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Synthesis of Type 2 Adenovirus DNA in the Presence of Cycloheximide

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Adenovirus type 2 DNA synthesis, either in permissive human cells or nonpermissive monkey cells, becomes independent of protein synthesis after the appearance of progeny viral DNA. In the presence of cycloheximide, semiconservative replication and initiation of progeny molecules can occur.

Chromosomal DNA replication in eukaryotic cells requires concomitant protein synthesis (2, 17). In two human tissue culture lines (HEp-2 and HeLa), DNA synthesis is rapidly terminated by protein inhibitors (2, 24). DNA synthesis within the livers of hepatectomized rats is similarly inhibited by cycloheximide (5). In a prokaryotic system (*Escherichia coli*) in which the two events of DNA initiation and elongation have been experimentally separated, new proteins are needed only for the initiation step (15). In eukaryotes, effects of protein inhibitors both on DNA initiation and elongation have been reported. In chick red blood cells, cycloheximide slows elongation (23); however, in L cells treated with puromycin, initiation of new DNA replicons is inhibited several hours before any effect on DNA chain elongation is evidenced (8). Viruses from the herpes (12), poxvirus (13), and papova (4, 14) groups, all of which infect eukaryotes, likewise need continued protein synthesis for DNA replication. This report, presented in part before the American Society of Immunologists (Fed. Proc. Abstr. 810, 1972), shows that adenovirus DNA synthesis becomes independent of protein synthesis 3 to 4 h after the appearance of new viral DNA. Semiconservative replication and initiation of progeny molecules can occur in the presence of cycloheximide.

MATERIALS AND METHODS

Cells and viruses. The source of HeLa cells, adenovirus type 2, and conditions of infection were previously described (9). All experiments in suspension cultures of HeLa cells were done at an input multiplicity of 4,000 virions per cell. Monolayer cultures of primary African green monkey kidney cells (AGMK) were infected either with 20 plaque-forming units (PFU) of simian vacuolating virus 40 (SV40) per cell, 2 PFU of adenovirus type 2 per cell, or a mixture of both viruses. After adsorption for 4 h, unadsorbed virus was removed, and the medium was replaced with 40 ml of Eagle medium containing 10% fetal calf serum.

Characterization of DNA. Band sedimentation of DNA on alkaline sucrose gradients with the use of whole cells to avoid fragmenting the macromolecules, DNA-DNA hybridization on nitrocellulose membranes, radioactive labeling of DNA, and the determination of acid-precipitable radioactivity were done by methods previously described (9).

Density labeling of DNA was accomplished by adding 100 μg of 5-bromo-2′-deoxyuridine (BUdR) and 125 μCi of ^{3}H-thymidine (14 to 21 Ci/mmol) in the presence of 10^{-5} M of 5-fluoro-2′-deoxyuridine (FUdr) to 20 ml of infected HeLa cells (2×10^5 cells per ml). A 5.5-ml solution of DNA, purified as previously described (21), was mixed with 7.9 g of CsCl, and the density was adjusted to 1.750 g/ml. The mixture was centrifuged to equilibrium for 62 h in a type 40 angle rotor (Spinco) at 38,000 rpm and 25 C. The gradient was fractionated by pumping oil onto the top and collecting fractions (10 drops) from a hollow needle at the bottom of the tube.

Characterization of proteins. Labeling and characterization of proteins on sodium dodecyl sulfate (SDS)-containing acrylamide gels was performed as previously described (10).

Reagents. BUdR was obtained from Schwarz/Mann; cycloheximide and puromycin from the Nutritional Biochemicals Co. FUdR was a gift from the Hoffmann-LaRoche Co. The sources of other chemicals and radioactive compounds have been described (9).

