JOURNAL OF VIROLOGY, Jan. 1969, p. 25-32

Vol. 3, No. 1
Printed in U.S.A.

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 SV40-transformed and Susceptible Cells

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Received for publication 23 September 1968

Infectious deoxyribonucleic acid (DNA) was extracted from green monkey kidney (CV-1) cultures at various times after the cultures were infected with simian virus 40 (SV40) at input multiplicities of 0.01 and 0.1 plaque-forming unit (PFU) per cell. A pronounced decrease in infectious DNA was observed from 3 to 16 hr after virus infection, suggesting that structurally altered intracellular forms may have been generated early in infection. Evidence is also presented that SV40 DNA synthesis requires concurrent protein synthesis. DNA replication was studied in the presence and absence of cycloheximide in: (i) SV40-infected and uninfected cultures of CV-1 cells; (ii) cultures synchronized with 1-β-D-arabinofuranosylcytosine (ara-C) for 24 to 30 hr prior to the addition of cycloheximide; and (iii) in heterokaryons of SV40-transformed hamster and susceptible monkey kidney cells. DNA synthesis was determined by pulse-labeling the cultures with ^{3}H-thymidine at various times from 24 to 46 hr after infection. In addition, the total infectious SV40 DNA was measured. Addition of cycloheximide, even after early proteins had been induced, grossly inhibited both SV40 and cellular DNA syntheses. The activities of thymidine kinase, DNA polymerase, deoxycytidylate deaminase, and thymidylate kinase were measured; these enzyme activities remained high for at least 9 hr in the presence of cycloheximide. SV40 DNA prelabeled with ^{3}H-thymidine before the addition of cycloheximide was also relatively stable during the time required for cycloheximide to inhibit further DNA replication.

The kinetics of simian virus 40 (SV40) deoxyribonucleic acid (DNA) replication have been studied by a number of methods: plaque assay of infectious DNA, radioautography, and band centrifugation or nitrocellulose chromatography of pulse-labeled DNA (16, 17, 19). These studies have shown that SV40 DNA replication begins at about 12 to 16 hr and continues at a rapid rate until about 44 hr after virus infection. In contrast, infectious virus production commences after a 20- to 24-hr eclipse period and virus titers increase until about 60 to 72 hr after infection. Also, infectious SV40 DNA was first detected in heterokaryons of transformed and susceptible cells at 19 hr after cell fusion, but was not detected in transformed cells before fusion or during the first 11 hr after fusion (19). Although the timing of SV40 DNA synthesis has been elucidated, the molecular events which occur during replication are poorly understood. Presumably, these events include: (i) conversion of closed circular, double-stranded SV40 DNA (form I) to a "nicked" circle (form II); (ii) formation of complementary copies of each of the parental DNA strands; and (iii) joining of ends of polynucleotide chains to form superhelical (form I) daughter molecules. It is possible that some of these processes take place at specific cellular sites, perhaps the nuclear membrane (4, 22, 27).

Two facets of SV40 DNA relication are described in the present study. First, it is shown that infectious SV40 DNA undergoes an "eclipse" soon after virus infection of green monkey kidney (CV-1) cells. Secondly, we have asked whether synthesis of essential proteins early in infection is a sufficient condition for the replication of SV40 DNA late in infection, or whether there is an additional requirement for the synthesis of proteins concurrent with replication of SV40 DNA. Our data show that the addition of cycloheximide either to cultures pretreated with 1-β-D-arabinofuranosylcytosine (ara-C) or to cultures not pretreated with ara-C, at times after early enzymes have been induced by virus infection, strongly inhibits SV40 DNA replication.

MATERIALS AND METHODS

Cell cultures and SV40 stocks. The cultivation of CV-1 and SV40-transformed hamster (TSV-5) cells has been described (6, 17). SV40 virus was propagated and assayed in monolayer cultures of CV-1 cells. Heterokaryons of transformed TSV-5 and susceptible (CV-1) cells were produced by treating mixtures of cells with ultraviolet light-inactivated Sendai virus, as described previously (6).

Extraction and assay of infectious SV40 DNA. Infectious SV40 DNA was extracted from cultures of infected cells and from heterokaryons of transformed and susceptible cells with *p*-aminosalicylate and phenol (16). The infectivity of the DNA extracts was determined by plaque titration on monolayer cultures of CV-1 cells (19).

