labeling period.

Effects of a "shift-up in cell growth" on SV40 DNA replication. The results presented in the two previous sections suggest that the conversion of

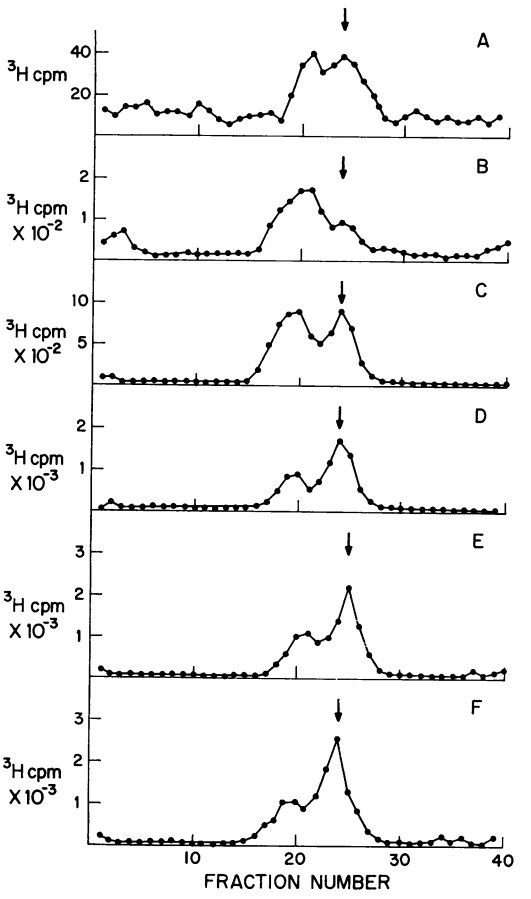

FIG. 2. *Sedimentation of SV40 DNA synthesized during recovery from cycloheximide inhibition. Cycloheximide (10 μg/ml) was added to five infected AGMK monolayer cultures for 3 hr. At that time, the drug was removed from four of these cultures. Viral DNA synthesis was measured by pulse-labeling with ^{3}H-thymidine (1 μCi/ml) for 30-min intervals. (A) Cycloheximide not removed; (B–E) cycloheximide was removed and the cultures were labeled at 0 to 0.5 hr, 0.5 to 1 hr, 1.5 to 2 hr, and 3 to 3.5 hr, respectively, after reversal of the inhibition. (F) No cycloheximide added. The procedures for sucrose gradient sedimentation are presented in figure 1. (●) ^{3}H counts per minute; the arrow represents the peak of a 21S ^{32}P-labeled SV40 DNA sedimentation marker.*

25S to 21S DNA requires a protein(s) that is reduced in amount by cycloheximide treatment. At 2 to 3 hr after removal of this drug, the protein(s) is present in sufficient quantities to permit the normal rate of conversion of 25S DNA to 21S mature DNA. These data, however, rest solely on the use of an inhibitor (cycloheximide) and therefore require confirmation by different experimental procedures.

TABLE 2. *Recovery of SV40 DNA synthesis from cycloheximide inhibition*

Cycloheximide concn	Labeling time	Sucrose gradient		BNC[b]	
		Per cent of control	Per cent 25S DNA[a]	Per cent of control	Per cent 25S DNA[a]
μg/ml	hr				
10 (not removed)	1–1.5[c]	1.8	49	0.9	52
10 (removed)	0–0.5[d]	21	74	25	68
10 (removed)	0.5–1	54	60	50	58
10 (removed)	1.5–2	68	39	68	47
10 (removed)	3–3.5	87	35	100	39
0 (not added)	3–3.5[e]	100[f]	33	100[f]	35

[a] Percentage of total DNA synthesized during the time interval indicated that sedimented at 25S or eluted with 2% caffeine from a BNC column.
[b] Benzoylated-naphoylated diethylaminoethyl cellulose.
[c] Labeling time 1 to 1.5 hr after cycloheximidide was added.
[d] Labeling time 0 to 0.5 hr after removal of cycloheximide.
[e] No cycloheximide was ever added; 3- to 3.5-hr labeling time refers to the same time interval as for the sample labeled 3 to 3.5 hr after removal of cycloheximide.
[f] The 100% value for the ^3H counts per minute as measured by sucrose gradients is 16,500 counts/min and by BNC is 136,000 counts/min.