RESULTS

Effect of cycloheximide on protein and DNA synthesis in adenovirus-infected and uninfected HeLa cells. The synthesis of viral DNA begins at 9 h postinfection (PI) and reaches a maximum rate at 16 to 18 h after virus addition (9). HeLa DNA synthesis is gradually

reduced to levels of 10 to 15% of control values by 16 h PI. At 18 h PI, cycloheximide was added at doses up to 300 μg/ml. After 30 min of drug treatment, the cells were pulse-labeled with a mixture of ³H-thymidine, ¹⁴C-valine, and ¹⁴C-threonine to measure DNA and protein synthesis, respectively. Uninfected HeLa cells were similarly treated. Figure 1 demonstrates the dose-dependent inhibition of protein synthesis. In both virus-infected and uninfected HeLa cells, protein synthesis is reduced 90 to 95% by 30 μg of cycloheximide per ml. Although HeLa DNA synthesis is reduced correspondingly by 83%, DNA synthesis in the infected cells is reduced only 9%. As shown below, most of the

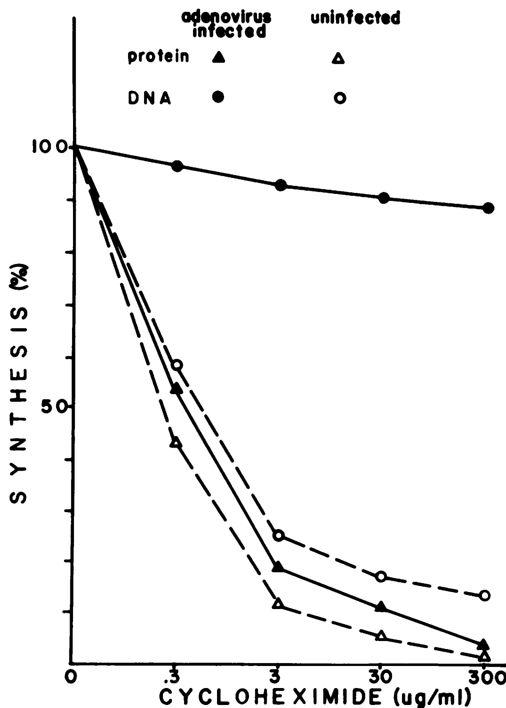

FIG. 1. *Effect of cycloheximide on DNA and protein synthesis in HeLa or adenovirus-infected HeLa cells. After 18 h of infection or mock infection, HeLa cells were centrifuged and suspended in Eagle minimal essential medium containing 0.05 the normal concentration of valine and threonine. A 5-ml amount of cells (2×10^5 cells per ml) was incubated for 30 min with varying doses of cycloheximide, and duplicate 2-ml samples were radioactively labeled with 2.5 μCi of ³H-thymidine, 0.25 μCi of ¹⁴C-valine, and 0.25 μCi of ¹⁴C-threonine for 60 min. The radioactive incorporation was stopped by dilution into cold medium, and the cells, centrifuged from solution, were analyzed for acid-precipitable radioactivity. The results are expressed as a percentage of the incorporation of thymidine (DNA) or valine and threonine (protein) in the absence of cycloheximide.*

newly synthesized DNA in the infected cell is viral DNA. Thus, there is clearly a dissociation between DNA and protein synthesis during the 1 h of observation in the infected cells. Quantitatively similar observations have been made with puromycin (Table 1).

Effect on DNA synthesis of cycloheximide added at different times in the infectious cycle. The kinetics of conversion of DNA synthesis to the cycloheximide-resistant state was measured at numerous times after the addition of virus. Mock-infected HeLa cells were investigated in parallel. Cycloheximide (30 μg/ml) was added for the 30 min preceding radioactive thymidine labeling. Figure 2 shows that, in the infected cells, thymidine incorporation becomes progressively resistant to the drug. At 14 h PI, DNA synthesis reached about 90% of the levels of the non-drug-treated, infected cells. The level of DNA synthesis in the uninfected cells was always reduced to approximately 10% by similar drug treatment.