Incorporation of ^{3}H-thymidine (^{3}H-dT) into DNA and separation of labeled SV40 DNA from cellular DNA. At various times after infection, cultures were inoculated with 0.1 or 0.2 ml of ^{3}H-dT (New England Nuclear Corp., Boston, Mass.). The cultures were incubated at 37 C for the times indicated in the legends to the tables and figures. Noninfected cultures were treated similarly, except that either growth medium or spent medium (medium removed from 4- to 7-day-old cultures) was substituted for the virus preparations. After the ^{3}H-dT labeling period, the cultures were harvested as previously described (16), and the total DNA content and specific activity of the DNA were determined on portions of the cell suspensions (18). The remainder of the cells were sedimented by centrifugation, washed with saline-glucose solution, and used for extraction of cellular and viral DNA. To determine the relative amounts of ^{3}H-dT incorporated into cellular and viral DNA, the nitrocellulose chromatography procedure of Kit et al. (16) and band centrifugation in CsCl gradients (16, 31) were employed.

Enzyme assays. Assays for thymidine kinase, thymidylate kinase, deoxycytidylate deaminase, and DNA polymerase were performed by standard methods employed in this laboratory (15, 17, 20, 21).

RESULTS

Decrease in DNA infectivity early after SV40 infection of CV-1 cells. When CV-1 cells were productively infected with SV40 virus, an increase in infectious SV40 DNA was observed, beginning at about 12 to 16 hr after infection, and maximal titers of DNA infectivity were attained at about 44 hr after infection. The initial increase in infectious SV40 DNA took place at 12 to 16 hr, whether cells were infected at high input multiplicities [10 to 50 plaque-forming units (PFU) per cell] or at low input multiplicities (0.01 to 0.1 PFU/cell). It apeared from previous experiments, however, that at low input multiplicities, but not at input multiplicities of 1 PFU/cell or greater, the infectious DNA content of cells was less at 7.5 hr than at 2.5 hr or at 20 hr after infection (19).

The observed decrease in infectious DNA early after SV40 infection suggested that structurally altered intracellular forms might be generated in virus-infected cells. As a first step in testing this idea, the early infection period was studied more closely. At low input multiplicities, a decrease in SV40 DNA infectivity was observed by 6 hr after virus infection (Fig. 1). When the input multiplicity was 0.1 PFU per cell, DNA infectivity diminished by 15.5 hr to almost one-tenth that at 3 hr after infection. At the input multiplicity of 0.01 PFU/cell, the low levels of DNA infectivity from 6 to 15.5 hr after infection represented the limits of detectable SV40 DNA. The data in Fig. 1 demonstrate that, early after virus infection, most of the parental SV40 DNA either is con-

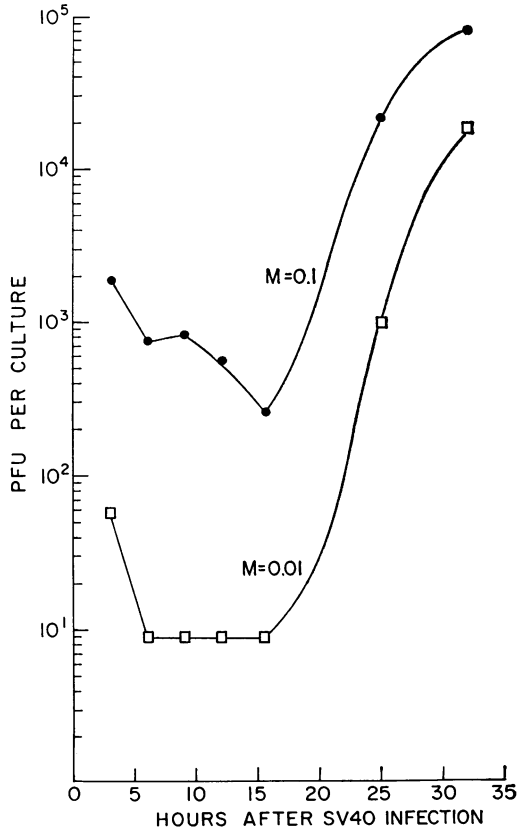

FIG. 1. *Assay of infectious SV40 DNA at various times after infection of CV-1 cells with intact virus. Confluent monolayer cultures (10^7 cells per culture) were infected with 1 ml of SV40 at input multiplicities of 0.1 and 0.01 PFU/cell. After an adsorption period of 2 hr at 37 C, unadsorbed virus was removed, 20 ml of new growth medium was added, and the cultures were further incubated at 37 C. At the times indicated, the cultures were trypsinized and DNA extracts were prepared from the resulting cell suspensions (16, 19).*

verted to a noninfectious form or to a form not extractable by the *p*-aminosalicylate procedure.