To determine whether protein synthesis plays a role in the 25S to 21S conversion, cultures of AGMK cells in two very different physiological states (growing and resting cells) were infected with SV40. Growing AGMK cells (about 40% of the petri dish surface contained cells) were infected with large-plaque SV40 in 2 ml of Dulbecco's medium containing 2% calf serum. After 3 hr of adsorption, the excess virus was removed, and 15 ml of Dulbecco's medium containing 10% calf serum was added to the culture. At the same time, monolayer cultures of AGMK cells (100% of the petri dish surface contained cells) were infected with large-plaque SV40 in 2 ml of Dulbecco's medium containing 2% depleted (21) calf serum. After the 3-hr adsorption period, the excess virus was removed, and 15 ml of Dulbecco's medium containing 2% depleted calf serum was added to the cultures. At 30 hr after infection, each of these cultures was pulse-labeled with ^3H-thymidine (25 μCi/ml) for a different length of time (4, 5, 8, 12, 20, or 25 min) depending on the experiment. The pulse-labeling period was terminated by washing the cell cultures with ice-cold PBS, and the cells were lysed with 0.6% SDS. A 0.2-ml sample of the 1 M NaCl-SDS soluble fraction was adjusted to 0.1 M NaOH (final concentration) and incubated at 37 C for 10 min. This sample was then centrifuged through an alkaline sucrose gradient, and the percentage of the total ^3H counts per minute that co-sedimented with a ^{32}P-labeled SV40 DNA sedimentation marker was determined (Fig. 3). In the infected monolayer cultures with depleted serum medium, there was a 7- to 8-min delay in the production of ^3H-labeled mature viral DNA. This delay is due to the time required to complete one replication of an SV40 genome (one doubling of a DNA molecule). It is clearly not due to the difficulty of getting the radioisotope into the cell, because the incorporation of ^3H-thymidine into viral DNA (25S and 21S) is approximately linear

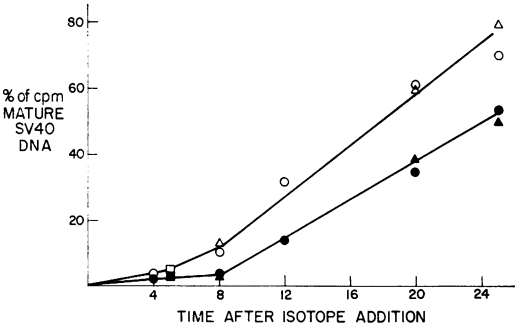

FIG. 3. *Kinetics of formation of ^3H-labeled mature viral DNA as a function of the length of exposure to ^3H-thymidine. At 30 hr after SV40 infection of stationary or growing cultures of AGMK cells, each culture was labeled with ^3H-thymidine for the length of time (in minutes) indicated on the abscissa. A sample of the 1 M NaCl-SDS soluble fraction was adjusted to 0.1 M NaOH and incubated at 37 C for 10 min. Alkaline sucrose gradients (5 to 20% sucrose in 0.1 M NaOH and 0.9 M NaCl) were run for 2 hr at 40,000 rev/min in an SW 50.1 rotor. The ordinate is plotted as the percentage of total counts per minute that co-sedimented with a ^{32}P-labeled SV40 closed circular DNA sedimentation marker.* (●, ■, ▲) *Stationary cultures;* (○, □, △) *growing cultures.*

with time over these short time periods (12). The lag in the production of mature labeled viral DNA has been attributed, therefore, to a slow or rate limiting step in the replication of SV40 DNA (12). This slow step has been shown to be the conversion of 25*S* to 21*S* DNA (12). In the infected growing cultures with 10% calf serum the lag in the production of labeled mature viral DNA was 4 to 5 min (Fig. 3). It can also be seen that the rate of production of mature viral DNA in the growing cultures (compare the slopes of the two curves) was greater than in the nongrowing cells. These results demonstrate that in infected growing cells, where the rate of protein synthesis is higher than in nongrowing infected cells, the conversion of 25*S* to 21*S* DNA takes less time than in monolayer cultures. This is consistent with the idea that active protein synthesis is required for the 25*S* to 21*S* conversion and that this is a rate-limiting step in confluent monolayer cultures. It is important to point out that even in infected growing cultures, where the rate of protein synthesis is very high, there is still a lag or delay in the production of labeled mature viral DNA.