The amount of radioactivity in viral DNA made at several critical times PI was measured by DNA-DNA hybridization. At 2, 4, 6, 7.5, and 23.5 h PI, 5 ml of adenovirus-infected HeLa cells, and, at 2 h, 5 ml of mock-infected cells were labeled with 12 μCi of ³H-thymidine for 60 min. The cells were harvested, the whole-cell DNA was purified, and samples were hybridized to 3 μg of adenovirus DNA on nitrocellulose filters. Table 2 shows that the first detectable viral DNA appeared at 8 h. Approximately 1% of the new DNA synthesized at 8 h was viral specific, whereas 100% of the newly labeled DNA at 23.5 h was viral. Thus, the increase in cycloheximide-resistant thymidine incorporation (26%) at 6.5 h seems to precede the onset of viral DNA synthesis, implicating an effect on

TABLE 1. *DNA and protein synthesis in the presence of puromycin*[a]

Cells	Protein synthesis (%)	DNA synthesis (%)
Uninfected HeLa	4.3	12.2
Adenovirus-infected HeLa	6.1	72.7

[a] At 18 h after adenovirus infection, the inhibition of DNA and protein synthesis by puromycin at various doses was tested as described in Fig. 1. The data from 40 μg/ml are shown. Percent refers to the counts per minute of radioactive amino acids (protein) or thymidine (DNA) incorporated in the presence of the drug as compared to untreated controls.

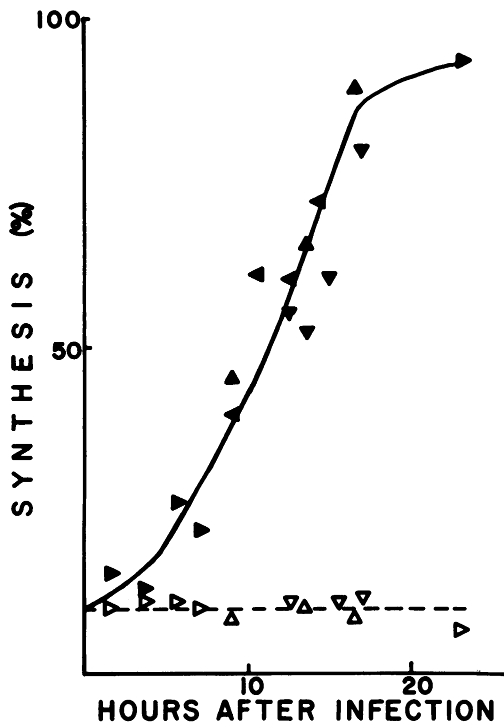

FIG. 2. *DNA synthesis in the presence of cycloheximide added at different times in the infectious cycle. Cycloheximide (30 μg/ml) was added to 2 ml of virus-infected HeLa cells, and 30 min later the cells were labeled with 1 μCi of ^{14}C-thymidine for 1 h. The amount of radioactivity incorporated was expressed as a percentage of the incorporation into cells similarly treated without cycloheximide. The results from several experiments are expressed in the solid triangles. Uninfected HeLa cells similarly treated incorporated radioactivity at levels shown in the open triangles. Each symbol shown is the result of duplicate determinations.*

host DNA within adenoviral-infected cells (Fig. 2).

Properties of DNA made in the presence of cycloheximide. Progeny adenoviral DNA synthesized in the presence of cycloheximide was examined to see if complete strands of DNA were made. Cycloheximide was added to adenovirus-infected HeLa cells at several times from 9 to 16 h PI. The infection was allowed to proceed, and all of the cultures were simultaneously labeled with radioactive thymidine for 1 h at 16 h PI. Radioactive labeling was terminated by the removal of ^{3}H-thymidine and by the addition of an excess of nonradioactive thymidine. The cells were harvested 30 min later. This "chase" period allowed any replicating viral or HeLa DNA molecules to fully elongate. Figure 3 shows that viral DNA made in the presence of cycloheximide has normal sedimentation properties. Sedimentation studies were performed on alkaline gradients on which single- or double-strand discontinuities would be detected.