[We also considered the hypothesis that the loss of SV40 DNA infectivity may have resulted from the progressive loss of virus adsorbed at the cell surface which never penetrated and was progressively detached from the cells. To test this hypothesis, CV-1 cultures were infected with SV40 at input multiplicities of about 20 and 0.2 PFU/cell, respectively. After a 2-hr adsorption period at 37 C, the unadsorbed virus was removed, the cultures were rinsed four times, and 20 ml of growth medium was added. At 2, 6, 10, and 16 hr. samples were removed from the medium and assayed for SV40. Contrary to the foregoing hypothesis, the virus concentration in the supernatant media decreased progressively from 2 to 16 hr after infection. The total PFU/culture in the supernatant media was in each case less than 10^{-3} that extracted from the cells in the culture at 16 hr after infection.]

Effect of cycloheximide on the incorporation of ^{3}H-dT into DNA of noninfected and SV40-infected CV-1 cells. To learn whether concurrent protein synthesis was required for incorporation of ^{3}H-dT into DNA, the effect of cycloheximide was studied in nonsynchronized cultures and in cultures in which DNA synthesis was synchronized by pretreatment of cells with ara-C. A pulse-labeling experiment involving ara-C pretreatment is shown in Fig. 2. SV40-infected CV-1 cells were incubated with 15 μg of ara-C per ml from 2 to 24 hr after infection. "Mock-infected" control cultures were similarly treated. It has been shown previously (14, 17, 21) that ara-C inhibits DNA synthesis and SV40 capsid protein formation, but not the induction of early enzymes or SV40-tumor antigen. Indeed, the activities of thymidine kinase and DNA polymerase were greater in either uninfected or SV40-infected monkey kidney cultures treated with ara-C than in untreated cultures.

The ara-C inhibition of DNA synthesis can be reversed by removing ara-C and adding deoxycytidine. After the addition of deoxycytidine, a rapid synthesis of DNA was initiated (Fig. 2). The synthesis of DNA, as measured either by the incorporation of tritium label for 2 hr into DNA or by colorimetric measurement of the total DNA content per culture, was greater in SV40-infected cultures than in noninfected cultures. When cycloheximide was added to the cultures at the time of the deoxycytidine reversal of the ara-C block, ^{3}H-dT was incorporated into DNA, but at a grossly inhibited rate. Similar results were obtained after cycloheximide addition to cultures not treated with ara-C.

The data in Fig. 2 suggested that cycloheximide, an inhibitor of protein synthesis (17), severely curtails DNA synthesis within 2 to 4 hr after addition of the drug. To learn more precisely the time required to produce the cycloheximide inhibition, the experiment shown in Fig. 3 was carried out. SV40-infected cultures of CV-1 cells