Effects of SV40 temperature-sensitive coat protein mutation on viral DNA replication. The previous results suggested that protein synthesis was required for the production of mature viral DNA (21*S*) from its 25*S* precursor. If this is correct, the protein(s) required for this process could be coded for by the virus, the cell, or both. The fact that a functional coat protein is required for the synthesis of single-stranded ϕX174 DNA (14) and the maturation of double-stranded linear lambda DNA (15) suggested the possibility that SV40 coat proteins might be involved in the conversion of 25*S* to 21*S* viral DNA.

The SV40 large-plaque mutant has been shown to possess a temperature-sensitive mutation in a coat protein gene (16, 17, 19, 20). To demonstrate the temperature-sensitive nature of the SV40 large-plaque mutant, the following experiment was performed. Two cultures of AGMK cells were infected with large-plaque SV40 and another set of two cultures with wild-type SV40. Adsorption was allowed to proceed for 3 hr at 36 C. At the end of that time, one culture infected with large-plaque SV40 culture infected with wild-type SV40 were shifted to 40 C. All four infected cell cultures were harvested at 72 hr after infection and the sonically treated cell lysates were titrated for infectious virus at 36 C (experiment A, Table 3). Wild-type SV40 replicated almost as well at 40 C as at 36 C. On the other hand, the large-plaque SV40 was restricted in its replication (greater than 99.9%) at 40 C.

To determine the time course of the SV40 large-plaque temperature-sensitive restriction, the same experiment was repeated but the infected cultures were shifted up to 40 C at 24 hr after infection. Infected cell lysates were prepared by sonic treatment at 10, 24, and 72 hr after infection, and infectious virus was titered at 36 C (experiment B, Table 3). Wild-type SV40 was made in normal quantities after the shift to 40 C. Little or no increase in infectious virus was observed when the SV40 large-plaque mutant was shifted to 40 C at 24 hr after infection. This indicates that the temperature-sensitive step occurs after viral DNA synthesis normally begins (16 to 20 hr after infection at 36 C). These data are in excellent agreement with those of others (16, 17, 19, 20) and are consistent with the results that indicate that the large-plaque SV40 is a temperature-sensitive mutant for a late function (coat protein; 16, 17).

To observe whether the SV40 large-plaque mutation in the viral coat protein had any effect on viral DNA replication, two monolayer cultures of AGMK cells were infected with large-plaque SV40 at 36 C. After 24 hr of incubation at the permissive temperature (36 C), one of the infected cell cultures was shifted to 40 C for 24 hr. ^{3}H-thymidine (1 μCi/ml) was then added to a culture at each temperature (36 and 40 C) for 45 min. At the end of this labeling period, the cells were washed with cold PBS and lysed with 0.6% SDS. A sample of the 1 M NaCl-SDS supernatant fraction was sedimented through a sucrose gradient for 3 hr at 40,000 rev/min. The results of this

TABLE 3. *Temperature-sensitive restriction of large-plaque SV40*

Virus	Expt A:[a] titer of virus replicated at		Expt B:[b] titer at time after infection		
	36 C	40 C	10 hr	24 hr	72 hr
Wild-type SV40	4.2×10^8	1.2×10^8	2.5×10^4	8.8×10^4	1.8×10^8
Large-plaque SV40	1.0×10^9	5.0×10^5	6.0×10^4	1.4×10^5	3.0×10^5

[a] Virus was adsorbed for 3 hr at 36 C and then shifted to 40 C. Virus production was titrated at 72 hr after infection at 36 C.

[b] Virus was adsorbed and incubated for 24 hr at 36 C. Cultures were then shifted to 40 C, and virus production was titrated at 36 C. Samples were obtained at 10, 24, and 72 hr after infection.