Duration of adenovirus DNA synthesis in the presence of cycloheximide. The duration of normal viral DNA synthesis after the addition of cycloheximide was measured. At 16 h PI, cycloheximide was added at a concentration of 30 μg/ml. At intervals after cycloheximide addition, radioactive thymidine was added for 1 h and the radioactivity incorporated into DNA was measured. Figure 4B shows that, for at least 6 h, drug-treated cells incorporate radioactive thymidine into DNA at levels greater than 85% of the infected controls. The decrease in the rates of DNA synthesis is due to the fact that the peak of viral DNA synthesis coincides with the first 2 h of the experiment. When cycloheximide and radioactive thymidine are simultaneously added

TABLE 2. *Onset of viral DNA synthesis in adenovirus-infected HeLa cells*[a]

Source of radioactive DNA	Time of ^{3}H-thymidine addition (h)					
	Infected HeLa					Uninfected
	2	4	6	7.5	23.5	2
Counts per minute hybridized to 3 μg Ad2 DNA[b]	50	36	32	182	1,722	4
Total counts/min in hybridization mixture	13,982	23,832	8,290	20,792	3,008	1,314
Hybridized (%)	0.36	0.15	0.39	0.88	57.2	0.3

[a] At various times after infection or mockinfection, 5 ml of cells (2×10^{5}/ml) were labeled with 12 μCi of ^{3}H-thymidine for 60 min. The DNA was purified from the whole cell lysate, denatured in NaOH, and hybridized to Ad2 DNA on nitrocellulose filters. The hybridization efficiency of radioactively labeled DNA purified from virion was 54%.

[b] Counts per minute sticking to blank filters was substrated.

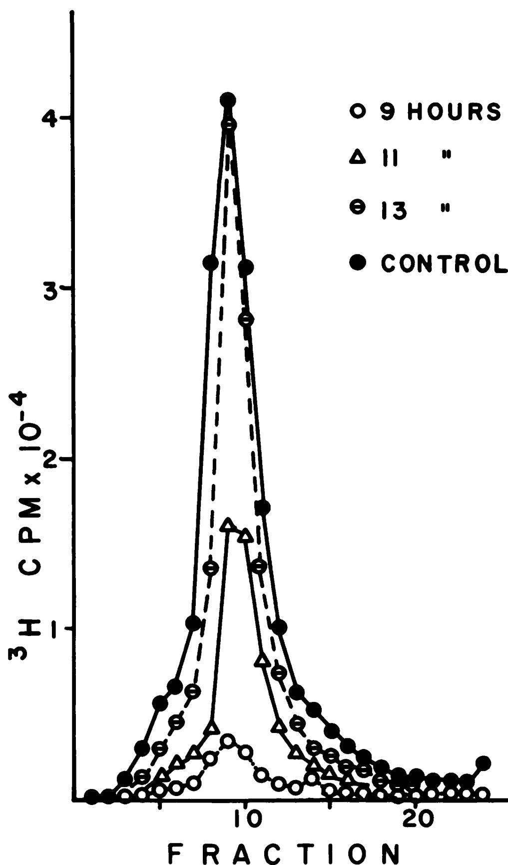

FIG. 3. *Sedimentation properties of DNA synthesized in the presence of cycloheximide. Cycloheximide (30 µg/ml) was added to 10-ml samples of infected cells at various times from 9 to 16 h PI. The incubation was continued until 16 h when all samples were simultaneously labeled with 40 µCi of ^{3}H-thymidine for 60 min. The cells were centrifuged, the radioactive supernatant fluid was removed, and the pellet was suspended in medium with 10^{-5} M thymidine and 30 µg of cycloheximide per ml for an additional 30 min. The cells were pelleted, suspended in 0.15 M NaCl containing purified virion labeled with ^{14}C-DNA, and placed intact onto alkaline sucrose gradients. Centrifugation was at 24,000 rpm for 16 h in a Spinco SW27 rotor as previously described (9). (The results of cycloheximide addition at 16 h were identical to the control.) ^{14}C-DNA sedimented to fraction 9. Cycloheximide added at: 9 h (○); 11 h (△); 13 h (⊖); control without cycloheximide (●). (^{3}H counts in pellet, 9 h, 7,990; 11 h, 8,970; 13 h, 4,980; control, 20,300.)*

to uninfected cells, the incorporation of radioactivity essentially ceases after a delay of 10 min (Fig. 4A). Simultaneous measurements with radioactive valine and threonine showed no lag in the inhibition of protein synthesis by cycloheximide in infected or uninfected cells.