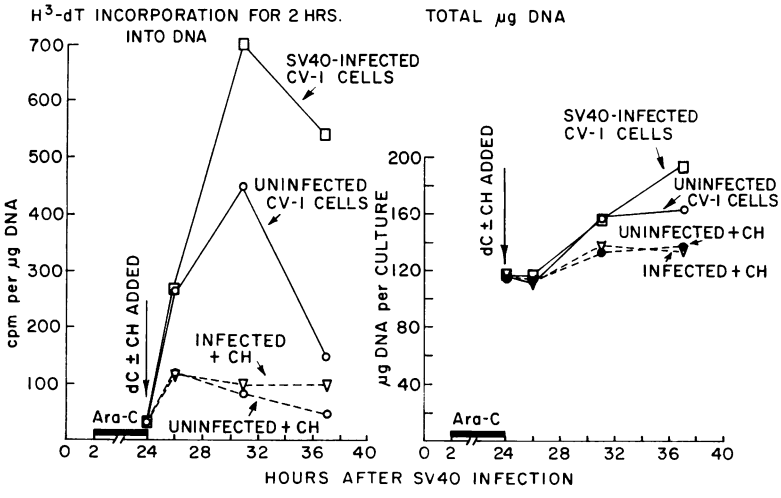

FIG. 2. *Effect of cycloheximide (3 μg/ml) on the incorporation, for 2 hr, of ^{3}H-dT into DNA of SV40-infected and uninfected green monkey kidney cultures. Seven-day-old CV-1 cells (9.3 × 10^6 cells/culture) were infected with SV40 at an input multiplicity of 90 PFU/cell. At 2 hr after infection, the unadsorbed virus was removed, and ara-C (15 μg/ml) was added. The cultures were incubated at 37 C for 24 hr and washed: 50 μg of deoxycytidine per ml, with or without 3 μg of cycloheximide per ml, was added. Cultures were harvested at the times indicated in the figure. At 22, 24, 29, and 35 hr after infection, 0.1 ml of ^{3}H-dT (0.5 μc and 2μg/ml) was added to each bottle.*

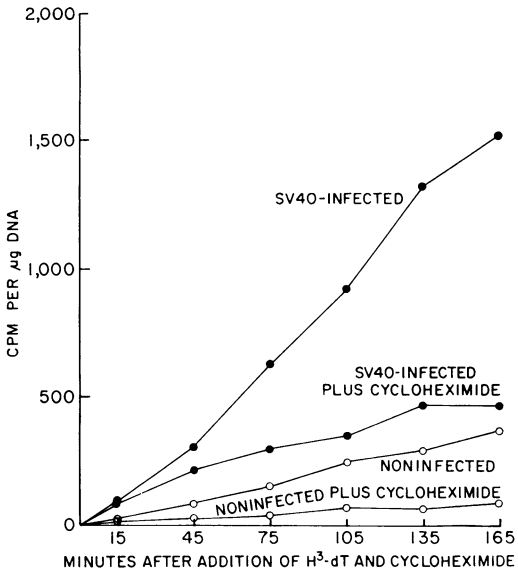

FIG. 3. *Effect of cycloheximide (3 μg/ml) on the kinetics of ^{3}H-dT incorporation into DNA of SV40-infected and uninfected CV-1 cell cultures. Replicate CV-1 cell cultures (11.4 × 10^6 cells/culture) were infected with SV40 at an input multiplicity of 50 PFU/cell. At 36 hr after infection, 0.2 ml of ^{3}H-dT (2 μc and 2 μg/ml) was added. Some of the cultures were also treated with cycloheximide. Cultures were harvested at the times shown on the figure.*

were incubated for 36 hr to permit the initiation of viral DNA synthesis. ^{3}H-dT was added to replicate cultures, either with or without cycloheximide. Cultures were harvested at the times shown in Fig. 3. Incorporation of ^{3}H-dT was severely inhibited within 1 hr of cycloheximide treatment and only limited incorporation of ^{3}H-dT into DNA occurred thereafter. The results suggested that a protein synthesized de novo is rate limiting for DNA synthesis in both uninfected and SV-40-infected cultures.

Separation of labeled cellular and SV40 DNA. The experiments in Fig. 3 show that SV40 stimulated DNA synthesis in CV-1 cell cultures and that the synthesis of DNA in either infected or uninfected cultures was inhibited by cycloheximide. To determine whether both SV40 and cellular DNA syntheses were inhibited by cycloheximide treatment, nitrocellulose chromatography and band centrifugation experiments were carried out (Fig. 4, 5). In the nitrocellulose chromatography experiment (Fig. 4), double-stranded DNA represents SV40 DNA and single-stranded DNA is cellular DNA. As shown previously (16), SV 40 infection stimulates cellular DNA synthesis in CV-1 cells 36 to 44 hr after infection. Cycloheximide treatment prevents this stimulation of cellular DNA synthesis and also markedly reduces SV40 DNA synthesis. The band centrifugation experiment (Fig. 5) further demonstrated the inhibitory effect of cycloheximide on both cellular DNA synthesis (fractions 1 to 22) and on SV40 DNA synthesis (fractions 23 to 30). Similar results were obtained when the ^{3}H-dT labeling periods were 34 to 38 hr, 40 to 44 hr, or 42 to 46 hr after infection.