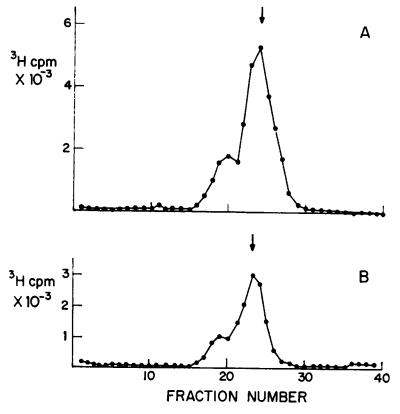

FIG. 4. *Sedimentation of large-plaque SV40 DNA synthesized at 36 or 40 C. Two monolayer cultures of AGMK cells were infected with large-plaque SV40 at 36 C; 24 hr later, one culture was shifted up to 40 C. At 48 hr after infection, each culture was labeled with ^{3}H-thymidine (1 μCi/ml) for 45 min. A sample of the 1 M NaCl-SDS soluble fraction was sedimented in a neutral sucrose gradient as described in Fig. 1. (A) 40 C; (B) 36 C. (●) ^{3}H-counts per minute; the arrow represents the peak of a ^{32}P-labeled 21S SV40 DNA sedimentation marker.*

experiment are presented in Fig. 4. SV40 DNA was synthesized at both the permissive and nonpermissive temperatures. About 1.9 times more viral DNA was made at 40 C than at 36 C. In both cases, however, 22 to 23% of the SV40 DNA made in a 45-min pulse-labeling period was 25S DNA. The only detectable difference between the two cultures was that about twice as much viral DNA was made at 40 C as at 36 C. Two additional experiments were performed to determine whether this difference was due to the absence of a functional coat protein at 40 C or just due to a difference in the incubation temperature of the two cultures. First, the experiment described above was repeated with the wild-type SV40 instead of the large-plaque virus. In this experiment, the wild-type virus was found to synthesize 1.7 times more viral DNA at 40 C than at 36 C. This is consistent with the interpretation that the large plaque SV40 synthesizes more viral DNA at 40 C than at 36 C because of a temperature difference and not because a functional viral coat protein is missing. In a second type of experiment, six monolayer cultures of AGMK cells were infected with large-plaque SV40 at 36 C. At 48 hr after infection, half of these cultures were shifted to 40 C for 1 hr. ^{3}H-thymidine was then added to each infected culture (at 36 and 40 C), and the amount of viral and cellular DNA synthesized during 4-, 7.5-, and 8-hr labeling periods was determined as described previously. In each case, the rate of viral DNA synthesis was 1.6 to 2.5 times greater at 40 C than at 36 C. The rate of cellular DNA synthesis in these same cells was 1.5 to 2.3 times greater at 40 C than at 36 C. Since a functional viral coat protein is clearly not required for cellular DNA synthesis, the higher levels of host-cell DNA replication at 40 C must be due to the difference in incubation temperatures. Thus, the rates of cellular and viral DNA synthesis were increased by about the same amounts (1.5 to 2.5 times) at 40 C. This is also consistent with the interpretation that the absence of a functional viral coat protein at 40 C is not responsible for the observed increase in the rate of viral DNA synthesis.

DISCUSSION

Figure 5 presents a model for SV40 DNA replication based on the results of Levine et al. (12). The mature SV40 closed circular and suprcoiled DNA is first nicked and replication begins. Because 16S, nicked circular DNA is never observed with either short or long pulses of ^{3}H-thymidine, replication must start very soon after the opening of the SV40 polynucleotide strand. At least one strand of the SV40 duplex must be open at some time during synthesis to permit a semiconversative mode of replication to occur (8, 22). Most of the replicating molecules observed in an infected cell have completed 90% of their replication (25S form; 12). This led to the suggestion that step I of this replication model was the fast step (50 sec), whereas step II was slower (5 to 8 min). Thus, a rate-limiting or slow step (II) occurs at the end of the replication of an SV40 circle (12).

Mutants of polyoma virus (1–3) have been isolated that do not synthesize viral DNA at the nonpermissive temperature. If these mutants are shifted up to the nonpermissive temperature during polyoma DNA synthesis, then polyoma DNA replication stops (4). This shows that the

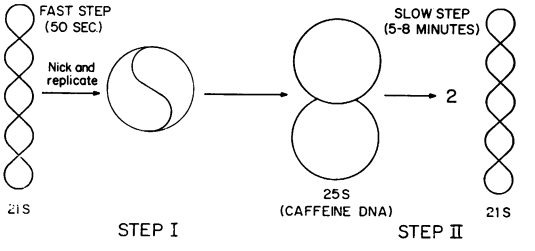

FIG. 5. *Model for the replication of SV40 DNA. Each line represents a double helix. The 21S DNA is closed circular and supercoiled DNA; 25S DNA is the predominant form of replicating molecules observed in an infected cell.*