Initiation of viral DNA molecules in the presence of cycloheximide. Further characterization of the DNA synthesized was accomplished by density labeling of the progeny molecules with BUdR and radioactive thymidine. Infected cells were treated with cycloheximide, BUdR, and tritiated thymidine at 9, 12, and 16 h PI. Nine hours corresponds to the onset of viral DNA synthesis, and the peak of DNA synthesis is at 16 to 18 h PI. If the progeny DNA is separated on CsCl equilibrium gradients, it can be seen that the addition of drug at 12 h does not prevent the efficient synthesis of DNA with a density (HH, completely heavy) expected for BUdR substitution in both strands (Fig. 5B). The results at 16 h (not illustrated) show equal amounts of radioactivity in the HH region of cycloheximide-treated and control cells. However, if the drug is added at 9 h, very little HH DNA is made in the cycloheximide-treated cells. The progeny DNA at

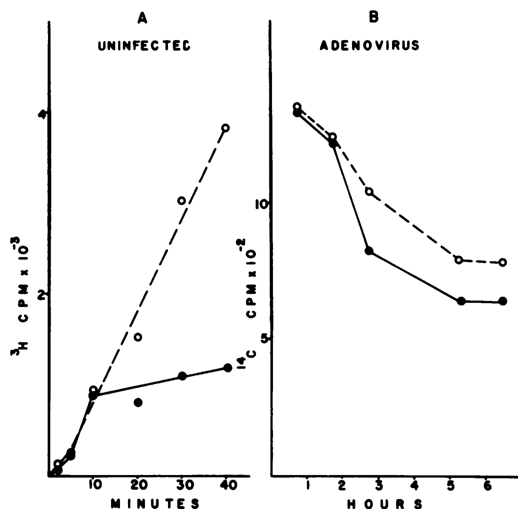

FIG. 4. *Kinetics of shut-off of viral or HeLa DNA after addition of cycloheximide. Panel A: 50 µCi of ^{3}H-thymidine and cycloheximide to achieve a final concentration of 30 µg per ml were added to 50 ml of uninfected HeLa cells (2 × 10^5 cells per ml) at 37 C. The controls were similarly processed without the inhibitor. At the designated times, 2 ml in duplicate were removed and rapidly chilled. The accumulation of acid-precipitable radioactivity in the cycloheximide-treated cells (●) is compared to the controls (○). Panel B: At 16 h PI, 30 µg of cycloheximide per ml was added to adenovirus-infected HeLa cells which were at a concentration of 2 × 10^5 cells per ml. At intervals, 2 ml of cells in duplicate were radioactively labeled with 1 µCi of ^{14}C thymidine for 60 min. Similar labeling of cells without the inhibitor provided control levels of incorporation of radioactive thymidine. The abcissa represents the time after addition of cycloheximide. Cycloheximide-treated cells (●); controls (○).*