Stability of "pulse-labeled" SV40 DNA during a "chase" period with or without cycloheximide. We considered the possibility that SV40 DNA synthesized during cycloheximide inhibition might be unstable. This instability could, perhaps, result from a failure to synthesize essential "maturation" proteins or capsid proteins in the presence of the drug. The "pulse-chase" experiment depicted in Table 1 contradicts this hypothesis. Infected and uninfected cultures of CV-1 cells were labeled with ^{3}H-dT from 36 to 40 hr after infection. The cultures were then washed, and excess "cold" thymidine was added either with or without cycloheximide. Cultures were further incubated at 37 C and harvested at 44 and 48 hr. The per cent tritium counts in cellular and viral DNA were

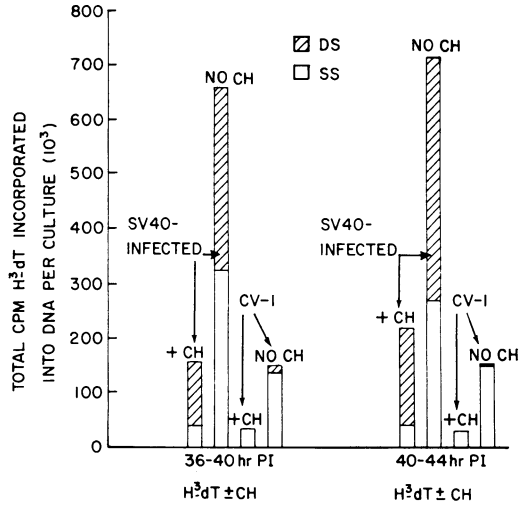

FIG. 4. *Estimation by nitrocellulose column chromatography of the relative amounts of SV40 DNA and cellular DNA synthesized in uninfected and SV40-infected CV-1 cell cultures. DNA samples were heated at 100 C for 10 min in 1 × SSC before chromatography. Single-stranded (SS) DNA is the denatured DNA which requires 0.1 × SSC and 0.01 N NaOH for elution. Double-stranded (DS) DNA is that DNA which is not denatured by the heating and is eluted from the columns with 2 × SSC (circular SV40 DNA). The intervals of ^{3}H-dT incorporation into DNA are shown below each column. Replicate cultures of 7-day-old CV-1 cells (10^7 cells/culture) were infected with SV40 at an input multiplicity of 100 PFU/cell.*

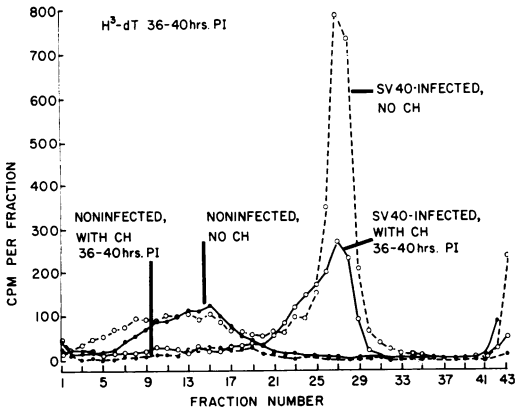

FIG. 5. *Sedimentation-velocity analysis (band centrifugation with CsCl, density 1.503 gcm⁻³, dissolved in 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 8) of DNA from uninfected or SV40-infected CV-1 cells labeled with ³H-dT from 36 to 40 hr after infection. DNA samples were from the same experiment shown in Fig. 4. A lamella of 0.1 ml, containing 3 μg of DNA, was added to each tube. Replicate cultures were treated with 3 μg of cycloheximide per ml at the time of ³H-dT addition. The centrifugation was for 150 min at 20 C and 100,000 × g in the SW39 rotor of the Spinco L2 centrifuge.*

determined by nitrocellulose column chromatography and band centrifugation in CsCl density gradients. The total ³H-dT in the DNA of both the uninfected and the infected cultures was about the same after the 8-hr chase period as at 40 hr. Cycloheximide treatment did not accelerate breakdown of either cellular or SV40 DNA. Thus, the inhibition of ³H-dT incorporation into DNA caused by cycloheximide cannot be ascribed to an enhancement of DNA turnover.