presence of a virus-specific protein is required concurrently with the synthesis of polyoma DNA. Although it has not been proven, this protein is most likely required for the initiation step of polyoma replication (a viral DNA intermediate is not accumulated; 4). The addition of cycloheximide to SV40-infected cells inhibits viral DNA replication (11) by 99% within 2 hr. One might expect that cycloheximide inhibits the synthesis of a virus-specific protein (SV40 protein analogous to that found with the polyoma mutants) that initiates the synthesis of SV40 DNA. As cycloheximide inhibition of SV40 DNA replication proceeds (3 to 4 hr after the addition of the drug), there is an increase in the proportion of 25S DNA synthesized (from one-fourth to one-half 25S DNA) in a 1-hr pulse. This would be expected if the initiation step was inhibited at a slower rate than the finishing step (step II). This is not observed during the first hour of cycloheximide treatment, indicating that for this time interval the protein(s) needed for the finishing step is inhibited or inactivated at the same rate as the initiator protein(s). It is important to point out that throughout the cycloheximide treatment (or recovery) lower than normal levels of 21S and 25S viral DNA were observed. These data indicate that both initiation and finishing steps are inhibited by cycloheximide.

Removal of cycloheximide from infected cells allows the recovery of SV40 DNA synthesis to normal levels. The first viral DNA accumulated (labeled with ^{3}H-thymidine) after removal of the drug (0 to 0.5 hr) is mainly 25S (75%). This is consistent with the expectation that initiator protein(s) recover from cycloheximide inhibition at a faster rate than the finishing protein(s). The presence of normal levels of finishing protein(s) can be observed by 3 hr after reversal of this drug. These data show that finishing protein(s) is inactivated or inhibited at a faster rate and recovers from cycloheximide inhibition at a slower rate than the initiator protein(s). By 0.5 to 1 hr after removal of cycloheximide from infected cultures, the rate of viral DNA synthesis is 50 to 55% of normal, and the levels of 25S DNA are 40 to 45% higher than normal.

The use of an inhibitor like cycloheximide suffers from the problem that the infected cell is most likely in an abnormal state during the inhibition or recovery stages of the treatment. It was therefore desirable to obtain an independent confirmation of a requirement for protein synthesis for the 25S to 21S conversion step. To do this, stage II of SV40 DNA replication was measured in infected growing and resting cells. The rate of protein synthesis was found to be three to five times greater in infected growing cells than in the infected confluent monolayer cells. The conversion of 25S to 21S DNA was also faster in growing cells. There was both a decrease in the delay of the production of ^{3}H-labeled 21S DNA and an increase in the rate of production of 21S DNA. These data demonstrate that at least a portion of the rate-limiting step (step II) in SV40 DNA replication can be eliminated by using infected growing cells. It is important to note, however, that a lag or delay in the appearance of ^{3}H-labeled 21S DNA is still observed with these cells (4- to 5-min delay), and therefore some of the factors needed for step II are still rate-limiting for SV40 DNA replication.

There are several difficulties in comparing events in growing and stationary cells. Virus production and the rate of incorporation of ^{3}H-thymidine into viral DNA are faster in the growing cell system. It is clear that 30 hr after infection with growing cells may be analogous to a later time period in infected monolayer cultures. To eliminate these objections, the rate of conversion of 25S to 21S DNA has been measured at different times after lytic infection of stationary cultures. At all times tested (24, 36, 48, and 60 hr), the rate of conversion of 25S DNA to 21S DNA was about the same (6 to 8 min). Since the ratio of replicating DNA to coat protein is probably different at 24 and 48 hr after infection, this result is consistent with the suggestion that SV40 coat protein has no effect on this step II conversion. Presumably, if one could look early enough in infection (when the replicating pool of DNA is very small), the delay in the 25S to 21S conversion would be eliminated. In addition, these data eliminate the possibility that the time after infection that one measures the 25S to 21S conversion may not be comparable in resting and growing cells.

The experiments presented in this paper suggest that a protein(s), or protein synthesis, is required at two stages in the replication of SV40 DNA molecules. A virus-specific initiator protein is probably required to start a round of replication (step I). A second protein, or proteins, appears to be needed either to finish the last 10% of the replication or to close the SV40 polynucleotide strands, or to do both. At least one SV40 coat protein, prescribed by the SV40 large-plaque mutation, is not involved in DNA replication.

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