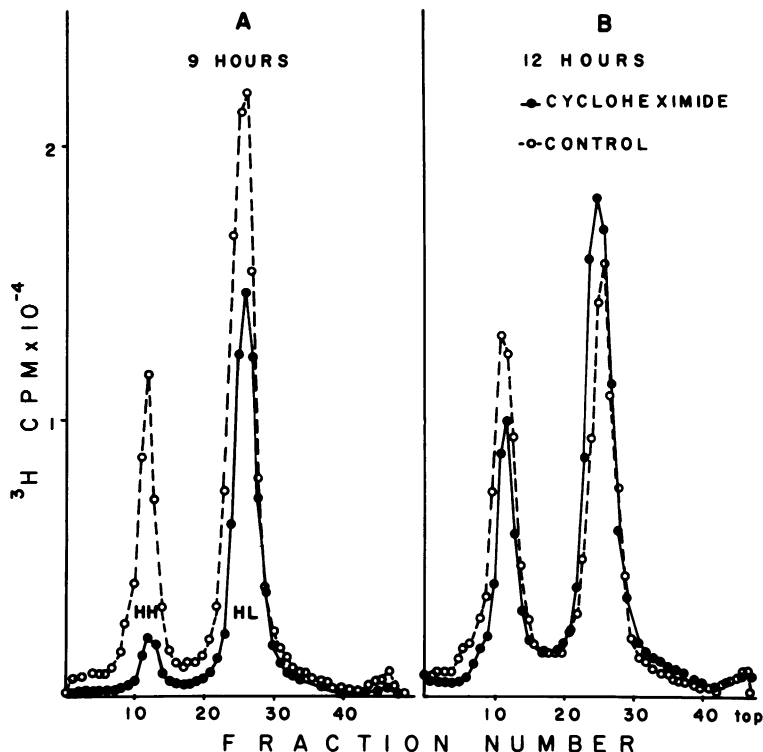

FIG. 5. Initiation of viral DNA molecules in the presence of cycloheximide. At 9 or 12 h after infection, samples of cells were density labeled with BUdR and ³H-thymidine as described in Materials and Methods. Cells were processed similarly, following a 15-min pretreatment with cycloheximide (30 μg/ml). After 6 h the labeling was terminated by centrifugation of the cells and suspension in 0.01 M Tris-hydrochloride, 0.01 M EDTA, 0.05 M NaCl, and 0.2% SDS. The DNA was purified and centrifuged to equilibrium, and the gradients were fractionated as described in Materials and Methods. HH and HL describe the location of completely "heavy" and hybrid density DNA molecules, respectively. Completely "light" DNA (LL), unlabeled in these experiments, is located in fraction 42.

that time can be converted with approximately 50% efficiency to heavy-light (HL) molecules indicating one round of DNA synthesis in the absence of new proteins. The density patterns after 12 h prove that incorporation of radioactivity in these experiments represents the synthesis of completely new molecules rather than repair replication or terminal addition of nucleotides.

Role of specific proteins in cycloheximide-resistant adenoviral DNA synthesis. Because the resistance of DNA synthesis to cycloheximide becomes most pronounced at 12 h PI which is also the time that structural viral protein synthesis begins, the role of the structural proteins in this process was studied. The polypeptides made in the presence of 30 μg of cycloheximide per ml were examined. The total radioactivity in the presence of cycloheximide was only 5% of the controls, and the separated polypeptides are shown in Fig. 6. Thus, even with a 100-fold change in the ordinate, there is no accumulation of a single cycloheximide-resistant polypeptide as detected by these techniques.

Human adenovirion production in primary AGMK is very inefficient. Although adenoviral DNA is made in approximately normal quantities, only small amounts (0.1%) of the capsid proteins (hexon, penton, and fiber) are synthesized (1). These observations suggest that adenoviral DNA production is independent of capsid protein synthesis. In addition, we have found viral DNA synthesis in adenovirus-infected AGMK cells resistant to cycloheximide similar to the results in HeLa cells (results not shown). This corroborates the previous impression that it is not an accumulation of capsid proteins that makes the adenoviral DNA resistant to cycloheximide.

As a control, we examined the replication of SV40 DNA. SV40 DNA replication was previously reported to be inhibited by cycloheximide (14), and we have confirmed this observation in

AGMK cells with our experimental conditions (Fig. 7A).

Cycloheximide in adenovirus and SV40 co-infected AGMK cells. We attempted to study the relationship between SV40 and adenovirus DNA replication to dissect some of the controls involved in the DNA initiation complex. Adenovirus and SV40 will co-infect monkey cells. SV40 corrects the defect in adenovirus protein synthesis and allows efficient completion of the adenovirus cycle (1).

However, co-infection of AGMK cells drastically reduces the amount of SV40 DNA compared to that made in singly infected control cells (7). The inhibition of SV40 DNA synthesis was identical whether the cells were simultaneously