Effect of cycloheximide on the replication of infectious SV40 DNA. The experiments in Fig. 2 through 5 depict the effect of cycloheximide on the "pulse-labeling" of SV40 DNA. The total SV40 DNA accumulated can be determined by measuring the infectious SV40 DNA at various times after infection. Figure 6 shows the effect of cycloheximide treatment on this process. Cultures were first incubated for 25 hr or more to permit the formation of "early proteins" and the initiation of DNA replication. Cycloheximide was added to replicate cultures 25, 28, or 34 hr after infection. Cycloheximide treatment inhibited the formation of infectious SV40 DNA, but did not induce loss of DNA infectivity. Experiments were also carried out in which CV-1 cell cultures were first treated with ara-C to permit formation of "early proteins" and to synchronize

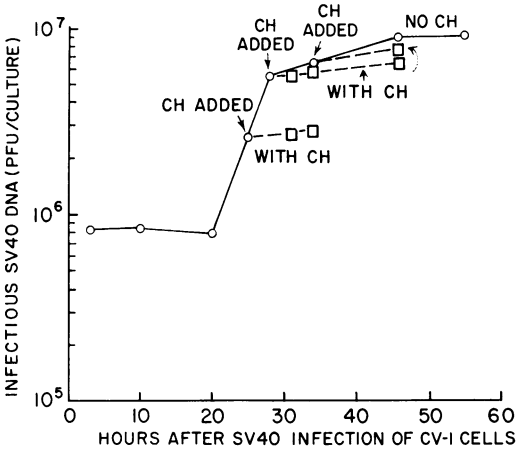

FIG. 6. *Effect of cycloheximide (CH) on the formation of infectious SV40 DNA in CV-1 cultures. Seven-day-old CV-1 cell cultures (11 × 10⁶ cells/culture) were infected with SV40 at an input multiplicity of 20 PFU/cell. Cycloheximide (3 μg/ml) was added at 25, 28, and 34 hr, and cultures were harvested at the times indicated in the figure.*

TABLE 1. *Stability of SV40 DNA labeled from 36 to 40 hr postinfection (PI) during subsequent incubation of infected cells from 40 to 48 hr PI with and without cycloheximide (CH)*[a]

Time of cell harvest (hr PI)	Total counts per min per culture				Total counts/min for SV40 DNA	
	Noninfected		SV40-infected			
	No CH	With CH	No CH	With CH	No CH	With CH
40	5 × 10⁵		1.59 × 10⁶		8.6 × 10⁵	
44	5.7 × 10⁵	5 × 10⁵	1.66 × 10⁶	1.48 × 10⁶	8.6 × 10⁵	8.6 × 10⁵
48	5.3 × 10⁵	4.8 × 10⁵	1.27 × 10⁶	1.15 × 10⁶	7.6 × 10⁵	6 × 10⁵

[a] Seven-day-old CV-1 cells (9.4 × 10⁶ cells/culture) were infected with SV40 at an input multiplicity of 100 PFU/cell. Replicate cultures were incubated with ³H-dT (2 μc and 0.25 μg/ml) from 36 to 40 hr PI. At 40 hr PI, cultures were washed, 20 μg of nonradioactive thymidine per ml was added, and cultures were further incubated at 37 C. Cycloheximide (3 μg/ml) was added at 40 hr PI to the infected and uninfected cultures.

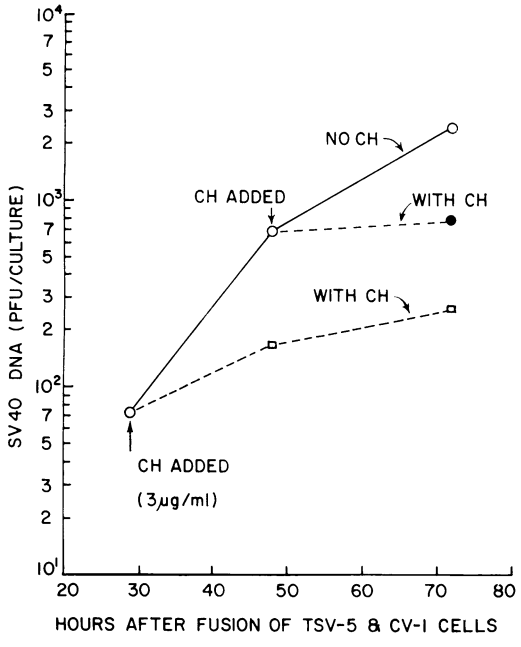

FIG. 7. *Effect of cycloheximide (CH) on the formation of infectious SV40 DNA in heterokaryons of transformed TSV-5 and susceptible CV-1 cells. Mixtures of CV-1 (10^7 cells) and TSV-5 (5.8×10^6 cells) were treated with 14,000 hemagglutinating units of ultraviolet-inactivated Sendai virus to promote fusion (19). A mixture equivalent to 2×10^6 CV-1 and 1.2×10^6 TSV-5 cells was then seeded in prescription bottles and incubated at 37 C for the times indicated. Cycloheximide (3 μg/ml) was added at 29 or at 48 hr after fusion.*