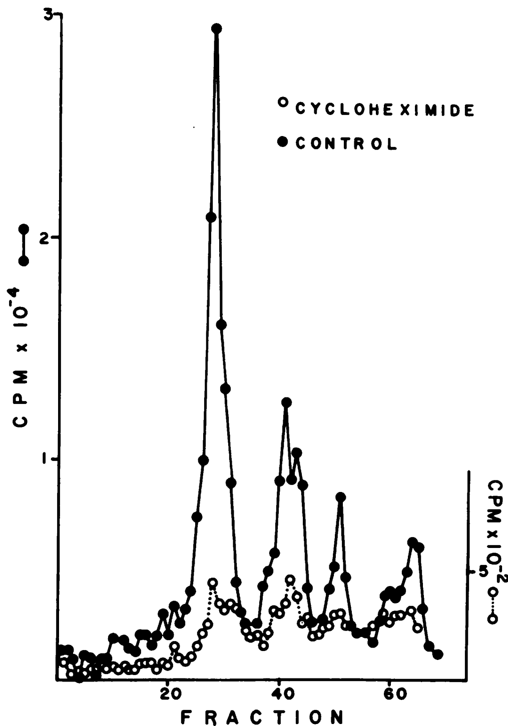

FIG. 6. *Residual protein synthesis in the presence of cycloheximide. A 20-ml amount of adenovirus-infected HeLa cells (2 × 10^5 cells/ml) was suspended at 16 h PI in 20 ml of Eagle medium containing 0.05 the normal amount of valine and threonine. Cycloheximide (30 µg/ml) was added to 10 ml of cells, and the remainder served as controls. Fifteen minutes later, 5 µCi of ^{14}C-threonine and 5 µCi of ^{14}C-valine were added to both cultures. After 1 h, the cells were harvested, dissociated with 2% SDS, 1% mercaptoethanol, and prepared for electrophoresis on 5% SDS-containing acrylamide gels. Separation, quantitation, and identification of each polypeptide was previously described (16). Migration is toward the anode on the right.*

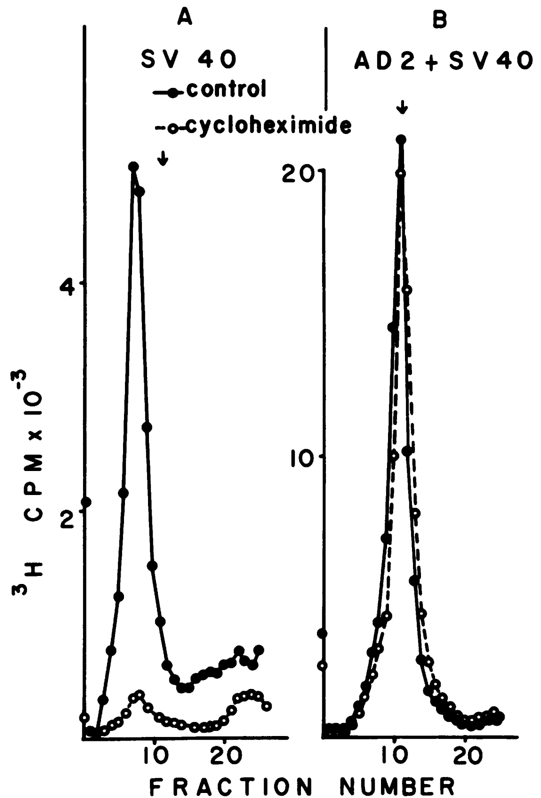

FIG. 7. *Effect of cycloheximide in AGMK cells on SV40 DNA production or combined adenovirus and SV40 DNA production. A, Confluent monolayers of AGMK cells were infected in duplicate with 20 PFU/cell of SV40. At 4 h PI, the unabsorbed virus was removed and the cells were incubated at 37 C. At 40 h PI, 30 µg of cycloheximide per ml was added to one bottle. Thirty minutes later, 250 µCi of ^{3}H-thymidine was added to each bottle for 4 h. The cells were scraped from the monolayer, centrifuged, and suspended on 0.15 M NaCl. Samples (0.5 ml of each culture) were placed onto alkaline sucrose gradients with purified adenovirion labeled with ^{14}C-DNA and centrifuged at 22,000 rpm for 16 h. B, A similar experiment done by co-infection of AGMK with SV40 and adenovirus type 2 (2 PFU per AGMK cell) is shown. The sedimentation of AD2 ^{14}C-DNA marker is designated by the arrow.*

infected with SV40 and adenovirus or there was a 24-h delay in the addition of adenovirions. Figure 7B shows that the addition of cycloheximide to AGMK cells infected with adenovirions and SV40 does not alter synthesis of DNA which is predominantly adenovirus specific.

DISCUSSION

The continued synthesis of adenovirus DNA in the presence of cycloheximide is in contrast to the inhibition of the replication of DNA of

numerous other viruses which infect eukaryotes. The production of viral DNA of SV40 (14), polyoma (4), rabbitpox (13), and pseudorabies (12) stops rapidly after the inhibition of protein synthesis. There are indications that adenovirus DNA is made and released from membranes (18) so that the participation of these structures seems to be similar to other DNA synthesizing systems in which new protein synthesis is necessary (11).

In prokaryotic cells, chromosomal initiation and phage replication likewise require continued protein synthesis (15). Several exceptions to this general requirement have been noted. Included is the recent observation that the colicinogenic episome (Col E_1) can replicate hundreds of copies of its double-stranded circular DNA in the presence of high doses of chloramphenicol (6). In the absence of new proteins, ΦX174 can convert single-stranded parental molecules to double-stranded replicating forms (22). Finally, initiation proteins which can be used for subsequent DNA synthesis, accumulate in *E. coli*, whose DNA synthesis has been inhibited either by thymine starvation (21) or by nalidixic acid (3).

Since the mechanism of adenovirus DNA replication is presently unknown, one cannot distinguish between some unique model of initiation and polymerization or the synthesis of large amounts of stable replication complexes.

There seem to be three stages in the replication of adenovirus DNA with respect to the need for protein synthesis. If cycloheximide is added before 9 h (onset of viral DNA synthesis), no viral DNA is made. If protein inhibitors are added from 9 to 12 h, few viral DNA molecules replicate more than once. Presumably, both the Crick and Watson strand replicate at that time. After 12 h there is semiconservative replication with initiation of new strands in the presence of cycloheximide. At all times PI, the viral DNA made in the presence of cycloheximide has normal sedimentation values indicating no delay in maturation. SV40 and polyoma DNA are inhibited under identical conditions, and a failure to covalently close the circular genome has been shown (4). The process of normal adenoviral DNA synthesis continues for more than 6 h and must represent strand initiation. Although the synthesis of HL molecules could conceivably represent the completion of chains initiated before the addition of cycloheximide to which only several nucleotides were added, the demonstration of HH molecules clearly indicates initiation in the absence of protein synthesis.

The proteins synthesized before 12 h are both host and viral nonstructural proteins. By the time viral structural proteins are made, viral DNA synthesis no longer requires new proteins. In agreement with these results in HeLa cells is the observation that viral DNA is synthesized in AGMK cells in which capsid proteins are made inefficiently.

The important relationship between the synthesis of adenovirus DNA and either HeLa or SV40 DNA remains incompletely defined. Since adenovirus infection reduces HeLa and SV40 DNA synthesis, we were unable to critically examine whether an adenovirus "initiator protein" or polymerase could function on another DNA template. However, there is a suggestion from Fig. 2 that HeLa DNA synthesis in the uninfected cell is more sensitive to cycloheximide than in the infected cell. Within a bacterial cell the ability of an episomal DNA (F-*gal*) to correct a replication defect in a temperature-sensitive second episome (F-*lac*) has been demonstrated. Presumably, a diffusable protein coded by F-*gal* was able to function on the temperature-sensitive F-*lac* genome, suggesting "positive" control for DNA replication (11).

Stable protein repressors of DNA synthesis have been proposed to explain the termination of DNA synthesis by protein inhibitors (20). Thus, when "derepressor-like" proteins are not made to facilitate DNA synthesis, a genome remains inhibited. Partial evidence against a diffusable stable protein repressor is derived from the SV40-adenovirus dual infection of AGMK. Although the SV40 genome functions sufficiently to enhance adenovirus protein synthesis, SV40 co-infection does not alter adenovirus DNA synthesis in the presence of cycloheximide.

One unique application of the adenovirus DNA synthesis system would be in studies of new inhibitors of protein synthesis. It is the only eukaryotic system in which pharmacology studies of protein inhibitors can be performed without major secondary effects on DNA synthesis.

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