subsequent SV40 DNA replication. Ara-C was removed and deoxycytidine was added 24 hr after infection. Cycloheximide was added to replicate cultures at 28 and 38 hr after infection. In this case, also, cycloheximide treatment inhibited infectious SV40 DNA synthesis. However, for 8 hr after cycloheximide addition, there was no appreciable decrease of infectious SV40 DNA. These experiments further demonstrated that SV40 DNA is relatively stable for several hours after protein synthesis is inhibited.

Experiments were also performed on the formation of infectious SV40 DNA in heterokaryons of susceptible CV-1 and transformed TSV-5 cells. Addition of cycloheximide to the cultures at 29 or 48 hr after cell fusion also retarded the rapid formation of infectious SV40 DNA in these cultures (Fig. 7).

Effect of cycloheximide treatment on enzyme activities. The induction of enzymes by SV40 is first detected at about 12 to 16 hr after virus infection, and maximal enzyme activities are usually observed by about 30 hr (14, 17, 19, 21). In the preceding experiments, cycloheximide was added at a time when enzymes in the DNA biosynthetic pathway had already attained high levels. We considered the hypothesis that one of the enzymes might undergo rapid turnover during the arrest of protein synthesis and that this enzyme deficiency might restrict DNA synthesis. Four of the enzymes catalyzing nucleotide interconversions and DNA replication were therefore studied, namely, thymidine kinase, thymidylate kinase, deoxycytidylate deaminase, and DNA polymerase. In some instances, cultures were pretreated for 30 to 32 hr with ara-C before the addition of cycloheximide. The results of these experiments failed to substantiate the hypothesis that cycloheximide initiated a rapid loss of enzyme activities. Thus, in SV40-infected cultures treated with cycloheximide at 36 hr, the activities of thymidine kinase, deoxycytidylate deaminase, and thymidylate kinase were as high at 42 hr as in cultures not treated with the drug. Similarly, when cycloheximide was added at 38 hr, the activities of thymidine kinase and DNA polymerase were about as high with cycloheximide as without drug treatment at 45 hr after infection. An experiment in which uninfected and SV40-infected cultures were pretreated with ara-C for 30 hr before the addition of cycloheximide is shown in Table 2. The cultures exhibited high levels of thymidine kinase activity for at least 9 hr after cycloheximide was added.

TABLE 2. *Thymidine kinase activities of uninfected and SV40-infected cultures of CV-1 cells treated with ara-C from 2 to 30 hr PI and subsequently treated with cycloheximide (CH)*[a]

Time enzyme assayed (hr PI)	Uninfected		SV40-infected	
	No CH	With CH	No CH	With CH
30	11.0		14.6	
32	10.2	9.6	13.5	16.1
35	12.2	11.5	18.0	14.3
39	13.2	12.7	19.0	16.0
42	12.3	9.1	19.2	12.6

[a] Enzyme activity is expressed as picomoles of ^{3}H-deoxyuridine monophosphate formed from ^{3}H-deoxyuridine per microgram of protein in 10 min at 38 C. Seven-day-old cultures of CV-1 cells (11×10^6 cells/culture) were infected with SV40 at an input multiplicity of 83 PFU/cell. Cultures were incubated with 20 μg of ara-C per ml from 2 to 30 hr after infection, the media were changed, and 50 μg of deoxycytidine per ml, and in some cases 3 μg of cycloheximide per ml, was added. Cultures were harvested at the times indicated.

DISCUSSION

The present experiments have shown that SV40 DNA infectivity undergoes an eclipse early after virus infection of CV-1 cells. This finding is reminiscent of the observation that phage λ DNA extracted from cells soon after infection retains very little infectivity in the Kaiser-Hogness transformation assay due to the formation of structurally altered intracellular forms (3, 5, 32). It is possible that the loss of SV40 DNA infectivity also results from the formation of structurally altered intracellular forms. The DNA of φX174 RF, which resembles SV40 DNA in conformation and molecular weight, is known to undergo structural changes during replication (22). Parental φX174-RF molecules are attached to a fast-sedimenting cell component; the "attached" RF is found in part as RFII and in part as RFI. Only attached RF molecules in the RFII form (nicked-circle) replicate, and daughter RF molecules are later found free in the cytoplasm of the bacterial cell.

We recognize, however, that the loss of SV40 DNA infectivity may have a less interesting explanation. Perhaps this loss results from intracellular nuclease action which occurs after virus particles are "uncoated" and while the SV40 DNA is "in transit" to replication sites. Additional studies, including experiments utilizing labeled virus, are required to ascertain the molecular conformation of the noninfectious SV40 DNA and its intracellular localization, and to learn whether it is, indeed, an obligatory intermediate in SV40 DNA replication.

The present experiments have also shown that protein synthesis must occur concurrently with SV40 DNA replication in productively infected CV-1 cells and in heterokaryons of transformed and susceptible cells. It is not known whether the requisite proteins are viral-specific or cellular proteins. This study and other investigations (25, 28, 30) have shown that drugs which prevent protein synthesis inhibit the acceleration of cellular DNA synthesis in synchronized cell cultures; it is conceivable that a specific class of host proteins is required for both cellular and viral DNA replication.

From the temporal point of view, the proteins required during SV40 DNA replication are "late" proteins. That is, in infected cultures not treated with ara-C, they are required several hours after progeny SV40 DNA and capsid protein syntheses have been initiated. Also, in cultures synchronized with ara-C, the new class of proteins is needed after the "early" class of proteins has accumulated. Data which suggest a stoichiometric relationship between DNA and protein synthesis have been obtained in several microbial and mammalian systems (23–25, 28). In virus-infected cells, it has been shown that protein synthesis late in phage development is necessary for the production of infectious λ DNA (5). The synthesis of a histidine-containing protein is necessary for the replication of the parental replicative form of φX DNA (9). Evidence has been presented that poxvirus DNA synthesis requires concurrent protein synthesis (1, 13). Moreover, although the enzymes catalyzing the incorporation of thymidine into DNA in cellular extracts from pseudorabies-infected cells are stable after addition of puromycin, DNA synthesis in vivo is rapidly inhibited by addition of the drug (2).

The nature and functions of the proteins required for SV40 DNA replication have not as yet been elucidated. Some possibilities that may be considered are that the proteins are: (i) enzymes exhibiting marked instability during inhibition of protein synthesis; (ii) proteins which stabilize progeny SV40 DNA; or (iii) proteins corresponding to the structural and initiator proteins (24, 26) postulated by Jacob et al. (11). Studies on the activities of the thymidine kinase, DNA polymerase, deoxycytidylate deaminase, and thymidylate kinase of infected cells indicate that these enzyme activities are relatively stable for several hours after cycloheximide treatment. It is, therefore, unlikely that one of these enzymes is the essential protein. However, these results do not necessarily exclude a role for another enzyme functioning in DNA biosynthesis. The data in Table 1 and those in Fig. 6 and 7 also suggest that the required protein is not one needed to stabilize SV40 DNA, since progeny DNA is relatively stable for several hours after cycloheximide addition. The third possibility, that protein synthesis is required either as a structural component of an SV40 DNA replication machine or as an initiator protein, merits further study (11, 22, 24, 26). Data supporting the hypothesis that DNA replication might occur in direct association with membrane structures have been obtained for the φX174 RF (22) and for microbial (8, 12) and mammalian cell systems (4, 27). The rate of cellular DNA replication is about 2 μm/min at 37 C for mammalian cells (10, 29) and varies from 6 to 40 μm/min for microbial cells, depending upon the growth media (7). Since SV40 DNA is less than 2 μm long, it is probable that once, initiated, a round of replication requires only a few minutes. The time intervals studied in the experiments shown in Fig. 2 and 3 are much longer than this. Thus, the observed inhibitions may reflect the failure to fabricate new viral DNA replication complexes, while existing replication complexes

may continue to function. This may permit continued synthesis of SV40 DNA at reduced rates.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grant GB 5917, American Cancer Society grant E-291E, Robert A. Welch Foundation grant Q-163, and Public Health Service grants CA-06656-06, 1-K6-AI-2352, and 5-K3-CA-25, 797.

We thank Marjorie Johnson, Carolyn Smith, Judith Rotbein, and Sun Ock Yim for able technical assistance.

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